DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

DSM Nutritional Products

45 Waterview Boulevard
Parsippany
NJ 07054
United States of America

Phone 1-973-257-8325
FAX 1-973-257-8414

Food and Drug Administration
Division of Animal Feeds (HFV-224)
Office of Surveillance and Compliance
Center for Veterinary Medicine
7519 Standish Place
Rockville, Maryland 20855

October 12, 2012

GRAS Notification Of RONOZYME HiPhos® by DSM Nutritional Products

Dear Mr. Wong

In response to the call for voluntary participation in the Notice of Pilot Program published in the Federal Register Vol. 75 31800-31803 on 4 June 2010, DSM Nutritional Products is hereby submitting in triplicate a Notification of the Generally Recognized As Safe (GRAS) use of the 6-phytase, Ronozyme HiPhos® , in poultry feed. This enzyme improves the availability of phosphorus found in plant based feeds by cleaving the myo-inositol - phosphate bond.

DSM Nutritional Products gathered the appropriate information on the safety and utility of the notified substance which was provided for evaluation to an independent panel of experts. The enclosed dossier contains information on the identity of the production organism, manufacture of the enzyme and commercial forms, safety and efficacy study data that was provided to the panel and the panel's signed conclusion statement. Also included are copies of the pertinent literature and the peer reviewed publications addressing the safety of Ronozyme HiPhos® and its performance in a variety of poultry feeds indicative of those normally fed in the United States.

DSM Nutritional Products has concluded that use of Ronozyme HiPhos® in poultry feed is GRAS through scientific procedures and is therefore exempt from the requirement for premarket approval noted in Section 201 (s) of the Federal Food Drug and Cosmetic Act.

The complete data and information that are the basis of this GRAS Notification are available to the Food and Drug Administration for review and copying upon request during normal business hours at our offices located at 45 Waterview Boulevard, Parsippany, NJ 07054.

Sincerely DSM Nutritional Products,

Alberto Davidovich, DVM, Ph.D.
Director Regulatory Affairs North America

RECEIVED
OCT 18 2012
By

HEALTH • NUTRITION • MATERIALS

RONOZYME® HiPhos

A 6-phytase preparation produced by an *Aspergillus oryzae* strain expressing a synthetic gene coding for a 6-phytase from *Citrobacter braakii* for use in poultry nutrition

SUMMARY OF DATA FOR AFFIRMATION OF GRAS STATUS

By
DSM NUTRITIONAL PRODUCTS

Jean-François Hecquet
DSM Nutritional Products
Basel, Switzerland

James La Marta
DSM Nutritional Products
Parsippany, New Jersey

TABLE OF CONTENTS

List of Tables

List of Figures

1 Executive Summary

The purpose of this document is to provide technical information that supports the Generally Recognized as Safe (GRAS) status of RONOZYME® HiPhos, a 6-phytase product for use in poultry based on scientific procedures.

Novozymes A/S and DSM Nutritional Products Ltd. are business partners and co-developed RONOZYME® HiPhos. Novozymes A/S is responsible for the manufacturing of the product (supplier) while DSM Nutritional Products Ltd. has market exclusivity for the product.

RONOZYME® HiPhos is an enzyme preparation that contains a 6-phytase [IUB number 3.1.3.26, CAS number 9001-89-2 (phosphatase, 6-phytase)]. The enzyme hydrolyses bonds between phosphate (P) and myo-inositol in phytic acid and its salt and thus increases the availability of phosphorus from plant based materials used in animal feed and decreases phosphate poultion. Both the nucleotide sequence of the gene encoding the 6-phytase as well as the resulting primary amino acid sequences of the phytase are known.

The subject 6-phytase will be marketed in three product forms. The first one is a micro granulated form (M form), the second one is a liquid form (L form) and the third form is a Granulated Thermo-tolerant form (GT form) under the trade names RONOZYME® HiPhos (M), RONOZYME® HiPhos (L) and RONOZYME® HiPhos (GT) respectively; additional product forms may be developed with feed grade ingredients according to marketing needs. RONOZYME® HiPhos is produced by Novozymes A/S, Copenhagen, Denmark and distributed by DSM Nutritional Products, Basel, Switzerland.

The safety of the enzyme has been established according to the published literature and guidelines described by Pariza and Foster (1983) Ref. 4, as updated by Pariza and Johnson (2001) Ref. 5, and recently by Pariza and Cook (2010) Ref. 29 to take into account genetic modifications. According to these latest guidelines, the safety of the production organism should be the prime consideration in assessing the safety of an enzyme preparation intended for use in food and thus feed. The microbial production strain is a genetically engineered variant of the fungus *Aspergillus oryzae* expressing a mimetic (synthetic) version of the *Citrobacter braakii* gene coding for 6-phytase. *Aspergillus oryzae* is non pathogenic. It is a species with a century-long history of safe use in food production. *Aspergillus oryzae* is used to produce fermented foods from rice and soya in Asia such as sake, shoyu, miso, soy sauce. Taking Japan an example, *Aspergillus oryzae* has been used for such food production there for over 400 years.

Aspergillus oryzae, along with *Aspergillus niger* and *Trichoderma reesei*, is one of the most important fungal production organisms in industrial fermentations [Blumenthal 2004] Ref. 2. Food enzymes produced from *Aspergillus oryzae* include amylase, hemicellulase, lipase, oxireductase, protease and pectin esterase. The production strain is derived from a safe strain line, which has been used by Novozymes A/S for over 40 years for food and feed enzyme production. Safety assessments for food enzymes from *Aspergillus oryzae* strains used by Novozymes A/S have been published [Greenough et *al.* 1996] Ref. 6. Any mycotoxin contamination of RONOZYME® HiPhos formulations arising from the production strain are effectively excluded, as described in detail further in this document.

Aspergillus oryzae has been combined with well-characterized genetic material mimetic to material from *Citrobacter braakii*. Metabolites of concern are (b) (4)

(b) (4) which are not produced by this *Aspergillus oryzae strain* as demonstrated by analysis. Enzymes preparations (e.g. lipase, alpha-amylase and glucoamylase) from *Aspergillus oryzae* are generally recognized as safe for use in food by the US Food and Drug Administration, and are the subject of extensive literature reports and are otherwise considered safe for animal feed uses by the Food and Drug Administration Center of Veterinary Medicine as evidenced by listings in Table 30.1 of the Official Publication of the American Association of Feed Control Officials, 2011.

The present preparation of 6-phytase is produced by (b) (4) fermentation of the production strain (*Aspergillus oryzae*) applying current good manufacturing practices for food (21 CFR 110). Since the host organism is safe and the incorporated DNA does not encode any known harmful or toxic substances, the resulting phytase preparation is considered safe. This safety was confirmed by a battery of *in vitro* and *in vivo* studies as described herein and published in Lichtenberg et al. 2011(Ref. 30). Lichtenberg et al. also describes the construction of the production strain. The subject phytase is substantially equivalent to other phytases that are recognized as GRAS and included in the AAFCO Official Publication Table 30.1.

The functionality of RONOZYME® HiPhos is demonstrated primarily by in vitro studies and controlled research studies conducted with poultry as summarized in this document in Section 7 and published in the International Journal of Poultry Science 10 (2): 160-168, 2011.

An independent panel of experts, qualified by their scientific training and national and international experience to evaluate the safety of food and food ingredients (the "Expert Panel"), was specially convened by DSM Nutritional Products, and asked to evaluate the safety and Generally Recognized as Safe (GRAS) status of the proposed uses of RONOZYME® HiPhos, a 6-phytase product for use in poultry, swine and fish feeds. The Expert Panel convened via telephone conference call on November 21, 2011,and unanimously concluded that RONOZYME® HiPhos, produced consistent with current good manufacturing practice (cGMP) and meeting appropriate specifications, is safe for its intended uses in poultry, swine and fish feeds. The Expert Panel further concluded that these intended uses are GRAS based on scientific procedures. It is also the opinion of this Expert Panel that other qualified experts would concur with these conclusions.

The swine and fish applications will be submitted separately.

1.1 Name and Address of Notifier

DSM Nutritional Products
45 Waterview Blvd.
Parsippany, New Jersey, 07054, USA
Tel:973-257-8500

Person responsible for the dossier:

Alberto Davidovich, DVM, Ph.D.
45 Waterview Boulevard
Parsippany, New Jersey 07054
Tel:973-257-8325

1.2 Name and Address of Manufacturer

Novozymes A/S
(b) (4)

Novozymes A/S
(b) (4)

Novozymes A/S
(b) (4)

(b) (4)

1.3 Name and Address of the Exclusive Distributor

DSM Nutritional Products
45 Waterview Blvd.
Parsippany, New Jersey, 07054, USA
Tel: 973-257-8500

1.4 Common or Usual Name of the Substance

DSM's phytase enzyme preparation is obtained from a Genetically Engineered strain of *Aspergillus oryzae* produced by (b) (4) fermentation. The common or usual name of the substance is "phytase". It is produced and sold in three forms; a liquid, a micro-granulate and a thermo-tolerant granulate. The trade name of the enzyme is RONOZYME® HiPhos.

1.5 Applicable Condition of Use

RONOZYME® HiPhos will be included in animal feeds of poultry for the nutritional purpose of increasing the digestibility of phytate. The recommended use level of RONOZYME® HiPhos is 250 FYT to 4000 FYT/Kg of poultry feed; where one FYT is the amount of enzyme that releases 1 micro mol of inorganic phosphorous from phytate per minute at 37°C and pH 6.5.

1.6 AAFCO Definition O.P. 2011 (Table 30.1)

Phytase derived from *Aspergillus niger* variants and *Aspergillus oryzae* variants are permissible as feed ingredients in swine and poultry diets. See reference 1.

Phytase	*Aspergillus niger,* var. *Aspergillus oryzae,* var.	Corn, soybean meal, sunflower meal, hominy, tapioca, plant by-products	Hydrolyzes phytate	Increases the digestibility of phytin-bound phosphorus in swine and poultry diets

1.7 Description of ingredient

Three product forms of RONOZYME® HiPhos will be available, two dry forms and a liquid form. RONOZYME® HiPhos (GT) is a granulated thermo-tolerant form with a minimum enzyme activity of 10,000 FYT/gram. RONOZYME® HiPhos (M) is a micro granulated form with a minimum enzyme activity of 50,000 FYT/gram. RONOZYME® HiPhos (L) is an aqueous liquid with a minimum enzyme activity of 20,000 FYT/g. Additional forms may be manufactured with feed grade ingredients if there are additional market needs.

2 Manufacturing Process

2.1 Pre-manufacturing—Description of the genetic engineering work

2.1.1 Description of the Production Strain

Production strain: The fungus *Aspergillus oryzae* expressing a synthetic 6-phytase gene.

Name and place of culture collection: (b) (4) .

Genetic modification: genetically modified.

The taxonomy of *Aspergillus oryzae* is:

Class:	*Eurotiomycetes*
Order:	*Eurotiales*
Family:	*Trichocomaceae*
Genus:	*Aspergillus*
Species:	*oryzae*

2.1.2 Host strain (recipient)

The host strain *Aspergillus oryzae* used for genetic engineering of the production strain was derived from the well-known, original wild type strain *Aspergillus oryzae* IFO4177 (syn. A1560). *Aspergillus oryzae* IFO4177 (synonym A1560) was genetically modified by means of site-directed disruption to cause inactivation of the (b) (4) aiming at preventing the expression of these enzymatic side activities. Only homologous gene manipulations exclusively using DNA from *Aspergillus oryzae* have been applied.

By means of classical mutagenesis aiming at the reduction of secondary metabolite formation capacity, a mutant deficient in ability to produce (b) (4) and (b) (4) was isolated. The mutant was termed BECh2.

Aspergillus oryzae BECh2 was genetically modified by means of (b) (4) leading to the host strain (b) (4).

The host strain, A. oryzae (b) (4), was constructed from A1560 through the following steps:

(b) (4)

(b) (4)

2.1.3 Gene template donor

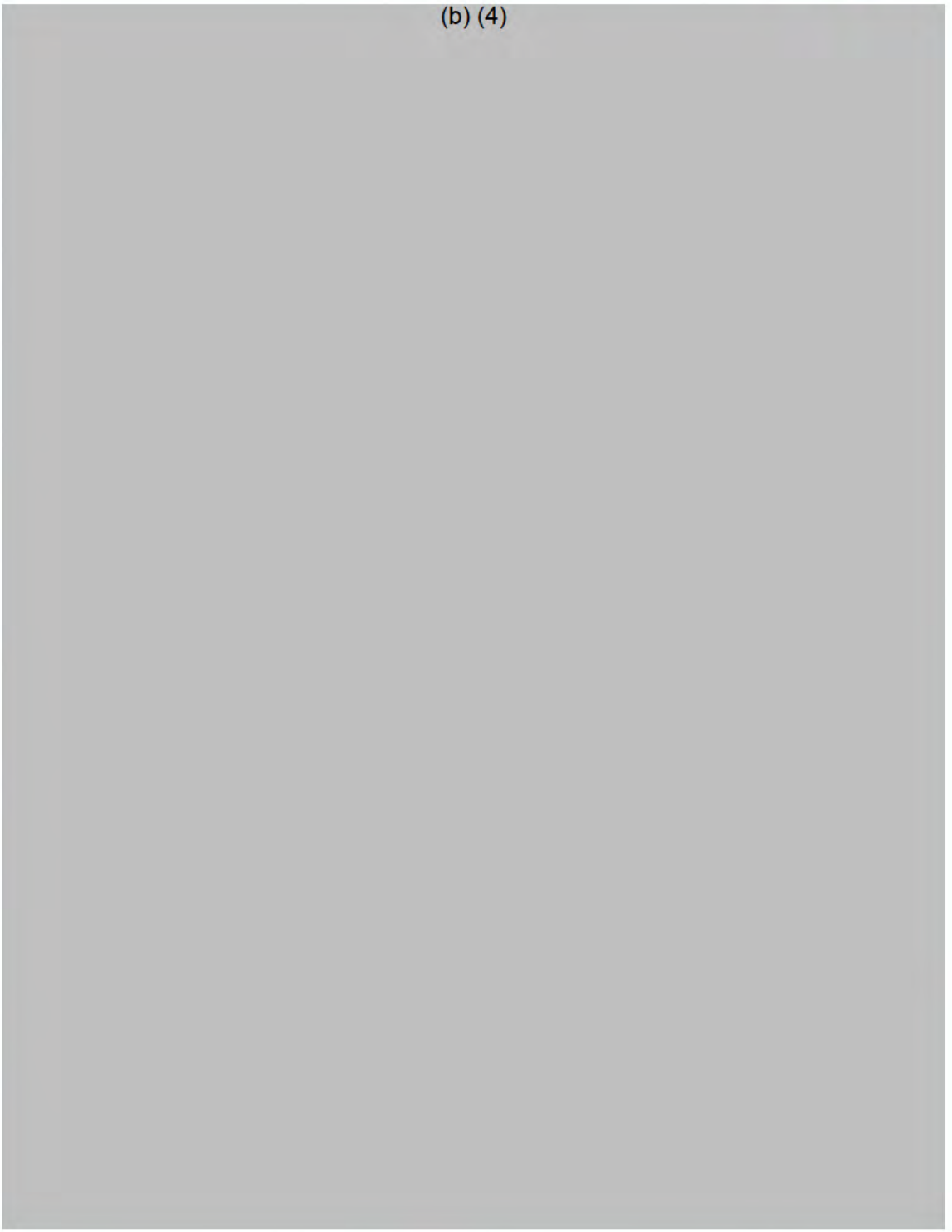
A phytase from the strain *Citrobacter braakii* ATCC51113 was characterized. The phytase gene from that strain was cloned and sequenced and from the DNA sequence, the protein sequence was deduced.

From the protein sequence, two synthetic genes, Cb-Phyt#1 and C-Phyt#4 were designed in silico from principles that would lead to optimal expression in *Aspergillus oryzae*. The two genes code for identical enzymes.

The two synthetic genes were subcloned in two expression plasmids named (b) (4) and (b) (4) respectively.

The two genes were resequenced and demonstrated to encode the same protein sequence. The protein sequence is shown below and aligned to the protein sequence deduced from the DNA sequence of the cloned gene from *Citrobacter braakii* ATCC51113 (Citrobacter phytase). The alignment is shown below.The protein sequence was confirmed experimentally by N-terminal sequencing (showing a start sequence of (b) (4) ;) Annex 40 and intact molecule mass spectrometry.

(b) (4)



The signal and prosequences are boxed. They are cleaved off when the protein is secreted to the medium so that the mature phytase starts with the sequence (b) (4) and the protein encoded by both (b) (4) and (b) (4) are identical with the protein sequence of the protein encoded by the gene cloned from Citrobacter (Citrobacter phytase).
The sequence of the mature phytase in 3 letter code is shown below.

(b) (4)

Glycosylation:

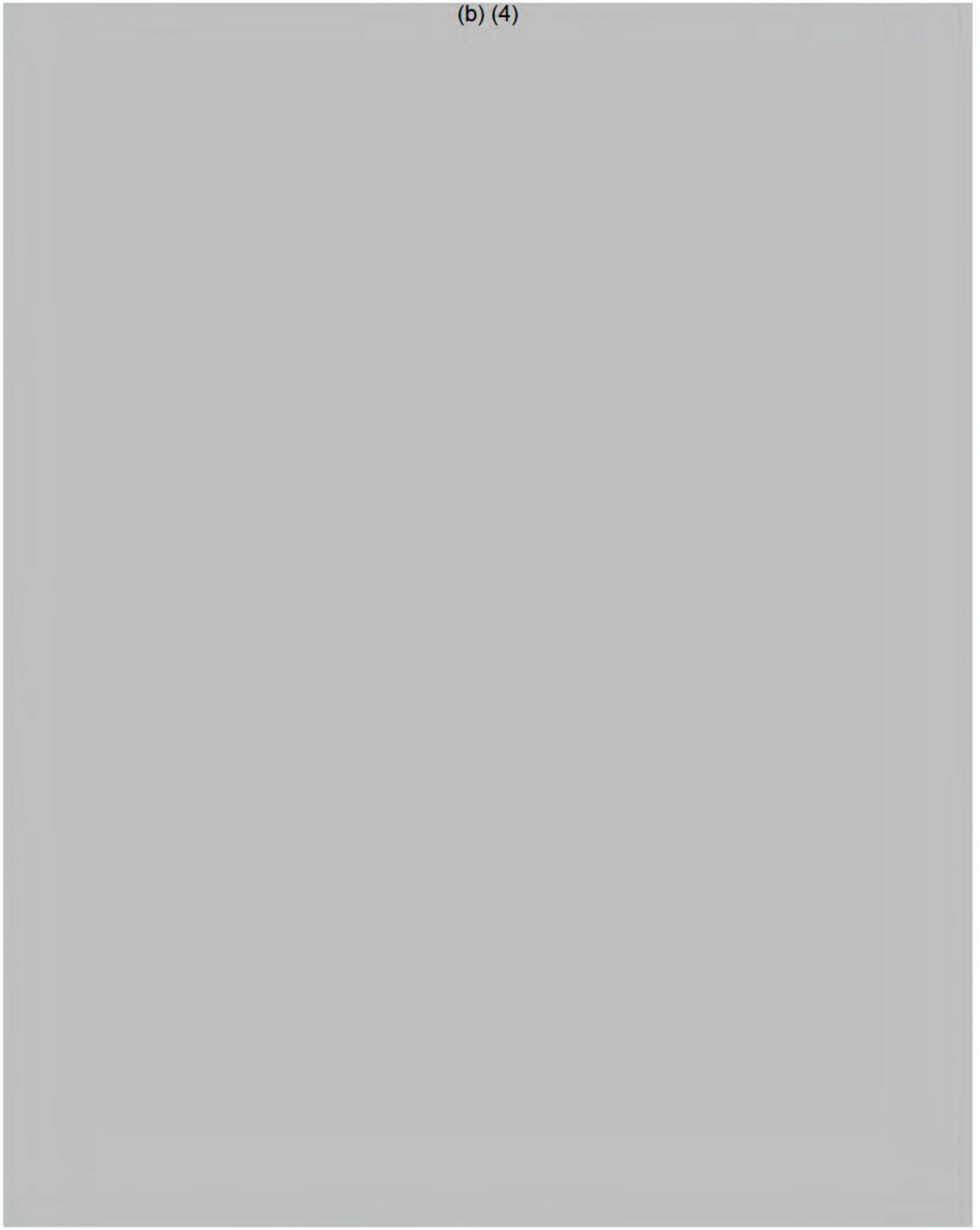
It is well known that *Aspergillus oryzae* can produce proteins that are N-glycosylated with the high-mannose type glycosylation. The protein sequence of the 6-phytase from *Citrobacter braakii* ATCC 51113 contains (b) putative N-glycosylated sites with the sequon "Asn-X-Ser/Thr": (b) (4) . However, only (b) and (b) (4) seem to be fully glycosylated. All of the work described in the dossier including all of safety and efficacy studies have been done using the phytase which is in a glycosylated form. Any effects from glycosylation would be shown in the results of the studies.

Structure of the expression vectors:

(b) (4)

(b) (4)

(b) (4)



(b) (4)

2.1.4 Description of Plasmid Expression Vectors

The expression construct carrying one phytase gene (b) (4) is based on the replication origin of the *E. coli* standard vector pUC19.
The expression construct carrying the other phytase gene (b) (4) is based on the replication origin of the *E. coli* standard vector pUC19.

None of these genetic elements are likely to raise safety concerns, because they do not encode any toxic or otherwise harmful substances. The intended nucleotide sequence of the vectors, fully revealed in the same document, was experimentally confirmed by re-sequencing.

2.1.5 Construction of the production strain *Aspergillus oryzae*

(b) (4)

2.2 Insert copies, genetic stability & genetic transfer capability

The plasmid DNA coding for phytase is integrated into the production strain chromosome in multiple copies (b) (4) for RONOZYME® HiPhos). Such transformants are generally mitotically stable.

The genetic stability was further assessed by Southern blot analysis. The approach consisted in comparing DNA prepared from a vial of the master cell bank of the strain to DNA from mycelium isolated after 4 plant fermentations (referred to as EOP mycelium – pilot batches HKF07, HKF 08 and HKF10). DNA was prepared from this strain material and the integration pattern of the phytase expression plasmids (b) (4) and (b) (4) was investigated by Southern blot analysis using the synthetic phytase genes Cb-Phyt#1 and Cb-Phyt#4 present on (b) (4) and (b) (4) respectively as probes. A negative control was also included in the test.

Result:
The band patterns of the DNA from all of the EOP mycelium and the master cell bank vial were identical.

Therefore, it is considered that the genetic modification is stable. See Annex 5.

Genetic transfer capability

Because the gene insert is chromosomally integrated in the production strain, it is poorly transferred to other organisms. The inserted recombinant DNA is also genetically stable during fermentation. Therefore, no increase of transferability is expected.

No antibiotic resistance marker genes or other sequences presenting safety concerns have been introduced into the organism with the recombinant DNA.

Genetic transfer capability of the recombinant DNA is thus low and of no practical relevance.

2.3 Antibiotic production

Out of the secondary metabolites reported for the species *Aspergillus oryzae*, violacetin would qualify as an antibiotic substance. Generation of antimicrobial activity has, however, not been observed for strains of the *Aspergillus oryzae* strain line derived from strain IFO 4177 (syn. A1560), of which the present *Aspergillus oryzae* production strain is a member.

No gene coding for antibiotic production has been added in the construction of the genetically modified production strain *Aspergillus oryzae*. It can therefore be assumed that the *Aspergillus oryzae* production strain is lacking the capability of antibiotic production.

This has been confirmed by the analysis of three batches of RONOZYME® HiPhos with (b) (4) . Culture plates of the organisms were challenged with paper discs of dilute enzyme broth and inhibition zones were measured vs. antibiotic controls. No antibiotic production could be detected according to the methodology referred to FAO/WHO (1992). See Annex 1 and Annex 3.

2.4 Antibiotic resistance

Antibiotic resistance markers have been present in some of the constructs used for DNA manipulations of the host strain. In all cases where they have been used, the transformation and selection systems have been designed in such a way that the antibiotic resistance markers were either not taken up in the first place or they were deleted again from the host cell pedigree in a later stage. All steps of the host strain development have been analyzed with Southern blot analysis and have confirmed that no antibiotic resistance markers (b) (4)) are present in the host strain (b) (4) as demonstrated in Annex 4. No antibiotic resistance markers are present on the expression plasmids. No antibiotic resistance genes have been added to the production strain through them.

2.5 Absence of the production micro-organism in the RONOZYME® HiPhos phytase products

Absence of the production organism is part of the purity criteria for all formulations of RONOZYME® HiPhos. Absence of the production micro organism has been shown on three lots of the product with a detection limit of 10 CFU/g or ml. See Annex 1.

2.6 Manufacturing Process

The manufacturing process is composed of the following steps: fermentation, purification, formulation, and finally quality control of the finished product. The process is described below.

2.7 Raw Materials Specifications

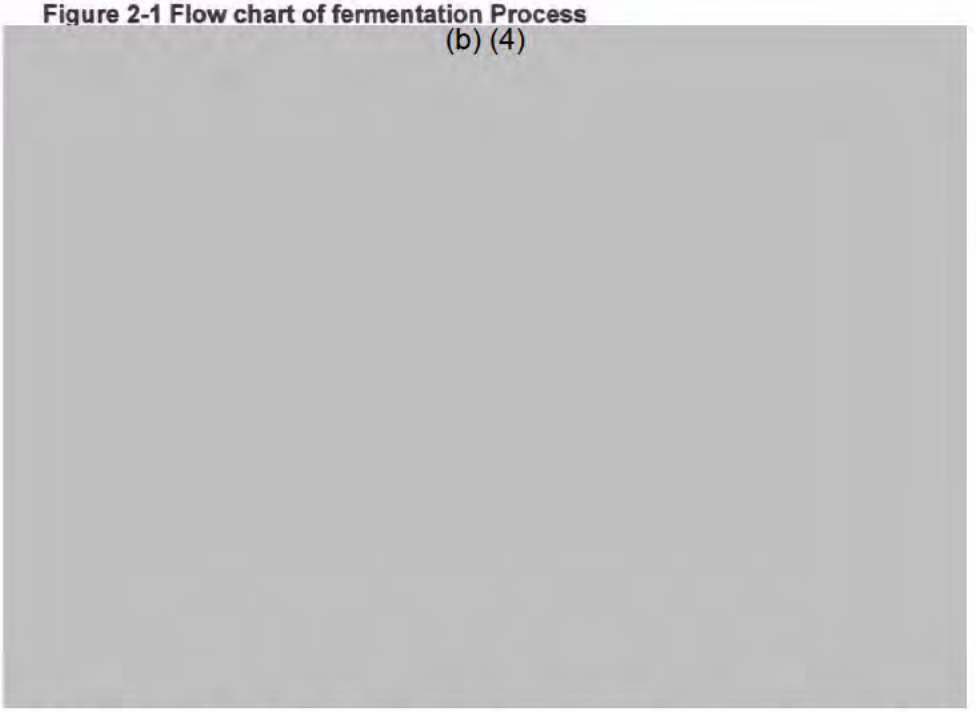
The raw materials are food or feed grade quality and have been subjected to appropriate analysis to ensure their conformity with the specifications.

A list of raw materials used in the manufacturing process and their specifications are provided in Annex 8. For the kaolin used in the granular formulation, absence of dioxin contamination is ensured by sourcing from non-contaminated deposits only as stated by Novozymes A/S in Annex 1.

2.8 The fermentation process

Figure 2-1 Flow chart of fermentation Process

(b) (4)



All equipment is carefully designed, constructed, operated, cleaned and maintained in order to prevent contamination by foreign micro-organisms. The raw materials of the fermentation medium are food or animal feed grade quality and are controlled for conformity with specifications. All media are effectively sterilized by the use of steam.
The seed culture is maintained in a master cell bank system, which ensures its consistency and uniformity for all production batches. Each new batch of production organism stock culture is controlled for identity, viable count, absence of contamination, and the ability to generate the 6-phytase enzyme.

The fermentation is initiated by inoculation of a shaking flask with the stock culture. It is controlled by temperature, agitation, aeration, pressure, pH and feed addition. Refractive index (RI), enzyme activity and microbiological purity are controlled at regular intervals during the process and before harvest. In the event of contamination, the process is terminated.

2.9 The purification process

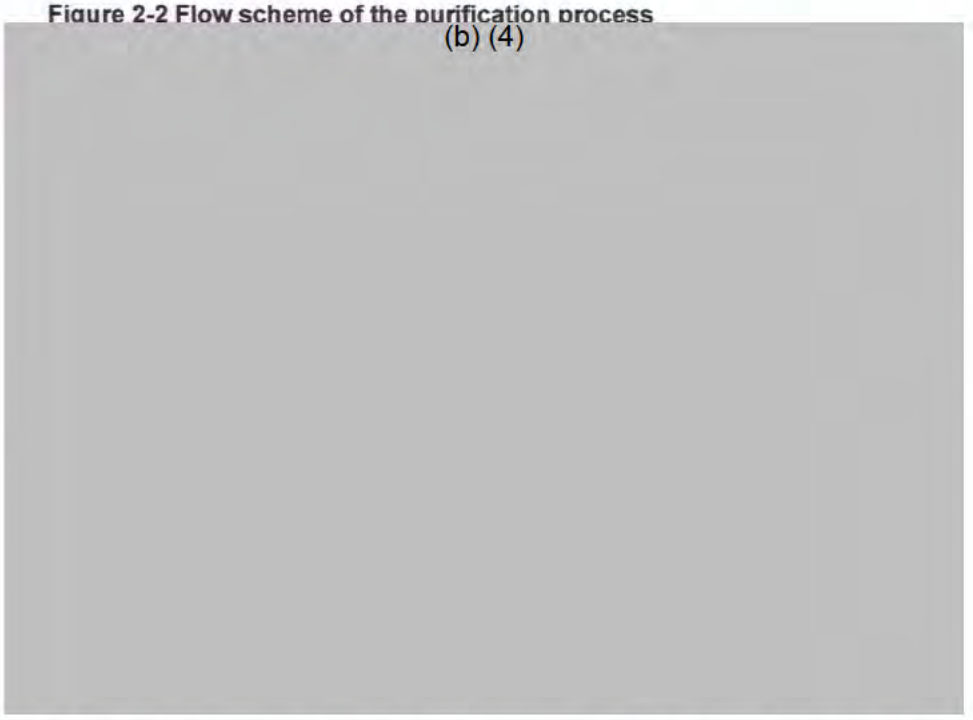
The purification is a multi-step operation designed to separate the phytase from the microbial biomass and partially purify, concentrate and stabilize the enzyme.

The process involves a series of unit operations:

- Pre-treatment
- Primary separation
- Concentration
- Preservation and stabilization.
- Pre and micro filtrations (if needed)

The following flow chart describes the purification process:

Figure 2-2 Flow scheme of the purification process



After pre-treatment with acids or bases and with flocculation agents (all of food or feed grade quality), the broth is separated from the cell mass by well-established techniques such as (b) (4) . The liquid is further purified and concentrated by (b) (4) and/ or (b) (4) . For removal of residual production strain cells

and as a general precaution against microbial degradation, a micro filtration is applied. Samples are checked after the micro filtration for total viable count, and final product samples are checked according to the product specifications. Preservatives and stabilizers are added to the concentrate immediately after the micro filtration in order to prevent microbial degradation.

2.10 The formulation process

M form

The stabilized concentrate is granulated with carbohydrate binder, dried and coated with kaolin and (b) (4) in order to obtain a dust free solid form. The final product is analyzed according to the specification. The next figure represents the granulation process.

Figure 2-3 Flow scheme of the granulation process (M form)

(b) (4)

GT form

The stabilized concentrate is granulated with carbohydrate binder and dried. A (b) (4) coating is then applied in a (b) (4), in order to obtain a dust free solid form. The final product is analyzed according to the specification. The next Figure represents the granulation process.

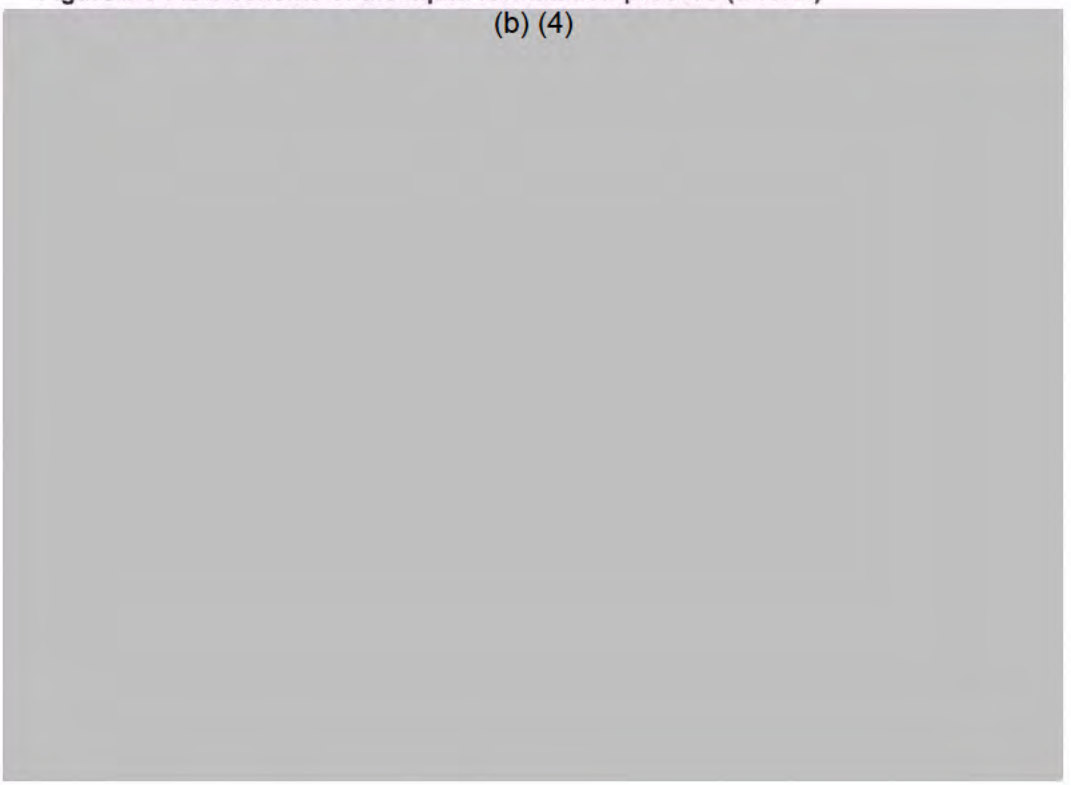
Figure 2-4 Granulation of RONOZYME® HiPhos (GT)

Liquid form

The stabilized liquid concentrate is blended with (b) (4) .

Figure 2-5 Flow scheme of the liquid formulation process (L-form)

(b) (4)



2.11 Methods used to control the product specifications

Test	Method	Dossier Annex
Heavy metals	UT.015Aa ver. 1.4	10
Lead	UT.015Aa ver. 1.7	10
Arsenic	UT.015Aa ver. 1.7	10
Total viable count	EB-SM-3001.02	11
Total coliforms	EB-SM-3091.02	12
Enteropathogenic *E. Coli*	EB-SM-3007.02	13
Salmonella sp.	EB-SM-3009.02	14
Production strain	EB-SM- 3000	15

Description of the methods for routine control of the active substance in premixtures and feed

Activity (FYT/g) as is and in feeds	PHY-101/05E	Annex 7
Activity (FYT/g) in premixtures	PHY-102/05E	Annex 16

The method PHY-101/05E is used to determine phytase activity in feed as well as in per se product samples. The method is not selective with respect to the origin of the phytase. Due to an effective extraction process and the nature of the known phytases all enzyme activity is recovered from organic matter. This method can be used in the feed industry to ascertain the phytase inclusion in feeds. This method was evaluated and validated by a multi-laboratory repeatability study. It was subsequently submitted on behalf of the FEFANA (European Association of Feed Additive Manufacturers) to become a CEN (European Committee for Standardisation) and an ISO standard under the number ISO 30024.

The method PHY-102/05E is used to determine phytase activity in premixtures. The method is similar to the method PHY 101/05E and differs only in the premix-adapted extraction procedure compared to method PHY-101/05E.

2.12 Quality control procedure scheme

It is the policy of DSM Nutritional Products Ltd. and Novozymes A/S that all enzyme preparations must conform to the purity criteria for enzyme preparations set up by the Food Chemicals Codex (FCC) Ref. 21 and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) Ref. 22. In the description of the impurity profile, it was shown that these criteria are met.

Novozymes A/S and DSM Nutritional Products Ltd. partners co-developed RONOZYME® HiPhos. Novozymes A/S is responsible for the manufacturing of the product (supplier) while DSM Nutritional Products Ltd. markets the product worldwide.

Some of the control processes are described below:

1/ Phytase activity: Novozymes A/S controls every production batch according to method EB-SM-0744.02. See Annex 6.
In order to secure that the product contains at least the declared activity at the end of the claimed RONOZYME® HiPhos shelf-life, the target activity of the product during production is higher than the declared activity for the product form, at least to the extent necessary to make up for the expected loss of enzymatic activity during storage, based on the stability data set. Any batch which does not meet the activity target (after analysis) is not released.

2/ Heavy metals, Pb and As: absence of unwanted metals is secured by selection of raw materials and suppliers meaning that Novozymes A/S use raw materials of human food or animal feed grade with defined maximum levels of these metals.

3/ Total viable count, Total coliforms, *E.coli, Salmonella sp.*:

Every batch is analysed in this respect.

Compliance of each batch to this set of tests confirms that the material recovered from the fermentation broth is the HiPhos phytase enzyme. This method of assurance is that same technique utilized by DSM / Novozymes for all the other enzymes they currently market that have been accepted for use in animal feed by the American Association of Feed Control Officials (AAFCO) and consequently have been reviewed by the Center for Veterinary Medicine (CVM).

In addition since 28 March 2005 Quality System at Novozymes A/S is covered by the FAMI-QS (Feed Additives and Premixtures Quality System) certificate. In January 2007, the Standing Committee on the Food Chain and Animal Health has formally adopted the Community Guide to Good Practice for Feed Additive and Premixture Operators – FAMI-QS.

3 Enzyme identity

3.1 Phytases

"The majority of phosphate in plant raw materials used as feed for monogastric animals is stored as phytate, the salt of phytic acid (myo-inositol hexakis dihydrogen phosphate or $InsP_6$), which presents a pool of almost indigestible phosphate to the monogastric animal. The action of a phytase on $InsP_6$ renders free phosphate to be utilized by the animal and at the same time the anti nutritional effect of $InsP_6$ is lowered when the molecule is de-phosphorylated to form inositol phosphates with less than six phosphate groups ($InsP_5$ – $InsP_1$).

Since the beginning of the 20th century when phytases were first discovered (Suzuki et al., 1907), phytases have been identified in numerous organisms including plants, microorganisms and animal tissues. Phytases belong to the enzyme class of phosphoric monoester hydrolases (EC 3.1.3) and are distinguished from other phosphatases by their ability to degrade $InsP_6$. Phytases may also degrade $InsP_5$ $InsP_4$, $InsP_3$, $InsP_2$, and $InsP_1$ even though $InsP_1$ is often accumulated as the end product rather than free myo-inositol (Tomlinson & Ballou, 1962; Wyss et al., 1999; Lassen et al., 2001). Based on their preference for the first phosphate group to be attacked, phytases are divided into 3-phytases (EC 3.1.3.8), 6-phytases (EC 3.1.3.26) and 5-phytases (EC 3.1.3.71). 3-phytases start hydrolysis of $InsP_6$ at the D-3 (L-1) position whereas 6-phytases start hydrolysis at the D-4 (L-6) position and 5-phytases start at position DL-5. The detailed degradation pathway of $InsP_6$ in vitro has been described for a number of different phytases including those from E. coli (Greiner et al., 2000), Saccharomyces cerevisiae (Greiner et al., 2001), Paramecium (Van der Kaay & Van Haastert, 1995) and wheat bran (Nakano et al., 2000). Such studies have demonstrated that phytases within e.g. the 6-phytase group may have different pathways for the continued degradation of the resulting $InsP_5$ molecule. Furthermore, it is also evident that phytases seldom have only one hydrolysis pathway, but rather conduct $InsP_6$ hydrolysis by one main and several alternative pathways (Greiner et al, 2001 and 2006). In this study we describe the $InsP_6$ degradation pathways of a phytase originating from the enterobacteria Citrobacter braakii under laboratory conditions (in vitro) as well as in the gastrointestinal tract of piglets (in vivo). "
(Pontoppidan et. al. 2012) Ref. 31

3.2 Ronozyme HiPhos

The enzyme activity in RONOZYME® HiPhos is that of a 6-phytase which catalyzes the reaction:

myo-inositol hexakisphosphate + H2O => 1L-myo-inositol 1,2,3,4,5 –pentakisphosphate + phosphate

as well as the hydrolysis of lower inositol phosphates.

From phytate-degradation studies, RONOZYME® HiPhos has been shown to have preference for the 6-position of the phytate molecule for the first hydrolysis step and it can therefore be classified as a 6-phytase (EC 3.1.3.26). The phytases from *Peniophora lycii* and *Escherichia coli* are also 6-phytases. Like the phytase from *Citrobacter braakii* they are histidine acid phosphatases (HAP) sharing the active site motif, RHGXRXP (Mullaney and Ullah, 2003) Ref. 32
Common name: Phytase

Generic name: Phosphoric monoester hydrolase

IUBMB nomenclature: 6-phytase

IUBMB number: 3.1.3.26

CAS number: 9001-89-2 (phosphatase, phytate 6-)

EINECS No: 232-630-9 (phosphatase, acid)

The 6-phytase is expressed in a genetically engineered *Aspergillus oryzae* production strain collection (b) (4). The host strain is developed from a strain line, which has been used in production at Novozymes A/S for more than 40 years.

4 Compositional analysis and specifications

The phytase formulation will be available in the following standard formulations:

RONOZYME® HiPhos (GT) (Thermo-tolerant granulate)
RONOZYME® HiPhos (M) (Micro granulate)
RONOZYME® HiPhos (L) (aqueous liquid)

The typical quantitative composition of the (GT), (M) and (L) forms are as follows:

Table 4-1 Composition of RONOZYME® HiPhos (GT), (M) and (L)

Components (%)	RONOZYME® HiPhos (GT)	RONOZYME® HiPhos (M)	RONOZYME® HiPhos (L)
	(b) (4)		

The parameter Total Organic Solids (TOS) is a means of standardizing the quantity of material derived from the enzyme source in order to assess its toxicological significance. TOS is defined as the sum of the organic compounds, excluding diluents, e.g. (b) (4) contained in the final enzyme preparation. The definition and use of TOS in safety assessments of enzymes is described in the publication of Pariza and Foster 1983 (Ref. 4)

Given that the recommended inclusion rate of the product into animal feeds is in the order of 0.825 mg TOS/kg body weight-day and the established safety of the enzyme (NOAEL of 860 mg TOS/kg body weight-day), as determined by the 13-week rat study, both granular and liquid products forms are considered safe.

The phytase in RONOZYME® HiPhos is produced from a non-pathogenic microbial source derived from a safe strain line for which the expression of residual mycotoxin-forming capacity is effectively

prevented. It is produced by methods and under culture conditions that ensure controlled fermentation. The introduction of contaminating micro organisms is therefore prevented. Furthermore, the production strain is absent in the final enzyme preparations. More details are provided in other sections of this document.

This enzyme preparation complies with the purity criteria recommended for enzyme preparations in the Food Chemicals Codex (FCC- Ref. 21), third supplement to the 7th edition, 2010 and also conforms to the "General specifications and considerations for enzyme preparations used in food processing" as recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), published in FAO Food and Nutrition Paper No. 52, 2001 (Ref. 22).

In addition, the compositional analysis of all 3 batches of the enzyme has been carried out. The analysis demonstrates consistent quality of the enzyme and compliance with the specifications as seen in Annex 1. A summary of purity specifications is shown below.

The final product forms of RONOZYME® HiPhos conform to the following purity specifications:

Table 4-2 Product specifications

Test	Method*	Limit
Phytase activity [0)] in M form Phytase activity [0)] in GT form Phytase activity [0)] in L form	Method 0744.02	>50,000 FYT/g >10,000 FYT/g >20,000 FYT/g
Heavy metals	UT.015Aa ver. 1.4	Not more than 30 ppm
Lead	UT.015Aa ver. 1.7	Not more than 5 ppm
Arsenic	UT.015Aa ver. 1.7	Not more than 3 ppm
Total viable count	EB-SM-3001.02	Not more than 50,000/g
Total coliforms	EB-SM-3091.02	Not more than 30 /g
E. Coli	EB-SM-3007.02	Not detected in 25 g
Salmonella sp.	EB-SM-3009.02	Not detected in 25 g

As mentioned previously in this document, the production strain does not have the capacity to form any toxic secondary metabolites under normal fermention conditions that could possibly be linked to the original *Aspergillus* oryzae wild type strain IFO4177. Elimination or minimization of potential toxin expression by enzyme production strains is an important development objective for Novozymes A/S. Early in the development of the production strain the aflatoxin gene cluster was removed. Intermediate strains deficient in CAP production were obtained. In a further round of classical mutagenesis another strain with severely compromised capacity to produce [(b) (4)] was developed (strain BECh2). Media composition also affects secondary metabolite production and some (b) (4) production was still seen with YES and Nakamura media, which differ significantly in their composition from the phytase production medium. Weak [(b) (4)] formation was seen only under very specific conditions and only in Nakamura and Raulin Thom media, which also differ substantially in their compositions from media used in phytase production.

Tests performed with RONOZYME® HiPhos have shown that those substances are not detected (below limit of quantification), see Annex 1. Any mycotoxin contamination of the phytase sales products (such as RONOZYME® HiPhos) arising from the production strain is therefore effectively excluded.

4.1 Analytical methods

- **Phytase activity as declared in units (FYT) per gram of product**

The method EB-SM-0744.02 is used as the standard method for product release of all RONOZYME® HiPhos formulations, at Novozymes A/S. It is run using a robot, designated (b) (4) as an indirect method, i.e. including a validated phytase standard for comparison. Apart from the enzyme standard, the method itself is the same as used for all other RONOZYME® phytase products marketed by Novozymes A/S and DSM Nutritional Products Ltd.

Phytase reacts with sodium phytate (phytic acid dodeca sodium salt $C_6H_6O_{24}P_6Na_{12}$) and releases inorganic phosphate. This phosphate is determined spectrophotometrically from a yellow complex formed by an acidic complex reagent containing molybdate/vanadate. The yellow complex is measured spectrophotometrically at a wavelength of 405 nm.

A true blank is included for each sample to take into account phosphate interference from the samples.

An analytical standard of known phytase activity is run in parallel during each analysis. The phytase activity of the sample is determined by comparison of its colour reaction to the colour value obtained from the analytical standard. The analytical standard was validated under the conditions defining the Phytase Unit (FYT).

One Phytase Unit (FYT) is defined as the amount of enzyme that releases 1 µmol of inorganic phosphate from phytate per minute under reaction conditions with a phytate concentration of 5.0 mM at pH 5.5 and temperature 37°C. For consistency and practical reasons, this phytase unit definition will remain the same for RONOZYME® HiPhos, as it is already used for the phytase products currently marketed. See the report of the method in Annex 6.

Alternatively, phytase activity of the products in FYT can also be determined by the method used at DSM Nutritional Products Ltd. PHY-101/05E, which is based upon the same test principle and activity unit definition. In contrast to the Novozymes method EB-SM-0744.02, method PHY-101/05E is run without the use of a robot and as a direct method, i.e. using an inorganic phosphate standard for comparison. Thus, the use of a phytase analytical standard is dispensable with this analytical setting. See Annex 7.

5 SAFETY EVALUATION

5.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in animal feed (Ref. 29). If the organism is nontoxigenic and nonpathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume (Ref. 28). Pariza and Foster (1983) define a nontoxigenic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances". *A. oryzae* meets these criteria for nontoxigenicity and nonpathogenicity. In addition, *A. oryzae* is not considered pathogenic by JECFA (Ref. 22).

Novozymes A/S has safely used *Aspergillus oryzae* enzyme production strains for over 40 years. The fungus *Aspergillus oryzae* is a species with a century-long history of safe use in food production. *Aspergillus oryzae* is used to produce fermented foods from rice and soya in Asia such as sake, shoyu, miso, and soy sauce. Taking Japan an example, *Aspergillus oryzae* has been used for food production there for over 400 years. *Aspergillus oryzae,* along with *Aspergillus niger* and *Trichoderma reesei,* is one of the most important fungal production organisms in industrial fermentations (Blumenthal 2004- Ref. 2). Food enzymes produced from *Aspergillus oryzae* include amylase, hemicellulase, lipase, oxireductase, protease, and pectin esterase. The food and industrial applications are described and listed in more detail in the proceedings of a 2004 seminar on *Aspergillus oryzae* (Tsukuba 2004- Ref. 3), as well as by Pariza and Foster (1983- Ref. 4) and Pariza and Johnson (2001- Ref. 5). Safety assessments for food enzymes from *Aspergillus oryzae* strains used by Novozymes A/S have been published (Greenough et *al.* 1996- Ref. 6, Barbesgaard et. al., 1992- Ref. 7 and Jorgensen 2007 – Ref. 8).

An evaluation of the genetically modified production microorganism for the phytase, embodying the concepts initially outlined by Pariza and Foster, 1983(Ref. 4) and further developed by IFBC in 1990 (Ref. 28), the EU SCF in 1991, the OECD in 1992, ILSI Europe Novel Food Task Force in 1996, FAO/WHO in 1996, JECFA in 1998 ,Pariza and Johnson, 2001 (Ref. 5) and finally Pariza and Cook 2010 (Ref. 29) demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the host strain, a description of the incorporated DNA, the sources and functions of the introduced genetic material, an outline of the genetic construction of the production strain, and some characteristics of the production strain and the enzyme derived from it are described.

Because the genetic modifications are well characterized and specific, and the incorporated DNA does not encode and express any known harmful or toxic substances, the enzyme preparation derived from the genetically modified *A.oryzae* is considered safe (Ref. 4, 30).

Prior reports of an extended history of safe industrial use
Aspergillus oryzae is used commercially today for the production of phytase, alpha-amylase, xylanase, hemicellulase, lipase, oxireductase, protease, pectinmethylesterase, laccase, glucoseoxidase, aminopeptidase, beta-glucanase, asparaginase, catalase, and phospholipase.

5.2 Safe strain lineage

The *Aspergillus oryzae production* strain was developed from the host strain (b) (4) which was derived from *Aspergillus oryzae* strain BECh2 (developed as a strain platform for the genetic construction of commercial food and feed enzymes producing strains). The *Aspergillus oryzae* strain BECh2 was itself derived from the wild type *Aspergillus oryzae* strain IFO 4177 (synonym A 1560).

Novozymes A/S has used *Aspergillus oryzae* production strains for over 40 years. A line of *Aspergillus oryzae* host strains, including BECh2, have previously been used as host strains for Novozymes' food and feed enzyme products. These production strains were constructed by standard transformation procedures using well-known plasmid vectors and well-characterized DNA sequences that were integrated into the *Aspergillus oryzae* host strain chromosome. Toxicological testing, confirming the safety of enzyme preparations derived from these *Aspergillus oryzae* production strains, was performed. No toxicological effects were observed for any of the test substances produced by strains derived from this *Aspergillus oryzae* lineage of host strains (Greenough et *al.* 1996- Ref.6 and Barbesgaard, 1991 Ref. 7).

Novozymes A/S has previously published safety studies on two products produced from *Aspergillus oryzae* strains developed from *Aspergillus oryzae* A1560 (Greenough et *al.* 1996) Ref.6. The safety of the enzyme products mentioned in the following table have been assessed according to the principles of the SCF guidelines and the products were approved in a wide range of countries (e.g. Australia, Canada, Denmark, France, the European Community (6-phytase and xylanase producing strains) or the subject of GRAS Notices or other reviews in the USA). GRAS Notices 8, 34, 43, 75, 90, 103, 106, 113, 122, 142, and 201; see References 9 – 19. The table demonstrates that *Aspergillus oryzae* BECh2 was used as the host strain in the construction of Novozymes' production strains for (b) (4) and the currently globally marketed RONOZYME® P and RONOZYME® NP *Peniophora lycii* 6-phytase. The latter are approved in the EU as feed additives and marketed under the trade names RONOZYME® P 5000 (CT), RONOZYME® P 20,000 (L) and RONOZYME® NP (CT), RONOZYME® NP (M) and RONOZYME® NP (L); respectively, see (http://ec.europa.eu/food/food/animalnutrition/feedadditives/comm_register_feed_additives_1831-03.pdf). Safety and efficacy of Ronozyme® HiPhos has been evaluated and received a positive opinion by the European Food Safety Authority (EFSA), see http://www.efsa.europa.eu/en/efsajournal/doc/2730.pdf and http://www.efsa.europa.eu/en/efsajournal/doc/2527.pdf.

Aspergillus oryzae strains are considered to be exempt from premarket notification under Section 5 of the Toxic Substance Control Act based upon a final risk assessment performed by the Environmental Protection Agency in 1997, Ref. 20.

Novozymes' food and feed enzymes derived from the same *A.oryzae* strain lineage

Enzyme	IUB no	Host strain	Donor strain	Safety studies[a]	Current use
Triacylglycerol lipase	(b) (4)		*Thermomyces lanuginosus*	Yes	Food
Mucorpepsin			*Rhizomucor miehei*	Yes	Food
Triacylglycerol lipase			*Rhizomucor miehei*	Yes	Food
Xylanase			*Thermomyces lanuginosus*	Yes	Feed
Triacylglycerol lipase			*Fusarium oxysporum*	Yes	Food
6-Phytase			*Peniophora lycii*	Yes	Feed
Triacylglycerol lipase			*Thermomyces lanuginosus*/ *Fusarium oxysporum*	Yes	Food
6-Phytase			*Peniophora lycii*	Yes	Feed
Xylanase			*Aspergillus aculeatus*	Yes	Food
Xylanase			*Thermomyces lanuginosus*	Yes	Food
Glucose oxidase			*Aspergillus niger*	Yes	Food
Phospholipase			*Fusarium venenatum*	Yes	Food
Asparaginase			*Aspergillus oryzae*	Yes	Food

a) At least the following: 13 week acute oral toxicity in rats; Ames test; In vitro human lymphocyte cytogenetic assay

(b) (4)

Safety studies, including a 13-week oral toxicity in rats, Ames bacterial mutagenesis test, and human lymphocyte cytogenetic assay, were performed on those preparations. In all these studies the conclusions were that oral administration to rats of the highest possible dosage level for 13 weeks did not reveal any signs of toxic effects related to treatment. No mutagenic activity was found in any of the test substances by the Ames' test or the human lymphocyte test.

These studies support the view that strains derived from *Aspergillus oryzae* (b) (4) can be used safely for the production of food and feed enzymes. Accordingly, production strains which are constructed from the host strain, *Aspergillus oryzae* (b) (4) where the genetic modifications are well characterized and specific, utilizing well-known plasmids, and for which the introduced genetic material does not encode for the expression of any known harmful or toxic substances, constitutes a safe strain lineage according to the outline by Pariza and Johnson 2001 (Ref. 5). The decision tree is in Annex 39.

The strain designed to produce the HiPhos phytase subject to this application, has been developed from a host strain derived from BeCh2.

It is concluded that the *Aspergillus oryzae* strains used for expression of a *Citrobacter braakii* 6-phytase, via a synthetic gene, is a member of the same safe strain lineage as the strains used to express previous approved phytases.

5.3 Assessment of mycotoxin forming potential of the *Aspergillus oryzae* production strain

Aspergillus oryzae is classified as a non-pathogenic microorganism. However, its relatedness to *Aspergillus flavus* and the potential for toxin production of some of the species included in the Flavi section of Aspergilli deserves a risk assessment on toxin production. It is understood that the domesticated *Aspergillus oryzae* fungus has lost a number of metabolic pathways that seriously compromise the capability to produce the mycotoxins typical for the *Aspergillus flavus* group. (Blumenthal 2004- Ref.2)

One of the major toxins produced by some of the members of this group of Aspergilli is aflatoxin. No aflatoxin has been detected during fermentation of the wild type *Aspergillus oryzae* strain IFO 4177 or derived production strains in the strain lineage.

Minimisation and effective control of the level of any secondary metabolite expressed by enzyme production strains from this lineage is an important development objective for Novozymes A/S. Therefore, a strain mutagenesis and screening program resulted in the identification of a strain, BECh1, that did not produce aflatoxin. Molecular characterization of BECh1 showed that a large deletion resulted in the removal of the genes of the aflatoxin biosynthetic cluster. Thus, BECh1 has lost the potential to produce aflatoxin. BECh1 was subsequently used to develop the production strain of RONOZYME® HiPhos. Therefore, no risk of aflatoxin production is associated with this strain. Consequently, it is not considered necessary to test RONOZYME® HiPhos production batches obtained from this strain for the presence of aflatoxin.

The large deletion present in strain BECh1 includes the genes required for production of the mycotoxin (b) (4) As for aflatoxin, (b) is not normally produced at detectable

levels by the host cells during fermentation. But with the deletion of the genes, the strain is rendered unable to produce (b)

In addition, following a subsequent mutagenesis step, a new strain (BECh2) showed a drastically reduced potential to produce (b) (4) This secondary metabolite is not produced at detectable levels by the cells during fermentation either. The deletion or mutation of the mycotoxins genes further increases the safety of the final production strain.

Apart from aflatoxin, other secondary metabolites reported for the species *Aspergillus oryzae* include violacetin, maltoryzine, (b) (4)

Violacetin production is not expected for the *Aspergillus oryzae* strain line IFO4177, because it has antimicrobial properties, which have not been observed for enzyme concentrates from this strain line. Further the substance is described in the literature as a broad spectrum antibiotic from *Streptomyces sp*. Though once reported allegedly from a strain of *Aspergillus oryzae*, and similarly (b) (4)) violacetin has never been isolated from any other microorganism outside the *Streptomyces* group.

Maltoryzyne was reported only for *Aspergillus oryzae* var. *microsporus*, which is not linked to the present strain line originating from *Aspergillus oryzae* strain IFO4177.
Possible concerns for the original wild type strain IFO4177 arise theoretically for the potential secondary metabolites (b) (4) which are regarded as mycotoxins, whereas the toxicological significance of (b) (4) is more remote.

Minimisation and effective control of the level of any secondary metabolites expressed by enzyme production strains from this lineage is an important development objective for Novozymes A/S. The *Aspergillus oryzae* mutant strain (b) (4) in which the aflatoxin gene cluster was removed, is also deficient in CPA production. In a second round of classical mutagenesis with strain (b) (4) another strain, BECh2, with severely compromised capacity to produce (b) (4) was developed. Strain BECh2 was thoroughly investigated for its potential to form secondary metabolites. It was grown on 5 optimal media known to elicit secondary metabolite formation. The resulting culture extracts were analysed by reversed-phase liquid chromatography with a diode array detector. As expected, neither (b) (4) aflatoxin nor maltoryzine were produced by BECh2 on any medium. Some (b) (4) production was seen with YES and Nakamura media, which differ significantly in their composition from the phytase production medium. As a result of the targeted mutagenesis and screening program, BECh2 produced less (b) (4) than the wild type strain IFO4177. Weak (b) (4) formation was seen only under very specific conditions and only in Nakamura and Raulin Thom media, which differ substantially in their compositions from media used in phytase production.

Consequently, only (b) (4) remained as substances of some, albeit limited, concern for strain BECh2 and production strains derived thereof. Tests performed with RONOZYME® HiPhos have shown that those substances are not detected (below limit of quantification). See Annex 1.

As a conclusion, any mycotoxin contamination of the commercial phytase products (M, L and GT formulations) arising from the production strain is therefore effectively excluded.

5.4 Safety of the Donor Organism

The organism where the 6-phytase was found is *Citrobacter braakii* which belongs to the genus *Citrobacter.* The genus *Citrobacter* was reclassified in the 1990's according to genetic relatedness and *Citrobacter braakii* refers to the genomospecies 6 of the *Citrobacter freundii* complex. *Citrobacter* are gram-negative bacilli which are found in water, soil, and also commensally within the human gastrointestinal tract. Thus, Citrobacter braakii is considered non-pathogenic although it is found as an opportunistic pathogen where other non-pathogenic microorganisms also are found (e.g., immunocompromised patients). It is therefore considered as a hazard group 2 organism.

However, as noted above, the gene was synthesized to mimic the phytase gene from *Citrobacter braakii.* By synthesizing the phytase genes, it is ensured that no genetic material (target gene or other DNA) from the donor organism is found in the production strain (Lichtenberg et al. 2011) Ref. 30.

5.5 Safety of the phytase enzyme

A wide variety of enzymes are used in animal feed and a selection are listed in the AAFCO OP table 30,1(Ref 1). Enzyme proteins do not generally raise safety concerns (Pariza and Foster, Ref. 4) and as noted by the Food and Drug Administration in Lipase Enzyme Preparation From *Rhizopus niveus*: Affirmation of GRAS status as a Direct Food Ingredient. Fed. Regist. 63:24416-24419, 1998. (Ref. 33) Food and Drug Administration. Statement of Policy: Foods Derived From New Plant Varieties. Fed. Regist. 57:22984-23005, 1992. (Ref. 34) Pariza and Foster (Ref. 4) note that very few toxic agents have enzymatic properties. The subject of this GRAS notification is a 6-phytase, EC 3.1.3.26. The safety of this phytase enzyme was confirmed by the toxicological studies published in Lichtenberg et al. 2011(Ref. 30).

5.6 Sequence homology of RONOZYME® HiPhos Phytase to known toxins and allergen as recommended by Joint Committee WHO/ FAO

In order to ensure that RONOZYME® HiPhos phytase is not a homolog to known toxins and allergens a study was performed with UNIPROT database entries (April 15, 2009).

Protein sequences that contain the word toxin in the description field were extracted from the UNIPROT Database; 5087 entries were found.

Each of the sequences was placed in its uniquely named Fasta file. The RONOZYME® HiPhos phytase sequence was placed in a separate file. The awk script was used to invoke the sequence alignment program ClustalW 1.83 to align each sequence to RONOZYME® HiPhos phytase. A summary file containing the length of each sequence and number of identical residues was also created.

From this, the identity percentage to the RONOZYME® HiPhos phytase sequence to the compared toxin sequence was calculated, whichever is longest. This was chosen because the toxin sequences have many different lengths, both much shorter and much longer than the

RONOZYME® HiPhos phytase sequence. By always using the longest sequence, artificial high scores from very short or very long toxins were avoided.

The largest homology encountered was 24.5%, indicating that the homology to any toxin sequence in this database is indeed very low. All available amino acid sequences of the SDAP allergen database (www.http://fermi.utmd.edu/SDAP/index.html) were downloaded.

For the 80 amino acid window search, no matches were found using a strict threshold of 35% identity as suggested in the "Evaluation of Allergenicity of Genetically Modied Foods" Report from the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (2001) - http://www.who.int/foodsafety/publications/biotech/ec jan2001/en/

The present investigation demonstrates that the RONOZYME® HiPhos phytase sequence is not homolog to any toxin of the UNIPROT database. Further details are found in Annex 2.

5.7 Safety of the Manufacturing Process

The phytase meets the purity specifications for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex (Ref. 21). The phytase preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in animal feed, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes (See Section 2).

5.8 Safety Studies

Toxicological tests on mutagenicity, cytogenetic effects and sub chronic toxicity, as well as a feeding safety test in target animals are described below. These are corroborative studies that support literature reports.

In all toxicological studies, carried out in vitro and in vivo, the enzyme concentrate batch was used. A certificate of Analysis is included for this batch in the micronuclei study report at page 40 (see Annex 32). No adverse effects of the test substance were found in these safety tests which are described in detail below. The studies were published in June 2011. Ref. 30

Summary of Safety Studies

Test	Test Object	Dose	Result	Report
Bacterial Mutation (Ames)	*Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 *Escherichia coli* strain WP2uvrApKM101	156 to 5000 µg /ml (plate) 7 to 225 FYT 156 to 5000 µg /ml (plate) 7 to 225 FYT	No significant increase in the revertant numbers No significant increase in the revertant numbers	20088064
Micronucleus Test	Cultured Human Peripheral Blood lymphocyte	3000 to 5000 µg /ml	No significant difference	20086022
13 Week Sub-Chronic Oral Toxicity	Crl: CD® (SD) rats	0, 1, 3.3, 10.0 ml/kg/day or 0; 50,000 ; 165,000 and 500,000 FYT units /Kg/day	no treatment-related macroscopic or histopathological findings	20086016
In vitro Skin Irritation (Episkin)	Episkin Standard Model	10 µl/ tissue or (0.5 FYT/tissue)	Negative	200905296
Acute Eye Irritation	Rabbit	0.11 ml or (5,000 FYT)	Negative	20096001

5.8.1 Genotoxicity studies including mutagenicity

5.8.1.1 Bacterial Mutation Assay (Ames test)

The objective of the study was to evaluate the mutagenic potential of RONOZYME® HiPhos by examining its effects on amino acid "growth dependant" bacteria (reversion effect).

The study was conducted in accordance with OECD Guidelines for testing of chemicals (No. 471, July 1997). The study was conducted in compliance with current GLP practices.

RONOZYME® HiPhos was examined for mutagenic activity in four histidine-dependent strains of *Salmonella typhimurium*, strains TA98, TA100, TA1535 and TA1537, and the tryptophan-dependent strain *Escherichia coli* WP2uvrApKM101.
The study was conducted in the presence and absence of an activating system derived from rat liver (S9 mix). All tests included solvent (purified water) and positive controls with and without S9 mix. All bacterial strains were tested at concentrations of the test article ranging from 156 to 5000 µg per ml (plate).

Results:

No treatments of any of the *Salmonella* and *E.coli* strains with RONOZYME® HiPhos, either in the presence or absence of S-9 mix, resulted in any increases in revertant numbers that meets the criteria for a positive response.

Conclusion:

The results of the experiments gave no indication of mutagenic activity of RONOZYME® HiPhos in either the absence or presence of S9, when tested under the conditions employed in this study. See Annex 31.

5.8.1.2 Micronucleus assay: Induction of micronuclei in cultured human peripheral blood lymphocytes

The objective of this study was to evaluate the clastogenic and aneugenic potential of RONOZYME® HiPhos by examining its effects on the frequency of micronuclei in cultured human peripheral blood lymphocytes treated in the absence and presence of S-9.

The test methodology is based on the current version of draft OECD guideline 487 [10] and accepted scientific/regulatory principles described in current guidelines for clastogenicity testing in vitro. The study was conducted in compliance with current GLP-practices. The highest dose level tested was 5000 μg/ml. (the recommended maximum for in vitro chromosome aberration studies according to current regulatory guidelines).

RONOZYME® HiPhos was added at 48 hours following culture initiation (stimulation by PHA). Cells were exposed to the test article for 3 hours in the absence and presence of S-9 (from rats induced with Aroclor). In addition, a continuous 24 hour treatment (equivalent to approximately 1.5 to 2 times the average generation time of cultured lymphocytes from the panel of donors used in this laboratory) in the absence of S-9 was included. All cultures were sampled 24 hours after the beginning of treatment (i.e. 72 hours after culture initiation).
Appropriate negative (vehicle) control cultures were included in the test system under each treatment condition. The proportion of micronucleated binucleate cells (MNBN) in these cultures fell within current historical vehicle control (normal) ranges.

Mitomycin C (MMC) and Vinblastine (VIN) were employed as clastogenic and aneugenic positive control chemicals respectively in the absence of rat liver S-9.

Cyclophosphamide (CPA) was employed as a clastogenic positive control chemical in the presence of rat liver S-9. Cells receiving these were sampled in the Main Experiment at 24 hours after the start of treatment; all compounds induced statistically significant increases in the proportion of cells with micronuclei.

The assay system was therefore considered as both sensitive and valid.

Results:

Treatment of cells with RONOZYME® HiPhos in the absence and presence of metabolic activation resulted in frequencies of MNBN cells, which were similar to and not significantly ($p \leq 0.05$) different from those observed in concurrent vehicle controls for all concentrations analysed. The MNBN cell frequency of all RONOZYME® HiPhos treated cultures fell within normal ranges.

Conclusion:
It was concluded that RONOZYME® HiPhos did not induce micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of a rat liver metabolic activation system. See Annex 32.

5.8.2 Sub-chronic oral toxicity study in rats

The objective of this study was to assess the systemic toxic potential of RONOZYME® HiPhos in the rat when administered daily by oral gavage over a period of 13 weeks.

The study was carried out in accordance with the OECD guideline 408 (1998). It was conducted in compliance with the requirements of current, international Good Laboratory Practice.

A total of 80 Crl: CD® (SD) rats (40 males and 40 females) were included in the study. The animals were allocated into four groups given each comprising ten male and ten female rats received RONOZYME® HiPhos at doses of 1.0, 3.3 or 10.0 ml/kg/day (10.0 ml/kg/day = 860 mg TOS/kg body weight/day) at a constant dose volume of 10 ml/kg bodyweight. A similarly constituted control group received the vehicle, purified water, at the same volume dose. The test article was administered daily by oral gavage.

During the study, clinical condition, detailed physical and arena observations, sensory reactivity, grip strength, motor activity, bodyweight, food consumption, ophthalmic examination, hematology, and blood chemistry were monitored. At termination of the study, all the animals were sacrified and subjected to a detailed necropsy; organ weight, macro pathology and histopathology.

Results:
There were no deaths during the study and there were no treatment related findings observed during the routine weekly physical examination, the arena observations or during post dosing observations. The functional observation battery investigation did not indicate any treatment related effects.

Slightly higher bodyweight gains were observed during the first two weeks of treatment for males receiving 10.0 ml/kg/day and the first week of treatment for females receiving 10.0 ml/kg/day. Food consumption was not affected by treatment. There were no treatment-related ophthalmic findings. The hematology and blood chemistry investigations did not indicate any toxicologically significant changes. There were no effects upon organ weight and no treatment-related macroscopic or histopathological findings.

Conclusion:
It was concluded that oral administration of RONOZYME® HiPhos to CD rats at doses up to 10.0 ml/kg/day for 13 weeks was well tolerated and did not cause any toxicologically significant change. Consequently, the no-observed-adverse-effect-level (NOAEL) was considered to be 10.0 ml/kg/day, the highest dose level administrated, which was equivalent to 860 mg TOS/kg body weight/day. See Annex 33.

5.8.3 Acute toxicity (skin and eye irritation)

5.8.3.1 Skin irritation test (Episkin in vitro assay for skin irritation)

The objective of the study was to provide data on the irritant effects of RONOZYME® HiPhos.

Introduction
The epidermis model; Episkin Standard Model, hereafter named Episkin has been validated by ECVAM. It is recognized as a stand alone in vitro assay for the assessment of skin irritation of chemicals. It is a three dimensional human epidermis model. Its use for skin irritation testing involves topical application of test materials to the surface of the epidermis and the subsequent assessment of their effects on cell viability.Acceptance criteria and a prediction model have been developed in order to evaluate the results of the assay and allow for a conclusion to be drawn. The results for the negative control (NC) and positive control (PC) of the present test meet the acceptance criteria for a valid test.

Protocol
Adult human derived epidermal keratinocytes are seeded on a dermal substitute consisting of a collagen type 1 matrix coated with type IV collagen. A highly differentiated and stratified epidermis model is obtained after 13 days culture period comprising the main basal, supra basal, spinal and granular layers and a functional stratum corneum. Its use for skin imitation testing involved topical application of RONOZYME® HiPhos to the surface of the epidermis and the subsequent assessment of their effects on cell viability.
RONOZYME® HiPhos was applied topically to the epidermal model for 15 minutes. Exposure was terminated by rinsing with PBS. Inserts were then incubated for 42 hours at 37 Celsius degrees.
Thereafter the viability was assessed by incubating the tissue inserts for 3 hours. Tissues inserts were used as positive and negative controls, respectively. For each treated tissue the viability was expressed as the percentage of the negative control, respectively. Values under 50% will qualify the test substance as irritant.

Results
The mean viability for RONOZYME® HiPhos was 92.3%, which is above 50%, hence it can be concluded that RONOZYME® HiPhos Phytase is not irritating in the present model under the applied conditions. See Annex 38.

Conclusion
RONOZYME® HiPhos is non-classifiable (non-irritant) according to the prediction model based on the viability results.

5.8.3.2 Acute eye Irritation/ corrosion to the Rabbit

The objective of the study was to provide data on the irritant effects of RONOZYME® HiPhos after one single application to the eye of albino rabbits. The study was performed according to OECD guideline 405. The study was conducted in accordance with GLP.
The study was carried out with three rabbits which were treated as follows: 0.1 ml of the test substance was instilled in the conjunctival sac of the right eye. After administration the upper and lower eyelid were carefully closed and subsequently held together for one second to prevent loss of test material. The left eye remained untreated and served as reference control. The reactions of the test eyes were judged at circa 1, 24, 48, 72 h after treatment.

Results

At 1, 24, 48 and 72h after removal, no signs of irritation were observed in any of the three rabbits. See Annex 37.

Conclusion
RONOZYME® HiPhos can be classified as a 'non irritant' to the rabbit eye according to the referred classification criteria..

5.8.4 Effects of RONOZYME® HiPhos phytase on the microflora of the digestive tract

RONOZYME® HiPhos is a 6-phytase preparation acting on phytate as a substrate. This substrate is typically found in plants and materials derived thereof.

RONOZYME® HiPhos does not contain the production organism and has no antimicrobial activity. This has been proved on 3 lots of enzyme concentrate.

Furthermore, the product conforms to the JECFA and FCC purity specifications for food enzymes, which stipulate limits for microbiological contaminants. In particular, absence of contamination by *Salmonella spp.* and enteropathogenic *E.coli* is ascertained.

No direct effects on the microflora of the digestive tract are therefore expected for this product.

5.8.4.1 Target animal studies (tolerance studies)

The poultry studies were published in February 2011, see Reference 26.

Summary of Target Animal Safety Studies

Target Animal	Number of Animals	Doses in FYT / kg	Study Period In Days	Parameters Compared	Observations	Report
Broilers Male/Female	192	0, 4000 & 40,000	35	Pathology Blood Chemistry Body Weight	No Abnormalities No Adverse Effects	00000961
Laying Hens	288	0, 500 to 40,000	56	Pathology Blood Chemistry Egg Production	No Abnormalities No Adverse Effects	00000960
Turkeys-Toms	480	0, 4000 & 40,000	42	Pathology Blood Chemistry Physical Characteristics	No Abnormalities No Adverse Effects	00003289

5.8.4.2 Tolerance study with IPA Mash phytase (RONOZYME® HiPhos) in broiler chickens (Czech Republic 2009)

The purpose of the trial conducted by Research Institute of Biopharmacy and Veterinary Drugs was to study the tolerance of broiler chickens towards diets supplemented with RONOZYME® HiPhos during 5 weeks. This study was conducted in compliance with the requirements of current, international Good Laboratory Practice. See Annex 34.

Experimental conditions

192 one-day-old sexed broiler chickens (Ross 308) were used and allocated to the treatments. There were 16 replicates (of four animals) per dietary treatment during the experiment which lasted 35 days.

Starter and grower diets based on maize and soybean meal as the main feed ingredients were formulated to meet NRC nutrient recommendations except for total and non-phytate P. Starter diet was provided from day 0 to day 13 of the experiment while the grower diet was provided from day 14 to day 35. Hematological and biochemical examinations were performed with one animal per box (16 chickens per group).

The treatment groups were the following:

Group A: Control non-treated group (basal diet, no enzyme added)
Group B.: basal diet + RONOZYME® HiPhos at 4000 FYT/kg diet
Group C: basal diet + RONOZYME® HiPhos at 40,000 FYT/kg diet

Composition of the diets

Main ingredients (%)	Starter	Grower
Maize	58.08	62.44
Soybean meal (48% CP)	34.97	32.00
Nutrients (%, calculated)		
Crude protein (%)	21.58	20.57
M.E. (MJ/kg)	12.75	12.75
Methionine + Cystine	0.96	0.93
Calcium	0.75	0.60
Total Phosphorus	0.56	0.50
Non-phytate Phosphorus	0.25	0.20

RONOZYME® HiPhos (M) recoveries in FYT/kg feed

Target	0	4000 FYT/kg feed (starter/ grower)	40,000 FYT/kg feed (starter/ grower)
Analyzed	<LOQ (< 50 FYT/kg)	4133/ 4750	45,400/ 36,460

LOQ: limit of quantification

Results

Performance parameters

Treatments	Mean body weight day 0 (g)	Mean body weight (g) day 35	Feed conversion ratio day 0-35
A (0 FYT/kg)	43.0	1720a	1.913
B (4000 FYT/kg)	43.3	1917b	1.838
C (40,000 FYT/kg))	43.4	1925b	1.796

a,b: means without a common letter are significantly different (P<0.05

Selected haematological and biochemical blood parameters

	RBC ($x10^6$/µl)	HCT (%)	MCV (fl)	PLT ($x10^3$/µl)	WBC ($x10^3$/µl)
A (0 FYT/kg)	2.5	29.9	120.5	24.5	27.45
B (4000 FYT/kg)	2.5	30.6	120.3	26.5	29.09
C (40,000 FYT/kg)	2.6	31.3	120.6	29.0	24.88
Physiolog. range	2.5-3.5	22-35	90-140	20-30	12-30

Selected haematological and biochemical blood parameters (next)

	ALB (g/l)	Glu (mmol/l)	P (mmol/l)	ALT (µkat/l)	CPK (µkat/l)
A (0 FYT/kg)	14.3	12.9	1.50	0.02	86.0
B (4000 FYT/kg)	13.4	12.5	1.95	0.01	77.1
C (40,000 FYT/kg)	13.2	13.0	1.93	0.02	87.2
Physiolog. range	9.8-30.9	7.7-18.6	1.4-2.9	0.0-0.3	13.7-88.8

Selected haematological and biochemical blood parameters (end)

	HGB (g/100ml)	EO (%)	BA (%)	LY (%)	MO (%)
A (0 FYT/kg)	9.20	4.0	3.06	63.7	5.44
B (4000 FYT/kg)	8.96	5.0	3.13	61.3	6.69
C (40,000 FYT/kg)	9.43	5.3	3.63	60.6	6.38
Physiolog. range	7.0-13.0	2-8	2-4	32-65	2-10

RBC: red blood cell, HCT: haematocrit, MCV: mean corpuscular volume, PLT: blood platelet, WBC: white blood cell, ALB: albumin, GLU: glucose, P: phosphorus, ALT: alanine transaminase, CPK: creatine phosphokinase, HGB: haemoglobin, EO: eosinophil, BA: basophil, LY: lymphocytes, MO: monocytes

Comments
Dietary administration of RONOZYME® HiPhos (M) resulted in beneficial effects on chickens performance. The final body weight of birds receiving the phytase at 4000 and 40,000 FYT/kg diet was significantly increased from 1720 g (control) to 1917 and 1925 g; respectively. Due to this increased growth rate in both phytase supplemented groups, the overall feed conversion ratio was numerically improved from 1.913 (control) to 1.838 and 1.796; respectively. No clinical signs of any health problems were noted and there was no mortality during this study. Furthermore, no pathological changes were observed in birds during the post-mortem necropsy.

Hematological and biochemical examination did not reveal any obvious changes due to dietary administration of RONOZYME® HiPhos. However, a significantly increased serum concentration of inorganic P was found in both treated groups and this finding is confirming the efficacy of RONOZYME® HiPhos (M).

Conclusion
At 10 times the highest dose recommendation, RONOZYME® HiPhos (M) was well tolerated by the broiler chickens and had some beneficial effects.

5.8.4.3 Dose response and tolerance study with IPA Mash phytase [RONOZYME® HiPhos (M)] in laying hens fed a maize-based diet (Spain 2009)

The purpose of the trial conducted by IRTA was to study the tolerance of laying hens fed diets based on maize and soybean towards increasing doses of RONOZYME® HiPhos during 8 weeks. See Annex 35.

Experimental conditions
288 Hy-Line brown laying hens 52 weeks old were used and allocated to the treatments. There were 16 replicates per dietary treatment during the experiment which lasted 56 days.
The feeding program consisted of a negative control diet low in non-phytate P (0.1%) supplemented or not with increasing doses of RONOZYME® HiPhos (500, 1000, 2000, 4000 and 40,000 FYT/kg feed). Experimental feeds were provided from 52 to 59 weeks of age and performance including weight gain, egg production, egg weight, feed consumption, and percentage of dirty, faulty and broken eggs were recorded. Phosphorus excretion was measured after seven weeks of feeding experimental diets and ileal P absorption and tibia ash and P concentrations and strength at week 59. Blood samples from one bird per cage from treatments fed 0, 4000 and 40,000 FYT/kg were taken for hematological and biochemical measurements.

Composition and nutrient content of the diet

Main ingredients	(%)
Maize	53.3
Soybean meal (48% CP)	22.9
Nutrients (calculated)	
Crude protein	17.0
M.E. (kcal/kg)	2700
Methionine + Cystine	0.687
Calcium	3.60
Total Phosphorus	0.33
Non-phytate Phosphorus	0.10

RONOZYME® HiPhos recoveries in FYT/kg feed

Target	0	500	1000	2000	4000	40,000
Analyzed	55*	635	1351	2098	4586	46,400

*: does not mean contamination but native activity in feed

Results

Performance from week 52 to week 59

Treatments	Rate of lay (%)	Egg mass (g/day)	Feed conversion	Broken eggs (%)	Faulty eggs (%)
T1 (0 FYT/kg)	85.7	55.4	1.946	0.68	0.83
T2 (500)	86.3	54.9	1.923	0.34	0.15
T3 (1000)	85.0	55.1	1.953	0.15	0.08
T4 (2000)	86.7	55.3	1.949	0.53	0.80
T5 (4000)	89.2	57.3	1.914	0.15	0.27
T6 (40,000)	89.2	57.2	1.917	0.30	0.30
P>F	0.322	0.457	0.938	0.152	0.315
4000 vs. 40,000 FYT/kg	0.880	0.964	0.949	0.565	0.813
Linear [3]	0.114	0.152	0.599	0.182	0.860
Quadratic [3]	0.607	0.462	0.612	0.798	0.738

[3] using treatments T1 through T5.

Other experimental parameters

Treatments	P content in tibia (% DM)	Bone strength (kg/mm^2)	Ileal P digestibility (%)	Ileal P digestibility (g/kg feed)	P in excreta (% of DM)
T1 (0 FYT/kg)	7.52	27.7	36.7d	1.15d	0.86a
T2 (500)	7.64	28.6	44.4c	1.40c	0.81ab
T3 (1000)	7.56	30.7	44.6c	1.40c	0.77b
T4 (2000)	7.48	29.4	49.6c	1.56c	0.76b
T5 (4000)	7.38	28.8	57.7b	1.82b	0.74b
T6 (40,000)	7.60	28.4	74.5a	2.34a	0.66c
P>F	0.827	0.656	<0.0001	<0.0001	<0.001
4000 vs. 40,000 FYT/kg	0.302	0.831	<0.0001	<0.0001	<0.05
Linear [3]	0.199	0.776	<0.0001	<0.0001	<0.01
Quadratic [3]	0.836	0.234	0.346	0.342	0.103

Values within a column not sharing a common superscript are statistically different (P<0.05)

Selected haematological and biochemical blood parameters

Treatments	Glucose (mg/dl)	ERY (X10^{12}/l)	HGB (g/dl)	GGT (U/l)	PROT (g/dl)
T1 (0 FYT/kg)	230	2.52	12.1	25.0	5.26
T5 (4000)	232	2.46	11.8	20.1	5.32
T6 (40,000)	233	2.46	11.7	22.1	5.41
Pr>F	0.937	0.596	0.408	0.081	0.796

Other selected haematological and biochemical blood parameters

Treatments	HCT (%)	MCV (fl)	P (mg/dl)	GPT (U/l)	GGT (U/l)
T1 (0 FYT/kg)	32.8	130	4.63b	1.6	25
T5 (4000)	31.6	129	6.13a	3.1	20
T6 (40,000)	31.7	129	6.37a	1.8	22
Pr>F	0.32	0.22	<0.01	0.14	0.08

ERY: erythrocytes count; HGB: haemoglobin; GGT: gamma glutamin transferase, PROT: total protein, HCT: haematocrit, MCV: mean corpuscular volume, GPT: alanine aminotransferase, GGT: gamma glutamyltransferase

Comments
Tibia ash and P concentrations and bone strength were not significantly affected by phytase supplementation. The apparent ileal P digestibility responded to phytase supplementation in a linear manner up to 4000 phytase FYT/kg ($P<0.0001$), from 36.7% in the negative control to 57.7% with 4000 phytase FYT/kg of feed. Further increase of phytase from 4000 to 40,000 FYT/kg increased P digestibility from 57.7% to 74.5% ($P<0.01$). The P excretion was reduced linearly ($P<0.01$) with the increase of phytase dose. Further increase of phytase from 4000 FYT/kg to 40,000 FYT/kg reduced excreta P content from 0.74% to 0.66% ($P< 0.05$).

Results of this experiment suggest that IPA Mash phytase (=RONOZYME® HiPhos) supplementation was efficacious in increasing apparent ileal P digestibility and in reducing phosphorus excretion of laying hens, fed a maize-soybean based diet, low in non-phytate phosphorus. The response to phytase supplementation was linear.

No significant changes related to phytase addition at 4000 FYT/kg or 40,000 FYT/kg feed (ten-fold maximum recommended dose) were detected in hematological and biochemical blood characteristics, with the exception of serum phosphorus concentration ($P<0.01$). Phytase increased significantly phosphorus concentration ($P <0.05$) and there was not difference between the supplementation of 4000 or 40,000 FYT/kg.

Conclusion
No adverse effects of the use of 40,000 FYT/kg level of phytase on performance, mortality or hematological-biochemical characteristics were observed, with respect to the maximum recommended level (4000 FYT/kg). The response beyond the 4000 FYT/kg of phytase level was in many cases considerable.

At 10 times the highest dose recommendation, RONOZYME® HiPhos (M) was well tolerated by the laying hens and even had beneficial effects.

5.8.4.4 Determination of the tolerance for IPA Mash Phytase in young turkeys (The Netherlands 2009)

The purpose of the trial conducted at Central Veterinary Institute (Lelystad) was to study the tolerance of turkeys towards RONOZYME® HiPhos in a corn/soy diet over a period of 42 days. The study was conducted in compliance with the requirements of current, international Good Laboratory Practice. See Annex 36.

Experimental conditions
480 male and female BUT Big 6 turkeys (day-old) were used over the 6 weeks of the trial and allocated to the three treatments. Each treatment was evaluated in eight replicates divided over two sexes with 20 birds each housed in floor pens. Feed intake, body weight gain, feed conversion ratio, blood haematology and selected biochemical blood parameters were evaluated as response parameters. The turkeys were fed the starter diet during days 0-14 (starter phase) and the grower phase diet during days 14-42 of the experiment. The starter and grower diets were based on maize and soybean meal as main ingredients and were formulated to be nutritionally adequate according to NRC (1998) Ref. 27.

The treatment groups were the following:

Group 1: Control non-treated group (basal diet, no enzyme added)

Group 2: diet + phytase at 4000 FYT/kg diet

Group 3: diet + phytase at 40 000 FYT/kg diet

Composition of the diets

Main ingredients (%)	Starter diet	Grower diet
Maize	42.8	51.3
Maize gluten meal	8.6	7.5
Soybean meal	40.0	35.0
Nutrients (g/kg - analysed)		
Crude protein	272	249
Calcium	1.56	1.34
Phosphorus	1.03	0.87
M.E. (MJ/kg)	11.0	11.1
Available P (calculated)	5.9	4.8
Phytate P (calculated) (g/kg)	2.7	2.6

RONOZYME® HiPhos recoveries in FYT/kg feed

Target	Starter 0 Group1	Starter 4000 Group 2	Starter 40,000 Group 3	Grower 0 Group 1	Grower 4000 Group 2	Grower 40,000 Group 3
Analyzed	<LOQ	4085	40,290	<LOQ	3736	38,940

Results

Performance parameters

Treatments	Initial body weight (g)	Body weight (g) Day 14	Final body weight (g) Day 42
1 (control)	56.7	290a	2087a
2 (4000 FYT/kg)	57.0	304b	2153a
3 (40,000 FYT/kg)	57.3	320c	2239b

[a,b,c] mean values with a different superscript differ significantly at P<0.05

Biochemical and hematological parameters on day 42

Treatments	Total Blood Cells (10^{12}/l)	Total White Blood Cells (10^{9}/l)	Alkaline Phosphatase (U/l)	Glucose (mM)
1 (control)	2.45	7.47	2609	16.6b
2 (4000 FYT/kg)	2.29	7.94	2589	16.1a
3 (40,000 FYT/kg)	2.36	7.54	2699	16.2a

a,b mean values with a different superscript differ significantly at P<0.05

Biochemical and hematological parameters on day 42 (next)

Treatments	Cholesterol (mM)	Albumin (g/l)	Total protein (g/l)
1 (control)	3.31	24.5	33.5
2 (4000 FYT/kg)	3.26	25.0	33.1
3 (40,000 FYT/kg)	3.50	24.7	32.6

a,b mean values with a different superscript differ significantly at P<0.05

Comments

The mean body weight (BW) of the birds was significantly influenced by the experimental treatments on day 14 (P<0.001) and 42 (P<0.01). On day 14, BW was higher in treatments 2 (4000 FYT/kg) and 3 (40,000 FYT/kg) compared to the control treatment and higher in treatment 3 compared to treatment 2 (P<0.05). On day 42, BW was higher in treatment 3 compared to treatments 1 (control) and 2 (P<0.05).

There were no differences between treatments in the concentration of total blood cells and white blood cells in the turkeys on day 42 of the study.

There was no influence of the experimental treatments on the concentrations of alkaline phosphatase, GOT, GPT, lactate dehydrogenase, bilirubine, albumin, total protein, cholesterol and urea. There was only a significantly higher concentration of glucose in the control treatment compared to treatments 2 and 3 (P<0.05). For blood glucose concentration, a significant sex effect was noted. Males showed a higher concentration of blood glucose than females.

Conclusion

The inclusion of RONOZYME® HiPhos Phytase in diets for turkeys up to a level of 40,000 FYT/kg over a period of 42 days (day 0 to 42 of age) did neither exert detrimental effects on performance (feed intake, body weight gain and feed conversion ratio), nor influenced the levels of total and white blood cells and the concentrations or activity of a number of clinical chemical blood parameters. The present study did not reveal any signs of intolerance of young turkeys for RONOZYME® HiPhos.

5.8.4.5 Target animal safety factors calculations

The product RONOZYME® HiPhos is intended for use in poultry feeds. The standard recommended dose range of the product is 500 - 4000 FYT/kg feed.
Based on the NOAEL of 860 mg TOS/kg bw-day derived from the 13 weeks study in rats and typical feed intake values as derived from NRC[1] feeding tables, the following safety margins can be calculated for the different categories of animals:

Table 5-1 Intake estimation and safety factors in target species

Target species	Body weight kg	Typical feed intake kg feed/ day [1] & [2]&[3]	RONOZYME® HiPhos highest use recommendation		Highest expected enzyme intake		Safety margin
			FYT/ kg feed	mg TOS/ kg feed	FYT/ day	mg TOS/ kg-bw day	(NOAEL / highest intake)
Broiler Chickens, 1st week	0.152	0.019	4000	6.6	76	0.825	1042
Broiler Chickens, 3rd week	0.686	0.070	4000	6.6	280	0.675	1274
Broiler Chickens, 6th week	2.088	0.163	4000	6.6	652	0.515	1670
Laying hens, 30 weeks old	1.50	0.110	4000	6.6	440	0.484	1777

[1] National Research Council, Nutrient Requirements of Poultry. Ninth Revised Edition, National Academy Press, Washington, D.C., 1994. **Ref. 27**.

Comments:
Among poultry, broiler chickens are considered as a worst case due to the ratio of typical feed intake versus body weight. The safety factors as derived from the NOAEL in rats are comfortably large, in excess of three to four orders of magnitude.

The safety in the target species was confirmed by tolerance studies in broiler chickens, laying hens, turkeys at 10 times the highest recommended dose in FYT. The excessive dose did not produce any adverse effect on body weight gain, reproductive parameters (litter weight), blood cell counts, blood chemistry and gross pathology.
The trials are discussed in more detail below.

As a consequence of the large safety margins, no regulatory maximum dose for RONOZYME® HiPhos in feed is necessary. However with cost-benefit and marketing considerations and in order to allow flexibility in feed formulation, the following upper dose is recommended: 4000 FYT/kg feed for poultry.

6 Stability of the enzyme

6.1 Shelf-life and stability of the preparations

The company guarantees that the minimum activity as given on the label is present in the product at the end of the indicated shelf life provided the product is stored in the unopened container and the storage temperature does not exceed 25°C (77 °F). RONOZYME® HiPhos (M) has a minimum guaranteed activity of 50, 000 FYT/g. RONOZYME® HiPhos (L) has a minimum guaranteed activity of 20, 000 FYT/g. RONOZYME® HiPhos (GT) has a minimum guaranteed activity of 10, 000 FYT/g when stored at recommended conditions.

A stability study of RONOZYME® HiPhos (L) was completed and the 24 months of data is available. A stability study of RONOZYME® HiPhos (M) was conducted and 24 months of data has been reported. A stability study of RONOZYME® HiPhos (GT) is being conducted and 24 months of data has been reported.

Based on the available data, it can be concluded that in the standard packaging the product forms RONOZYME® HiPhos (M), and RONOZYME® HiPhos (GT) will maintain the declared activity for at least 24 months at 25°C and RONOZYME® HiPhos (L) for at least 9 months at 25°C .

6.1.1 RONOZYME® HiPhos (L)

A stability study was carried out with 3 batches of RONOZYME® HiPhos (L) at storage temperatures of –18°C, 10°C, 25°C, 35°C, 40°C and 50°C for 104 weeks. A stress test exposing the product to higher than recommended storage temperature of 40 °C was carried out over 12 weeks.

Results
All 3 batches of RONOZYME® HiPhos (L) showed good stability when stored at temperatures up to 25°C for 24 months with average residual activity of 69%. The stress test showed an average 88% residual activity of the 3 batches after 4 weeks of exposure of the enzyme formulation to air at 40 °C. Results of the study are shown in the table below.

Table 6-1 RONOZYME® HiPhos (L): Storage at 25°C

	Batch PPQ28432		Batch PPQ28459		Batch PPQ28460	
Activity in FYT/g; in % of initial activity	FYT/g	%	FYT/g	%	FYT/g	%
Reference (10°C)	26,900	100.0%	23,950	100.0%	25,400	100.0%
13 weeks (25°C)	25,200	93.7%	21,600	90.2%	22,900	90.2%
26 weeks (25°C)	22,900	85.1%	20,000	83.5%	22,850	90.0%
39 weeks (25°C)	22,750	84.6%	19,500	81.4%	22,000	86.6%
52 weeks (25°C)	21,550	80.1%	18,100	75.6%	20,900	82.3%
104 weeks (25°C)	18,600	69.1%	16,600	69.3%	17,650	69.5%

Conclusion
The minimum expected shelf life of RONOZYME® HiPhos (L) stored at room temperature is 6 months. The stress test revealed that RONOZYME® HiPhos (L) could withstand a short term stress by heat and remain to the great extent intact. The complete stability report is provided in Annex 17.

6.1.2 RONOZYME® HiPhos (M)

A stability study was carried out with 3 batches of RONOZYME® HiPhos (M) at storage temperatures of –18, 10, 25, 35 and 40°C for 24 months according to the standard Novozymes study design. The main results are shown in the table below.

Table 6-2 Storage stability of RONOZYME® HiPhos (M)

	PPQ 28656 Batch		PPQ 28683 Batch		PPQ 28684 Batch	
Activity in FYT/g; in % of initial activity	FYT/g	%	FYT/g	%	FYT/g	%
Initial (-18°C)	60950	100.0	60950	100.0	62650	100.0
6 months (-18°C)	61500	100.0	60200	100.0	64750	100.0
9 months (-18°C)	57400	100.0	58500	100.0	64400	100.0
12 months (-18°C)	64100	100.0	65900	100.0	64600	100.0
24 months (-18°C)	60700	100.0	57300	100.0	62600	100.0
6 months 25°C	56000	91.1	57400	95.3	63000	97.3
9 months 25°C	56500	98.4	54400	93.0	55900	86.8
12 months 25°C	62800	98.0	55100	83.6	56900	88.0
24 months 25°C	53500	88.1	47400	82.7	50100	80.0
6 months 35°C	55600	90.4	45100	74.9	47800	73.8
6 months 40°C	47300	76.9	39900	66.3	40600	62.7

Results
The average measured residual activity in RONOZYME® HiPhos (M) after 12 months and 24 months storage at 25°C are 89.9% and 83.6%; respectively.

Conclusion
The storage stability of RONOZYME® HiPhos (M) is acceptable at 25°C. RONOZYME® HiPhos (M) when stored at room temperature will maintain the declared activity for at least 12 months. See Annex 18.

6.1.3 RONOZYME® HiPhos (GT)

A stability study was carried out with 3 batches of RONOZYME® HiPhos (GT) at storage temperatures of –18, 10, 25, 35 and 40°C for 12 months according to the standard Novozymes study design. The main results are shown in the table below.

Table 6-3 Storage stability of RONOZYME® HiPhos (GT)

	09FLB0031-1FV Batch		09FLB0031-2FV Batch		09FLB0031-3FV Batch	
Activity in FYT/g; in % of initial activity	FYT/g	%	FYT/g	%	FYT/g	%
Initial (+10°C)	12400	100.0	12700	100.0	13600	100.0
3 months 25°C	13900	112.1	11250	88.6	12350	90.8
6 months 25°C	13200	106.5	11000	86.6	14050	103.3
9 months 25°C	-	-	11600	91.3	12800	94.1
12 months 25°C	13800	111.3	11650	91.7	13050	96.0
18 months 25°C	12900	104.0	11500	90.5	13500	99.3
24 months 25°C	11300	91.1	10200	80.3	11800	86.8
6 months 35°C	11250	90.7	8680	68.3	11200	82.4
6 months 40°C	9100	73.4	7240	57.0	8700	63.8

Results

The average measured residual activity in RONOZYME® HiPhos (GT) after 24 months storage at 25°C is above 100% of label claim. The stress test showed an average 83.4% residual activity of the 3 batches after 4 weeks of exposure of the enzyme formulation to air at 40 °C as shown in Annex 19.

Conclusion

The storage stability of RONOZYME® HiPhos (GT) is very good at room temperature. RONOZYME® HiPhos (GT) when stored at room temperature will maintain the declared activity for at least 24months.

6.2 Mixability and stability in premixtures and feeds

6.2.1 RONOZYME® HiPhos (L)

Stability studies in premixtures are not relevant to RONOZYME® HiPhos (L) because it is applied to the complete feed just prior to allocation to the animals.

A stability study in pelleted compound feeds broiler chicken was performed using 3 lots of RONOZYME® HiPhos (L) where the product was applied by spraying onto the feed at 1500 FYT/kg feed.

The initial recovered activity was on average 105% (100%) in the broiler feed.
During storage of the feed, RONOZYME® HiPhos (L) phytase retention decreased by 2% per month in the broiler feed. After the three months storage, the enzymatic activity retention was 98% in the broiler feed. It can thus be concluded that RONOZYME® HiPhos (L) is quite stable while stored during three months in feeds. See Annex 20.

6.2.2 RONOZYME® HiPhos (M)

Stability studies looking at premixtures and compound feeds were performed using 3 lots of RONOZYME® HiPhos (M).

In the first experiment, RONOZYME® HiPhos (M) was added to two types of premixtures: 1) a complete premix for broilers containing trace minerals and choline chloride; 2) a vitamin premix. For the first premixture, 1.25g RONOZYME® HiPhos was added to one kilogram of premixture 1. For the second premix, 63 g RONOZYME® HiPhos were added to one kg premixture 2. Those rates correspond to target doses of 75000 FYT/ kg in premix 1 and 3.15 million FYT/ kg in premix 2. Samples were subsequently stored for 6 months at 25°C. The method PHY-102/05E was used for the determination of the phytase activity in the premixtures.

The initial recovery in both premixtures was greater than 100% of the added enzymatic activity. After 6 months premixture storage, the enzymatic recoveries in the vitamin premixture and mineral premixture were 70% and 61%; respectively.

It can therefore be considered that RONOZYME® HiPhos (M) is not affected by mixing with other substance in premixtures and its stability in premxitures is moderate.

In the second experiment, 30 mg RONOZYME® HiPhos (M) were added to one kilogram of one broiler mash feed (target phytase activity of 1500 FYT/kg feed). The method PHY-101/05E was used for the determination of the phytase activity in the feeds. The enzymatic recoveries after mixing with the feeds were 100% in both feeds. After 3 months storage of the feeds, recoveries were calculated as 96% and 90%, respectively.

It can therefore be considered that RONOZYME® HiPhos (M) is not affected by mixing with other substance in feeds and is stable in feeds stored for up to three months at room temperature.

In the third experiment, RONOZYME® HiPhos (M) was tested in a commercial broiler feed. The product was pelleted at two temperatures 75°C and 85°C. Thirty mg RONOZYME® HiPhos (M) were added to one kilogram feed (target enzymatic activity: 1500 FYT/kg feed). The method PHY-101/05E was used for the determination of the phytase activity in the feed.

When the broiler feed was processed at 75°C and 85°C, the enzymatic activity recoveries were 99% and 21%; respectively. It can thus be concluded that RONOZYME® HiPhos (M) is stable while processed up to 75°C. In general, it is recommended to use the GT form for pelleted feed. See Annex 20.

6.2.3 RONOZYME® HiPhos (GT)

Stability studies looking at premixtures and commercial feeds were performed using 3 lots of RONOZYME® HiPhos (GT).

In the first experiment, RONOZYME® HiPhos (GT) was added to a mineral premixture containing trace minerals and choline chloride. 3.0g RONOZYME® HiPhos (GT) was added to one kilogram of premixture. This rate corresponds to target doses of 30,000 FYT/ kg Samples were subsequently stored for 6 months at 25°C. The method PHY-102/04E was used for the determination of the phytase activity in the premixtures.

The initial recovery in premixture was greater than 100% of the added enzymatic activity. After 6 months premixture storage, the enzymatic recovery was calculated to 99%.
It can therefore be considered that RONOZYME® HiPhos (GT) is not affected by mixing with other substance in premixtures nor by its storage during six months at room temperature.

In the second experiment, 150 mg RONOZYME® HiPhos (GT) was added to one kilogram of one broiler and one piglet mash compound feeds (target phytase activity of 1500 FYT/kg feed). The method PHY-101/04E was used for the determination of the phytase activity in the feeds. The enzymatic recoveries after mixing with the mash feeds were greater or equal to 100% in both feeds. After 3 months feeds storage, recoveries were greater than 100% in both mash compound feeds. It can therefore be considered that RONOZYME® HiPhos (GT) is not affected by mixing with other substance in feeds and is also stable in feeds stored for up to three months at room temperature.

In the third experiment, RONOZYME® HiPhos (GT) was tested in broiler feed. The product was pelleted at 80°C. 150 milligrams of product were added to one kilogram feed (target enzymatic activity: 1500 FYT/kg feed). The method PHY-101/04E was used for the determination of the phytase activity in feeds.

After pelleting at 80°C, the enzymatic activity recovery was 99%. After 3 months feeds storage, recoveries was 98%. It can therefore be considered that RONOZYME® HiPhos (GT) is not affected by pelleting at 80°C under the conditions of the test nor by its storage in feed for up to three months at room temperature.

In the fourth experiment, RONOZYME® HiPhos (GT) was tested versus one commercial feed.
The product was pelleted at two temperatures (80 °C and 90°C). 150 mg RONOZYME® HiPhos (GT) were added to one kilogram feed (target enzymatic activity: 1500 FYT/kg feed). The method PHY-101/04E was used for the determination of the phytase activity in the feed. When the broiler

feed was processed at 80°C and 90°C, the enzymatic activity recoveries were 106% and 94%; respectively. It can thus be concluded that RONOZYME® HiPhos (GT) is practically stable while processed up to 90°C. See Annex 19.

Information on resistance of the additive to moisture when mixed in premixtures and compound feed

Moisture is not an independent variable during the preparation of feeds, but a consequence of applying temperature in the form of steam during the pelleting process. For this reason, our stability studies report on both parameters and the evaluation of the results is done on temperature as the main influencing factor. During the storage of feed its moisture falls within a very narrow range (12 – 14%) and is covered by feed storage in environment where only temperature is controlled.

6.3 Physical properties of the formulations

The physical properties of the commercial formulations are described in the table below.

Table 6-4 Physical properties of the formulations

Formulation	L	M	GT
Physical Form	Liquid	Granulate	Granulate
Color	Yellow to Brown	Beige	Yellow to Brown
Enzyme Activity	20,000 FYT/g	50,000 FYT/g	10,000 FYT/g
Ave. Particle Size	NA	230 µm	495 µm
Bulk Density	NA	0.87 Kg/L	0.98 Kg/L
Density	1.21 - 1.23 Kg/L	NA	NA
Viscosity @ 25C	10 mPas	NA	NA

6.4 Incompatibilities with other feed ingredients

An enzyme acts specifically on its corresponding substrate. As a phytase, RONOZYME® HiPhos acts upon phytic acid, hydrolyzing its phosphate-bonds.

The history of use of various phytases in animal nutrition on a large industrial scale has not given rise to any concern of significant incompatibilities.

No adverse effects were detected in the comparative feeding studies performed with RONOZYME® HiPhos in any of the target animals receiving feeds of various ingredient compositions.

The potential adverse effects of certain aggressive ingredients of compound feed and premixtures (e.g. choline chloride, minerals) on the activity of RONOZYME® HiPhos have been investigated. The results of stability testing of RONOZYME® HiPhos in premixtures and feed showed acceptable stability of the phytase activity even when mixed in an aggressive vitamin-mineral premix containing choline chloride; i.e., no significant loss of 6-phytase activity was recorded during 3-6 months storage at room temperature. These results involving various premixes and feed matrixes gave no indication of any incompatibilities with any of the product forms of RONOZYME® HiPhos. See Annex 21.

Conclusion: significant incompatibilities with other commonly used non-phytase feed ingredients are not expected.

6.5 Feed Homogeneity:

The homogeneity of a mixture of powders is influenced by several well known factors; particle size, density and cohesiveness, order of ingredient addition, mixer design, impellor speed and mixing time. The last four factors are outside of the control of the feed ingredient manufacturer and appropriate parameters can only be provided as recommendations to the users. The influence of particle size, density and cohesiveness has been studied extensively by the engineering community because they affect a broad number of industries from food, feed and pharmaceuticals to plastics, fertilizers and ceramics. (Bridgewater, 1976 and Chowhan & Linn, 1979), Ref. 36 & Ref. 37 .

One of the first publications to address the mixing of small quantities of ingredients in animal feed was Merck's "A Guide To Mixing Microingredients in Feed" first printed in 1959, Ref. 38. In the section headed 'Particle Size' they noted the importance of small particle size for improved biological response. Feed particle size impacts the digestibility of the feed and consequently measures of livestock performance. Goodband et. al, (Ref. 39) reported at the 2006 Manitoba Swine Seminar that the production rate increased as grain particle size decreased for swine feed with a particle size between 650 – 750 microns being optimum. The ISA a multispecies breeder of livestock recommends for their laying hens a diet with 75% of the particles 500 to 3200 microns and 15% <500 microns (Ref 40). Amornthewaphat et. al. 1998 (Ref. 41) noted that as the mean particle size approached 400 microns mixing time increased however the possibility of segregation during handling went down. Animal feed, even if mixed on the farm is subjected to vibration during conveyance, transport and distribution that can cause segregation.

Ronozyme HiPhos (M) & (GT) have been designed to meet the size and density values, see Table 6-4, that have been reported in the above cited references to be suitable for typical feed formulations used in the United States. The particle characteristics of Ronozyme HiPhos are within the same range as other Ronozyme enzyme products that were the subject of AAFCO Ingredient Definitions and therefore were reviewed and accepted by the FDA Center for Veterinary Medicine. The AAFCO reviewed ingredients are published annually and are referenced by not only the individual States but several foreign governments.

Therefore, since Ronozyme HiPhos (M) and (GT) forms meet the criteria for good mixing taught in agricultural universities, that are published in peer review journals and reference books and have particle characteristics similar to other Ronozyme enzymes in the marketplace there is no reason to believe that a practitioner of the art of feed mixing would not general accept that Ronozyme HiPhos would perform in a similar fashion with respect mixing homogeneity.

Ronozyme HiPhos has a margin of safety greater than 1000 fold as reported in section 5.8.4.5, consequently an error in addition or non-uniformity due to under or over mixing that would result in a portion of a batch of feed that does not possess the expected enzyme activity does not affect the safety of the feed.

The published efficacy studies support that Ronozyme HiPhos performs as expected in commercial feeds.

7 Functionality

RONOZYME® HiPhos (in some reports is also designated phytase HK or IPA mash phytase) hydrolyses bonds between phosphate (P) and myo-inositol in phytic acid and its salt. Phytate phosphorus is an essential component of all seeds and phytate phosphorus accounts for 2/3-3/4 of the total plant phosphorus. Phytate phosphorus is not bioavailable for monogastric animals.

RONOZYME® HiPhos used in animal feeds increases the availability of phosphorus from typical plant based diets. Forage, cereals and legumes used in animal feed contain the anti-nutrient phytic acid in concentrations of 0.5 to 1.75%. See Hidvegi & Laszitity (Ref. 35) RONOZYME® HiPhos is therefore classified as a digestibility enhancing additive.

RONOZYME® HiPhos is produced in three forms using well-established formulation processes. The liquid formulation (L) contains minimum 20,000 FYT per gram. The micro granulated feed form (M) contains minimum 50,000 FYT per gram. The GT (granulated thermo-tolerant form) contains minimum 10,000 FYT per gram. As the claimed efficacy is attributed to the presence in all formulations of the same 6-phytase enzyme protein, the results of the efficacy studies are also valid for all formulations.

Efficacy is demonstrated for RONOZYME® HiPhos as typically for phytases by significant increases in phosphorus digestibility and utilization in mineral balance studies in the target animal species.

Phytase is a well-established enzyme product used in animal feeds and review papers on the use of phytase in poultry nutrition have been published by Selle & Ravindran (Ref.23); Broz and Ward (Ref.24); Singh (Ref.25); and Aureli et *al.* (Ref.26).

Over the last ten to fifteen years, DSM Nutritional Products Ltd. and Novozymes A/S have performed several dozen efficacy studies with phytases expressed in *Aspergillus oryzae*, that substantiated its efficacy and quantified the utility in the target animals. A number of these efficacy and utility studies have been published as listed in the previous paragraph.

Likewise, addition to animal diets of the phytase, RONOZYME® HiPhos - expressed in *Aspergillus oryzae*, the subject of the present application, increases the availability and utilization of phytin-bound phosphorus from plant materials. This in turn allows for a reduction of inorganic phosphorus supplementation in the animal diets without compromising performance. Lower phosphorus content in the diets helps to overcome environmental phosphorus contamination problems arising in areas with high production animal concentration.

The tables on the next pages summarize the study results obtained specifically with RONOZYME® HiPhos and support existing literature and commercial use of phytases in addressing 6-phytase functionality.

7.1 Summary of Efficacy Studies

Table 7.1 Summary of Efficacy Studies

Target Animal	Number of Studies	Number of Animals	Study Period In Days	Dose FYT / Kg of feed	Observations	Report *
Broilers Male/Female	3	96 - 550	14 - 28	0, 250 to 8000	Improved feed conversion ratio No Adverse Effects	00000101 00001790 00001184
Laying Hens	3	240 - 480	28 - 63	0, 500 to 40,000	Improved P digestibility No Adverse Effects	00000960 00000959 00000099
Turkeys-Toms	3	150 -240	21 - 28	0, 500 to 4000	Increased Final Weight No Adverse Effects	00001628 00002585 00003287

*Reports are Annexes # 22 through #30

7.2 OVERVIEW OF EFFICACY STUDIES IN BROILER CHICKENS

Report	Animal Numbers / Species	Trial Duration (wks)	Dosage FYT per kg feed		Negative Control→ RONOZYME® Treatments Effect on Phosphorus Utilization Measured by			
			as targeted	As analyzed				
00000101 Effect of graduated amounts of the RONOZYME® HiPhos phytase on growth performance and phosphorus utilization of broiler chickens fed low-phosphorus diet based on maize and soybean meal (BE-15/08) (France 2009) Maize and soybean meal diet	480 broiler chickens	2			FCR day 8-22	Apparent P utilization (% of intake)	P in excreta (g/kg DM)	Apparent Ca Utilization (%)
	n=48		0 (NC)	78*	1.576a	51.8f	8.3c	39.8g
	n=48		250	255	1.402b	60.8d	6.9d	52.1f
	n=48		500	505	1.329bc	69.0c	5.5e	61.2cd
	n=48		1000	1035	1.339bc	73.6b	4.6f	65.2bc
	n=48		2000	1878	1.270c	74.9ab	4.3f	67.8ab
	n=48		4000	3605	1.382bc	77.3ab	4.0f	70.2ab
	n=48		8000	8019	1.368bc	78.2a	3.9f	72.6a
	n=48		0 (PC)		1.366bc	57.2de	10.0b	55.5ef
	n=48		0 (PC)		1.378bc	57.6de	9.9b	60.3cde
	n=48		0 (PC)		1.361bc	54.6ef	11.2a	59.0de
00001790 Dose response study with RONOZYME® HiPhos in broiler chickens Hohenheim (De 2009) Maize, soybean diet	550 broiler chickens	4			P precaecal digestibility (%)	Ca precaecal Digestibility (%)	P excreted amounts mg/day	Tibia ash (g/kg DM)
	n=100		0	<50	32a	47a		
	n=100		500	466	43b	57b		
	n=100		1000	1012	53c	62bc		
	n=100		2000	1939	60d	68c		
	n=100		4000	3644	73e	75d		
	n=10		0	<50			97a	425d
	n=10		500	466			86ab	452c
	n=10		1000	1012			78b	473bc
	n=10		2000	1939			66c	486ab
	n=10		4000	3644			51d	505a
00001184 Comparison of two formulations of a microbial 6-phytase included at graduated levels on growth performance and phosphorus utilization of broiler chickens (BE- 07/09) (France 2009) Maize and soybean meal diet	96 broiler chickens	2			FCR	Apparent P Utilization (% of intake)	P in excreta (g/kg DM feces)	Toe ash (%)
	n=12		0 (NC)	<LOQ	1.988a	44.5d	9.5b	20.5b
	n=12		500	500	1.461b	64.5c	5.9c	32.2a
	n=12		1000	983	1.446b	70.7b	4.9d	32.7a
	n=12		2000	2170	1.426b	77.9a	3.7e	35.0a
	n=12		500	531	1.492b	62.1c	6.4c	33.4a
	n=12		1000	1445	1.458b	69.5b	5.1d	33.7a
	n=12		2000	1900	1.430b	71.1b	4.8d	37.3a
	n=12		0 (PC)	<LOQ	1.463b	47.8d	10.9a	33.1a

abcd: means within one column not sharing a common letter index differ with statistical significance
* means no contamination but some native activity in feed

7.3 OVERVIEW OF EFFICACY STUDIES IN LAYING HENS

Report	Animal Numbers / Species	Trial Duration (wks)	Dosage FYT per kg feed		Negative Control→ RONOZYME® Treatments Effect on Phosphorus Utilization Measured by			
			as targeted	As analyzed				
00000960 Dose response and tolerance study with IPA Mash phytase [= RONOZYME® HiPhos (M)] in laying hens fed a maize-based diet (Spain 2009) Maize and soybean meal diet	288 laying hens	8			FCR Week 52 – 59	Rate of lay (%)	P in excreta (% DM)	Apparent ileal P digestibility (%)
	n=48		0	55*	1.946	85.7	0.86a	36.7d
	n=48		500	635	1.923	86.3	0.81ab	44.4c
	n=48		1000	1351	1.953	85.0	0.77b	44.6c
	n=48		2000	2098	1.949	86.7	0.76b	49.6c
	n=48		4000	4586	1.914	89.2	0.74b	57.7b
	n=48		40000	46400	1.917	89.2	0.66c	74.5a
00000959 IPA mash phytase (RONOZYME® HiPhos) improves ileal P and Ca-absorption in laying hens (The Netherlands 2009) Maize, soybean diet	480 laying hens	9			Apparent ileal P absorption coefficient (%)	Tibia ash Content (g/kg DM)	Total P absorption (g/kg diet)	Laying Rate (%) Week 26-28
	n=96		0 (NC)	<LOQ	25.8d	518b	0.84c	94
	n=96		500	556	46.3b	527ab	1.50b	96
	n=96		1000	1086	53.7a	532a	1.74a	97
	n=96		2000	2583	57.6a	530a	1.87a	96
	n=96		0 (PC)	<LOQ	34.2c	523ab	1.42b	96
00000099 Effect of graduated levels of 6 phytase (= RONOZYME® HiPhos) on apparent ileal digestibility of phosphorus in laying hens fed a maize-based diet low in phosphorus content (H 01/09) (France 2009) Maize, barley diet	240 laying hens	4			Apparent ileal P digestibility (%)	P in excreta (g/kg DM)	Tibia Strength (N)	P in plasma (mMol/l)
	n=48		0 (NC)	<LOQ	45.8b	9.23	39a	1.23a
	n=48		500	562	54.8a	8.93	44a	1.50a
	n=48		1000	1114	56.1a	8.75	48a	1.36a
	n=48		2000	2097	58.7a	8.05	54a	1.20a
	n=48		0 (PC)	<LOQ	35.8c	13.6	42a	1.41a

abcd: means within one column not sharing a common letter index differ with statistical significance
* means no contamination but some native activity in feed

7.4 OVERVIEW OF EFFICACY STUDIES IN TURKEYS

Report	Animal Numbers / Species	Trial Dura-tion (wks)	Dosage FYT per kg feed		Negative Control→ RONOZYME® Treatments Effect on Phosphorus Utilization Measured by			
			as targe-ted	As ana-lyzed				
00001628 Efficacy of IPA PHYTASE (= RONOZYME® HiPhos) in Turkeys (Spain 2009) Maize and soybean meal diet	216 turkeys	3			Final weight Day 21 (g)	Aver. Daily gain (g)	Tibia ash (%)	Blood P (mg/100ml)
	n=36		0 (NC)	59*	368c	13.8c	37.2e	7.69a
	n=36		500	522	442b	17.3b	41.1d	6.15bc
	n=36		1000	1040	464b	18.2b	44.6c	7.17ab
	n=36		2000	1966	458b	17.9b	47.7b	7.27ab
	n=36		4000	4397	515a	20.4a	49.8a	7.10ab
	n=36		0 (PC)	61*	438b	17.0b	43.8c	5.23c
00002585 Efficacy of a novel phytase product (= RONOZYME® HiPhos) in young turkeys poults (USA 2009) Maize, soybean diet	150 turkeys	3			P retention** (%)	Ca retention (%)	Body weight gain (g)	
	n=30		0	63*	50.76d	44.08c	436b	
	n=30		250	216	64.16c	59.52b	445b	
	n=30		500	448	64.04c	54.99b	510a	
	n=30		1000	799	71.76b	65.53a	526a	
	n=30		2000	2024	74.32a	66.81a	542a	
0003287 Efficacy of IPA phytase (=RONOZYME® HiPhos) in turkeys (France 2009) Maize, soybean meal, wheat diet	240 turkeys	4			Apparent P utilization (% DM)	FCR Day 9 – 29	Ash in bone (% DM)	P in serum (mgl/l)
	n=30		0 NC	44*	50.98f	1.685a	30.13e	32e
	n=30		0 (PC)	55*	52.02f	1.598bc	34.40d	33de
	n=30		0 (PC)	44*	52.44ef	1.616b	38.53c	41d
	n=30		0 (PC)	45*	54.34e	1.584bc	41.27ab	53c
	n=30		500	581	60.95d	1.584bc	36.84c	36de
	n=30		1000	919	66.00c	1.563bcd	40.40b	51c
	n=30		2000	2327	72.26b	1.543cd	43.37a	63b
	n=30		4000	4075	76.96a	1.519d	44.78a	80a

abcd: means within one column not sharing a common letter index differ with statistical significance
* means no contamination but some native activity in feed
**: P retention is equal to apparent P utilization

7.5 Effect of graduated amounts of RONOZYME® HiPhos phytase on growth performance and phosphorus utilization of broiler chickens fed low-phosphorus diet based on maize and soybean meal (BE-15/08) (France 2009)

The purpose of the trial conducted at Village-Neuf (France) was to determine the effects of graduated amounts of RONOZYME® HiPhos on growth performance and phosphorus utilization of broiler chickens during 2 weeks. See Annex 22.

Experimental conditions

Day-old male broiler chickens (ROSS "PM3") were fed a low phosphorus pelleted basal diet supplemented with 50 µg vitamin D_3/ kg until day 8, when the trial started. On day 8,the chickens were divided by weight into groups, each comprising 8 birds, which were allocated to one of the different treatments. Each treatment was replicated with 6 groups. The groups were weighed on days 8, 15, and 22. Feed consumption for the intermediate periods was determined and body weight gain (WG) and feed conversion ratio (FCR) were calculated. The basal diet was based on maize and soybean meal as main ingredients.

Apart from the control treatment without phytase supplementation, all other treatments were supplemented with the RONOZYME® HiPhos phytase at one of the following doses: 250, 500, 1000, 2000, 4000 and 8000 FYT/kg feed, and with additional DCP (Dicalcium Phosphate) to provide 4.9, 5.3 and 5.7 g total P per kg feed, that means 0.8 g, 1.2 g and 1.6 g P more than the negative control diet, per kg feed.

Table 7-2 Composition and nutrient content of the diets

Main ingredients (%)	Basal diet
Maize	60.20
Soybean meal (50% CP)	35.50
DL-Methionine	0.20
L-Lysine	0.05
Calculated content	
Crude protein (%)	21.6
Methionine + Cystine (%)	0.90
Metabolizable energy ME_N (MJ/kg) (calculated)	12.7
Analysed content	
Ca (g/kg)	5.2
Phytate P (g/kg)	2.3
Total P (g/kg)	3.9
Non Phytate-P (g/kg)	1.6

Table 7-3 Analyzed P and Ca concentration in samples of the experimental diets

Treatments	Product	Total P (g/kg feed)		Ca (g/kg feed)	
		expected	measured	expected	measured
A	Negative control	4.1	3.9	6.0	5.2
P	Positive control	4.9	5.0	6.0	5.6
Q	Positive control	5.3	5.2	6.0	5.7
R	Positive control	5.7	5.6	6.0	5.6

Table 7-4 RONOZYME® HiPhos recoveries in FYT/kg feed

Treatments	Product	Target (FYT/kg)	Analysed (FYT/kg feed)
A	Negative control	0	78*
B	phytase	250	255
C	phytase	500	505
D	phytase	1000	1035
E	phytase	2000	1878
F	phytase	4000	3605
G	phytase	8000	8019

*: does not mean contamination but native phytase activity in feed

Results (next page)

Performance of broiler chickens (day 8 to day 22)

Product	Negative control	RONOZYME® HiPhos						Positive control		
Treatment	A	B	C	D	E	F	G	P	Q	R
Dose (FYT/kg)	-	250	500	1000	2000	4000	8000	4.9 g P/kg	5.3 g P/kg	5.7 g P/kg
Weight gain (g/bird)	451^{E}	727^{D}	778^{BCD}	809^{BC}	880^{A}	837^{AB}	806^{BC}	730^{D}	753^{CD}	756^{CD}
Feed intake (g/bird)	708^{D}	1018^{BC}	1033^{BC}	1080^{ABC}	1114^{AB}	1156^{A}	1101^{ABC}	998^{C}	1037^{BC}	1028^{BC}
Feed conversion	1.576^{A}	1.402^{B}	1.329^{BC}	1.339^{BC}	1.270^{C}	1.382^{BC}	1.368^{BC}	1.366^{BC}	1.378^{BC}	1.361^{BC}

Newman-Keuls test: Means within a row, not sharing a common superscript, are significantly different ($p<0.05$)

Table 7-5 *Apparent utilization of phosphorus and calcium in male broiler chickens, resistance of the tibia and tibia ash*

Product	Negative control	RONOZYME® HiPhos						Positive control		
Treatment	A	B	C	D	E	F	G	P	Q	R
Dose (FYT/kg)	-	250	500	1000	2000	4000	8000	4.9 g P/kg	5.3 g P/kg	5.7 g P/kg
Apparent P utilization (% of intake)	51.8 ^{F}	60.8 ^{D}	69.0 ^{C}	73.6 ^{B}	74.9 ^{AB}	77.3 ^{AB}	78.2 ^{A}	57.2 ^{DE}	57.6 ^{DE}	54.6 ^{EF}
P in excreta (g/kg DM)	8.3 ^{C}	6.9 ^{D}	5.5 ^{E}	4.6 ^{F}	4.3 ^{F}	4.0 ^{F}	3.9 ^{F}	10.0 ^{B}	9.9 ^{B}	11.2 ^{A}
Apparent Ca utilization	39.8 ^{G}	52.1 ^{F}	61.2 ^{CD}	65.2 ^{BC}	67.8 ^{AB}	70.2 ^{AB}	72.6 ^{A}	55.5 ^{EF}	60.3 ^{CDE}	59.0 ^{DE}
Tibia strength (N)	76 ^{C}	167 ^{AB}	234 ^{A}	214 ^{A}	243 ^{A}	234 ^{A}	229 ^{A}	128 ^{BC}	169 ^{AB}	182 ^{AB}
Tibia ash (%)	40.7 ^{C}	46.8 ^{B}	50.2 ^{AB}	51.2 ^{A}	51.9 ^{A}	52.0 ^{A}	53.6 ^{A}	46.2 ^{B}	49.7 ^{AB}	49.8 ^{AB}

Newman-Keuls test: Means within a row, not sharing a common superscript, are significantly different ($p<0.05$)

Comments

Adding dicalcium phosphate (DCP) to the negative control diet gave a significant improvement of the weight gain and the FCR, clearly indicating that the negative control diet was P-deficient. At a supplementation level of + 1.1 g (analyzed) DCP per kg feed, the WG and the FCR were improved by 62 % and 13.3 %, respectively compared to the negative control diet.

Phytase supplementation resulted in a significant improvement of the weight gain and the feed conversion ratio compared to the negative control diet. The lowest phytase inclusion level of 250 FYT/kg already resulted in a significantly higher WG (+ 61.2%) and better FCR (- 11%). Increased phytase supplementation from 250 to 8000 FYT/kg resulted in a significant improvement of the WG and the FCR with significant differences between the supplemented treatments. The weight gain and the feed conversion ratio were improved in a logarithmic dose response manner with increasing phytase. The response of weight gain and feed conversion ratio to the addition of phytase to the diet can be described by non-linear regressions.

The mortality observed throughout the trial was higher for the control treatment (12.5 %) than the other treatments, but was within an acceptable range (results not displayed in the summary).

The apparent utilization of phosphorus was significantly improved by the action of the phytase. The apparent P-utilization increased with increasing dietary levels of the phytase. Compared to the negative control diet, an improvement in a range of 17 % to 51 % was obtained with graduated levels of phytase. The effect of the phytase supplementation on P utilization for all supplementary levels was further confirmed by a significant reduction in P excretion, in which reductions of about 44.6 % and 48.2 % were obtained at 1000 and 2000 FYT/kg feed supplementations, respectively. The utilization of P in the negative control diet was 51.8 %, and it was increased to an estimated asymptotic value of 78.2 % with phytase supplementation.

The improvement of P-utilization in the phytase treatment indicated that phytate-P was liberated due to the action of the phytase. More than 0.8 g additional available phosphorus was released due to the action of 1000 FYT phytase per kg feed. P-utilization of the positive control groups was lower than of all other groups.

The apparent Ca-utilization was significantly improved in all treatments compared to the negative control diet. Similar to the P-utilization, the effect was dose-dependent with significant differences between the dosages. Utilization of Ca in the negative control diet was 39.8%, and it was increased up to an estimated asymptotic value of 72.6% by including different levels of phytase. The results indicate that in addition to P release there is an additional availability of calcium caused by the phytase.

Supplementing phytase, irrespective of the dose, significantly improved tibia strength compared to the negative control diet. Tibia strength values increased in a pattern corresponding to supplementation levels. At levels above 1000 FYT/kg the bone strength was improved more than 2 fold.

The effects of phytase supplementation on tibia ash, a parameter that indicates the extent of bone mineralization, were significant for all treatments compared to the negative control diet. With increasing levels of phytase, great improvements ranging between 15 % and 32 % were noticed.

Conclusion
The results of this study demonstrate that the supplementation of low P diet with the RONOZYME® HiPhos phytase significantly improved the weight gain and the feed conversion ratio of male broiler chickens at 22 days of age. The utilization of phosphorus was significantly increased and consequently the amount of P excreted in the feces was reduced. P-utilization is dependent on level of phytase and the relationship is a perfect fit to an exponential function with P utilization approaching an asymptote at 80%. The phytase is efficacious in releasing phytate-P. More than 0.8g additional available phosphorus was released due to the action of phytase at 1000 FYT/kg feed.

7.6 Dose response study with a new phytase (IPA Mash Phytase = RONOZYME® HiPhos) in broiler chickens (Germany 2009)

The purpose of the trial conducted at the University of Hohenheim (Germany) was to determine the effects of graduated amounts of RONOZYME® HiPhos on phosphorus utilization of broiler chickens. See Annex 23.

Experimental conditions
Six hundred day-old broiler chickens (Ross 308) were allocated to 50 pens of 12 birds each. From day 1 to 13 the birds received a starter diet based on maize and soybean meal that was calculated to be adequate in available energy and all nutrients including P according to the recommendations. On day 14 of the experiment 50 birds (the one with the highest body weight from each pen, 10 per treatment) were housed individually in balance cages. From day 14 to 24 the respective experimental diet was fed slightly restricted (50 g per bird and day) in order to avoid feed refusals. On day 25 the experimental diet was offered *ad libitum*. The experimental basal diet was based on maize (541 g/kg) and solvent-extracted soybean meal from dehulled seed (400 g/kg) without a mineral P supplementation in order to achieve a sufficiently low basal P level. From day 19 to 24 excreta were quantitatively collected, pooled for each bird and stored. Later the excreta were mixed and oven-dried prior to analysis. Broilers were weighed at the beginning and the end of the collection period. On day 26 the birds were killed and the tibia bones removed and stored until further handling. A total of 500 broilers remained in their pens for determination of precaecal (pc) digestibility of P and Ca. Ten pens were allocated to each of the five experimental diets. The respective experimental diet was offered *ad libitum* for 7 days until slaughter.

Phytase activities and nutrient content in the experimental diets

Diet	Target activity	Analysed activity (FYT/kg)	Crude protein (g/kg DM)	P (g/kg DM)	Ca (g/kg DM)
Starter			260	7.6	13.1
A	0	<50*	275	4.7	10.8
B	500	466	247	4.6	9.8
C	1000	1012	247	4.7	12.1
D	2000	1939	250	4.3	10.2
E	4000	3644	243	4.8	10.4

*: does not mean contamination but native phytase activity in feed

Results

Precaecal (pc) digestibility, excreted amounts and utilisation of P and Ca of broiler chickens

Phytase FYT/kg	Treatment	P digestibility %	Ca digestibility %	P utilization %	Ca utilization %	P Excreted amounts (mg/d)	Ca Excreted amounts (mg/d)
0	A	32a	49a	47a	28a	97a	311a
500	B	43b	52ab	57b	38b	86ab	292ab
1000	C	53c	55bd	62bc	40bc	78b	283abc
2000	D	60d	51a	68c	43bc	66c	266bc
4000	E	73e	56cd	75d	48c	51d	248c
P=		<0.001	0.002	<0.001	<0.001	<0.001	0.05

Means are significantly different from the un supplemented treatment A according to Dunnett test.
abc values without a common superscript are significantly different according to t-test ($p \leq 0.05$)

Content of crude ash, P and Ca of the tibia

Phytase FYT/kg	Treatment	Ash g/kg dry matter	Ca mg/bone	P mg/bone
0	A	425d	96d	45d
500	B	452c	126c	59c
1000	C	473bc	133bc	62bc
2000	D	486ab	147b	70b
4000	E	505a	170a	81a
P=		<0.001	<0.001	<0.001

Comments

The prececal digestibility of P increased from 32% to 73% with increasing phytase supplementation. Each level of phytase supplementation led to a significant increase in precaecal P digestibility. The precaecal digestibility of Ca was 49% in the unsupplemented basal diet. Phytase supplementation also led to a significant increase in precaecal Ca digestibility.

The excretion of P was significantly reduced ($P < 0.001$) by phytase supplementation. Correspondingly, the effect of phytase on utilization of P also was highly significant.

The excreted amounts of Ca were significantly lower at the two highest levels of supplementation compared to the control. The utilization of Ca increased from 28 to 48 % with increasing phytase supplementation.

Tibia contents of ash, P and Ca were significantly improved by RONOZYME® HiPhos supplementation. The ash concentration of the tibiae from the birds fed the un-supplemented control diet was 42.5% (on dry matter basis), and it was increased up to an estimated value of 50.7%, respectively. Concentrations of Ca and P in tibia ash were only slightly affected by phytase supplementation. P concentration in tibia ash increased from 172 to 177 g/kg ash.

7.7 Comparison of two formulations of a microbial 6-phytase (RONOZYME® HiPhos) included at graduated levels on growth performance and phosphorus utilization of broiler chickens (BE-07/09) (France 2009)

The purpose of the trial conducted at Village-Neuf (CRNA) was to determine the effects of two forms of RONOZYME® HiPhos at graduated levels on growth performance and phosphorus utilization of broiler chickens from day 8 to day 22 of life. See Annex 24.

Experimental conditions

Day-old male broiler chickens (ROSS "PM3") were used in the experiment and fed with a low phosphorus basal diet until day 8, when the trial started. The low-phosphorus diets were based on maize and soybean meal. On day 8, the chickens were divided by weight into groups, each comprising 8 birds, which were allocated to one of the different treatments. Each treatment was replicated with 12 groups. The groups were weighed on days 8, 15, and 22. Feed consumption for the intermediate periods was determined and body weight gain (WG) and feed conversion ratio (FCR) were calculated. The two forms of RONOZYME® HiPhos were included at 500, 1000 and 2000 FYT/kg feed,. On day 22, blood samples from 4 male chickens randomly chosen from each group were collected. The concentrations of inorganic phosphate (Pi) and calcium (Ca) in the plasma were determined. In addition at day 23, tibiae and toes were collected from randomly selected chickens.

Composition and nutrient content of the diets

Main ingredients	(%)
Maize	59.1
Soybean meal (50% CP)	36.8
Nutrients (% – calculated)	
Crude protein	22.2
$M.E_N$. (MJ/kg)	12.6
Calcium	0.56
Total Phosphorus	0.38 (Negative Control) , 0.49 (Positive Control)
Non-phytate Phosphorus	0.08

RONOZYME® HiPhos recoveries in FYT/kg feed

Diet	Treatment Target activity	Analysed
Negative control	0	<LOQ
HiPhos (L)	500	500
HiPhos (L)	1000	983
HiPhos (L)	2000	2170

RONOZYME® HiPhos recoveries in FYT/kg feed

Diet	Treatment Target activity	Analysed
HiPhos (solid)	500	531
HiPhos (solid)	1000	1445
HiPhos (solid)	2000	1900
Positive control	0	<LOQ

Results

Performance of broiler chickens between day 8 and day 22

Phytase FYT/kg	Treatment	FCR	Apparent P utilization (% of intake)	P in excreta (g/kg DM feces)	Apparent Ca utilization (% of intake)	Tibia ash (%)	Toe ash (%)
0	Negative control	1.988a	44.5d	9.5b	32.8d	37.2d	20.5b
500	HiPhos (L)	1.461b	64.5c	5.9c	56.0b	46.6c	32.2a
1000	HiPhos (L)	1.446b	70.7b	4.8d	61.3ab	50.0ab	32.7a
2000	HiPhos (L)	1.426b	77.9a	3.7e	66.7a	50.8a	35.0a
500	HiPhos (on salt)	1.492b	62.1c	6.4c	48.8c	45.7c	33.4a
1000	HiPhos (on salt)	1.458b	69.5b	5.1d	56.8b	48.7ab	33.7a
2000	HiPhos (on salt)	1.430b	71.1b	4.8d	67.1a	50.5a	37.3a
0	Positive control	1.463b	47.8d	10.9a	50.3c	48.3b	33.1a

Comments

Increased phytase supplementation from 500 to 2000 FYT/kg feed resulted in a significant improvement in Weight Gain and the FCR ratio compared to the negative control diet. The two forms of RONOZYME® HiPhos tested at the lowest inclusion level already resulted in a significantly higher WG and better FCR compared to the negative control diet. The WG was improved by 97 % and 84 % with the RONOZYME® HiPhos in liquid form and in salt coated form, respectively. The FCR was improved by 26.5 % and 24.9 %, respectively.

Compared to the negative control diet, an improvement in a range of 45 % to 75 % and 40 % to 60 % was obtained with graduated levels of the RONOZYME® HiPhos (L) and the RONOZYME® HiPhos phytase (s. coated), respectively. The effects on P-utilization were comparable between the two forms of RONOZYME® HiPhos Phytase included at 500 and 1000 FYT/kg feed. The effect of the phytase supplementation on P utilization for all supplementary levels was further confirmed by a significant reduction in P excretion over the negative control diet. The concentration of phosphorus in excreta recorded for the two phytases included at 500 and 1000 FYT/kg feed was comparable. The apparent Ca-utilization was significantly improved in all treatments compared to the negative control diet. Similar to the P-utilization, the effect was dose-dependent with significant differences between the dosages.

The P-concentration in the plasma was significantly increased by all treatments (results not summarised above) compared to the negative control diet. The Pi concentration in the plasma

increased with increasing dietary inclusion level of either RONOZYME® HiPhos (L) or RONOZYME® HiPhos phytase (salt coated).

Supplementing phytase, irrespective of the dose, significantly improved tibia strength compared to the negative control diet. Tibia strength values increased in a pattern corresponding to supplementation levels (results not summarised above). The effects of phytase supplementation on tibia ash, a parameter that indicates the extent of bone mineralisation, were significant for all treatments compared to the negative control. With increasing levels of phytase, important improvements ranging between 25 % to 37 % and 23 % to 36 % were noted with RONOZYME® HiPhos phytase (L) and with RONOZYME® HiPhos phytase (s. coated), respectively. An exponential dose-dependent relationship was found for the tibia ash, in which the slope rose very fast with increasing levels of phytase in the diet. The inclusion of either RONOZYME® HiPhos (L) or RONOZYME® HiPhos phytase (salt coated) resulted in a comparable improvement of the tibia ash and tibia strength.

In this experiment, phytase supplementation was effective in improving toe ash compared to the negative control. The effects on toe ash were comparable between both forms of the RONOZYME® HiPhos phytase. In the present study, no significant differences between dosages were noted.

Conclusion

The results of this study demonstrate that the supplementation of low P diet with the RONOZYME® HiPhos phytase in liquid or in salt coated form significantly improved the weight gain and the feed conversion ratio of male broiler chickens at 22 days of age.

The utilization of phosphorus was significantly increased and consequently the amount of P excreted in the faeces was reduced. P-utilization was improved dependent on level of phytase and could be described by an exponential function. The RONOZYME® HiPhos phytase was effective in releasing phytate-P according to the effects obtained on tibia and toe ash.

The efficiency of RONOZYME® HiPhos Phytase (salt coated) recorded in this trial was comparable to that of IPA Phytase (L) for growth parameters, bone parameters and for mineral utilization.
In most parameters, the treatments supplemented with higher dosages of RONOZYME® HiPhos phytase performed equally or even outperformed the treatment supplemented with additional mineral P (positive control).

7.8 Dose response and tolerance study with IPA Mash phytase [= RONOZYME® HiPhos (M)] in laying hens fed a maize-based diet (Spain 2009)

The purpose of the trial conducted by IRTA was to study the efficacy of the product and the tolerance of laying hens fed diets based on maize and soybean meal involving increasing doses of RONOZYME® HiPhos during 8 weeks. See Annex 25.

Experimental conditions
288 Hy-Line brown laying hens 52 weeks old were used and allocated to the treatments. There were 16 replicates per dietary treatment during the experiment which lasted 56 days.

The feeding program consisted of a negative control diet low in non-phytate P (0.1%) supplemented or not with increasing doses of RONOZYME® HiPhos (500, 1000, 2000, 4000

and 40 000 FYT/kg feed). Experimental feeds were provided from 52 to 59 weeks of age and performance; including weight gain, egg production, egg weight, feed consumption, and percentage of dirty, faulty and broken eggs were recorded. Phosphorus excretion was measured after seven weeks of feeding experimental diets and ileal P absorption and tibia ash and P concentrations and strength at week 59. Blood samples from one bird per cage from treatments fed 0, 4000 and 40,000 FYT/kg were taken for haematological and biochemical measurements.

Composition and nutrient content of the diets

Main ingredients	(%)
Maize	53.3
Soybean meal (48% CP)	22.9
Nutrients (% – calculated)	
Crude protein	17.0
M.E. (kcal/kg)	2700
Methionine + Cystine	0.687
Calcium	3.60
Total Phosphorus	0.33
Non-phytate Phosphorus	0.10

RONOZYME® HiPhos recoveries in FYT/kg feed

Target	0	500	1000	2000	4000	40,000
Analyzed	55*	635	1351	2098	4586	46,400

*: does not mean contamination but native activity in feed

Results

Performance from week 52 to week 59

Treatments	Rate of lay (%)	Egg mass (g/day)	Feed conversion	Broken eggs (%)	Faulty eggs (%)
T1 (0 FYT/kg)	85.7	55.4	1.946	0.68	0.83
T2 (500 FYT/kg)	86.3	54.9	1.923	0.34	0.15
T3 (1000 FYT/kg)	85.0	55.1	1.953	0.15	0.08
T4 (2000 FYT/kg)	86.7	55.3	1.949	0.53	0.80
T5 (4000 FYT/kg)	89.2	57.3	1.914	0.15	0.27
T6 (40,000 FYT/kg)	89.2	57.2	1.917	0.30	0.30
P>F	0.322	0.457	0.938	0.152	0.315
P-value Linear [3]	0.114	0.152	0.599	0.182	0.860
P-value Quadratic [3]	0.607	0.462	0.612	0.798	0.738

[3]using treatments T1 through T5.

Other experimental parameters

Treatments	Apparent ileal P digestibility (%)	% P in excreta (dry matter basis)
T1 (0 FYT/kg)	36.7d	0.86a
T2 (500 FYT/kg)	44.4c	0.81ab
T3 (1000 FYT/kg)	44.6c	0.77b
T4 (2000 FYT/kg)	49.6c	0.76b
T5 (4000 FYT/kg)	57.7b	0.74b
T6 (40,000 FYT/kg)	74.5a	0.66c
P>F	<0.0001	<0.0001
P-value Linear [3]	<0.0001	<0.01
P-value Quadratic [3]	0.346	0.103

Values within a column not sharing a common superscript are statistically different (P<0.05)

Comments
Tibia ash and P concentrations and bone strength were not significantly affected by phytase supplementation. The apparent ileal P digestibility responded to phytase supplementation in a linear manner up to 4000 phytase FYT/kg (P<0.0001), from 36.7% in the negative control to 57.7% with 4000 phytase FYT/kg of feed. Further increase of phytase from 4000 to 40,000 FYT/kg increased P digestibility from 57.7% to 74.5% (P<0.01).

The P excretion was reduced linearly (P <0.01) with the increase of phytase dose. Further increase of phytase from 4000 FYT/kg to 40,000 FYT/kg reduced excreta P content from 0.74% to 0.66% (P< 0.05).

Conclusion
Results of this experiment suggest that phytase IPA Mash (RONOZYME® HiPhos) supplementation was efficacious in increasing apparent ileal P digestibility and in reducing phosphorus excretion of laying hens, fed a maize-soybean based diet, low in non-phytate phosphorus. The response to phytase supplementation was linear. The response beyond the 4000 FYT/kg of phytase level was in many cases considerable.

7.9 IPA mash phytase (RONOZYME® HiPhos) improves ileal P and Ca-absorption in laying hens (The Netherlands 2009)

The purpose of the trial conducted at Schothorst Feed Research was to determine the dose-response relationship between RONOZYME® HiPhos and the absorption of P and Ca, tibia ash and laying hens performance. The laying hens were fed a maize/soy diet. See Annex 26.

Experimental conditions
A total of 480 laying hens (Isa White) 16 week of age were used for the trial. After arrival the hens received a commercial corn/soypre-layer diet. When egg production started, the diet was switched to a commercial layer diet (mash). Starting in week 26 the experimental diets were fed after a two day transition period in which a 50/50 mixture of the commercial layer diet and the experimental diets was fed. Subsequently, the experimental diets (mash) were fed until the end of the experiment (15 days).

Five dietary treatments were included in the study and there were six replicates per treatment for calcium and phosphorus absorption. Each replicate comprised four cages with four hens per cage (16 laying hens per replicate). Graduated levels of the test product were added to a

phosphorus deficient basal diet. The P-deficient basal diet was also fed as such (negative control diet) and was supplemented with 1.0 g P from di-calcium phosphate (DCP) as positive control diet.

Composition and nutrient content of the diets

Main ingredients (%)	Neg. control Low available P	Positive control
Maize	50.0	50.0
Soybean meal	14.3	14.3
Sunflower seed meal	9.1	9.1
Calculated nutrients (g/kg)		
Crude protein	156	156
Methionine + Cystine (digestible)	5.7	5.7
Crude fat	50	50
Ca	37	37
Phytate P	2.5	2.5
Total P	3.6	4.6
Absorbable P	1.2	2.0
AME_N (Kcal/kg)*	2800	2800

*Apparent Metabolizable Energy, nitrogen corrected

Analyzed nutrients and phytase activity in the experimental diets

Treatment No.	Dose FYT/kg	DM g/kg	CP g/kg	P g/kg	Phytate-P g/kg	Ca g/kg	Activity FYT/kg
1	0	897	153	3.24	2.10	33.60	<50
2	500	896	n.a.	n.a.	n.a.	n.a.	556
3	1000	890	n.a.	n.a.	n.a.	n.a.	1086
4	2000	898	n.a.	n.a.	n.a.	n.a.	2583
5	+ 1 g P	898	n.a.	4.16	n.a.	32.80	<50

n.a. = not analyzed, as all diets were obtained from the same basal diet, without further supplements of these nutrients. CP: crude protein

Results

Laying rate of hens, apparent ileal absorption coefficient of P and tibia ash, total apparent ileal P absorption and calculated P excretion of laying hens in the 28th life week

Treatment No.	Laying rate % week 26-28	Apparent Ileal P absorption coeff. %	Tibia ash content g/kg DM	Total P absorption g/kg diet	Calculated P excretion g/kg diet
1	94	25.8d	518b	0.84c	2.41
2	96	46.3b	527ab	1.50b	1.74
3	97	53.7a	532a	1.74a	1.50
4	96	57.6a	530a	1.87a	1.37
5	96	34.2c	523ab	1.42b	2.74
P value	NS	<0.001	0.02	<0.001	

NS = non significant *(P>0.10)*

a,b,c Mean values without a common letter indicate significant differences (P≤0.05)

Comments
A significant positive response to the phytase supplementation was found for P absorption. Compared to the negative control group, the group with the lowest phytase supplementation level of 500 FYT/kg improved P absorption significantly from 26% to 46%. A dose level of 1000 FYT/kg resulted in a further significant improvement of P absorption up to 54%. Doubling the dose level to the highest inclusion level of 2000 FYT/kg gave further improvement of P absorption to 58%, but this additional increase could not be shown to be significant versus the previous level.

Total P absorption as well as P excretion was calculated. Assuming that the increased P absorption in the phytase supplemented diets is fully accounted for by phytate P degradation, it can be calculated that the degradation coefficient was increased to 32, 43 and 49% for the dose levels 500, 1000 and 2000 FYT/kg respectively.

P absorption of the positive control group was significantly higher compared to the negative control group. For the absorption of P from DCP a coefficient of 64% could be calculated. In broilers the retainable P content (as a percentage of total P) for DCP (dihydrate) is 78%.The lower value in layers is most probably due to differences in Ca and P metabolism in layers compared to broilers [Van der Klis et al 1997 also published a lower P absorbability from MCP in layers compared to broilers (59-70 versus 83%)].

Phytase supplementation did not improved calcium absorption, resulting in a significantly higher tibia ash content when 1000 or 2000 FYT/kg phytase was added to the diet. The lowest phytase inclusion level or extra DCP-P gave a numerical improvement of tibia ash contents compared to the negative control. The small response on tibia ash content will most probably become more pronounced in a long-term study.

Conclusion
From this experiment with laying hens it was concluded that the dietary supplementation of RONOZYME® HiPhos phytase improved apparent ileal P absorption and tibia ash contents in the 28th week test period.

7.10 Effect of graduated levels of bacterial 6-phytase (= RONOZYME® HiPhos) on apparent ileal digestibility of phosphorus in laying hens fed a maize-based diet low in phosphorus content (H 01/09) (France 2009)

The purpose of the trial conducted at CNRA Village-Neuf (France) was to determine the dose-response relationship between RONOZYME® HiPhos and mineral digestibility at the ileum in laying hens. The laying hens were fed a maize/soybean meal diet. See Annex 27.

Experimental conditions
240 laying hens (Isa Brown), 23 weeks of age, were divided into groups of two hens per cage. The 120 groups were randomly allocated to five treatments with 24 replicates per treatment.

In a 14-day pre-experimental period the laying hens were fed the low phosphorus basal diet without enzyme supplementation. The basal diet in mash form was formulated based on maize (65.0 %) and soybean meal (23.6%) as main ingredients to contain 2.6 g P /kg diet, and 34.5 g Ca /kg diet.
Besides a low available P content the supply of other nutrients, minerals and vitamins with the diet to the hens were adequate to meet the hen's requirement.

Beside the control treatment without enzyme supplementation, graduated levels of the phytase were added to a phosphorus deficient basal diet. The unsupplemented P-deficient basal diet was also fed as such (negative control diet) and was supplemented with 1.0 g P from dicalcium phosphate dihydrate (DCP) as positive control diet. The 6-phytase in liquid form was added at 500, 1000 and 2000 FYT/ kg feed, respectively.

The groups of hens were weighed at the beginning and at the end of the experimental period of the trial. Feed consumption was determined for the experimental period of four weeks. The eggs were collected daily and the number of broken eggs was noted for each group. Once a week, the collected eggs were weighed per group. Total egg production, egg weight and rate of broken eggs were calculated per group.

Excreta from six cages from each treatment were collected by a total collection method after three weeks of feeding experimental diets. The excreta were quantitatively collected once per day.
At 29 weeks of age blood samples from six selected groups of hen per treatment were taken from the vena jugularis to determine the inorganic P and Ca concentrations in plasma.
At the end of the trial, the hens were euthanized and the content of the terminal part of the ileum, were sampled for chemical analysis. The contents of Ca and P were determined in the digesta samples and in the feed. The bone quality was assessed by measuring tibia strength and tibia ash percentage. Toe samples were also obtained.

Composition and nutrient content of the diets

Main ingredients (%)	Neg. control Low available P	Positive control
Maize	65.0	65.0
Soybean meal (50% CP)	23.6	23.6
Calculated and analysed nutrients (g/kg)		
Crude protein	161	161
Methionine + Cystine (%)	0.73	0.73
Phytate P (analysed)	2.11	2.15
Total P (analysed)	2.85	3.90
Available P	0.74	1.75
ME_N (MJ/kg)	11.9	11.9

RONOZYME® HiPhos recoveries in FYT/kg feed and total P in g/kg feed

Treatment Target	A 0	B 500	C 1000	D 2000	E Pos. control 0
Analyzed	<LOQ	562	1114	2097	<LOQ
Total P	2.5	3.0	3.0	2.9	3.9

Results

Inorganic P in plasma, tibia strength, apparent ileal P digestibility and toe ash

Treatment No.	Inorganic P in plasma (mMol/l)	P in excreta (g/kg DM)	Tibia strength (N)	Apparent ileal P digestibility (% of intake)	Toe ash (%)
A	1.23a	9.23	39a	45.8b	31.7a
B	1.50a	8.93	44a	54.8a	31.6a
C	1.36a	8.75	48a	56.1a	30.1a
D	1.20a	8.05	54a	58.7a	31.3a
E	1.41a	13.6	42a	35.8c	33.4a

a,b,c Mean values without a common letter indicate significant differences ($P \leq 0.05$)

Comments

No significant differences among treatments were observed in egg weight, egg production and percentage of broken eggs. Nevertheless the egg production was improved by 2.7 % and 5.7 % with the addition of 500 and 1000 FYT/kg feed, respectively, over the negative control.

The apparent ileal digestibility of phosphorus was clearly improved by phytase supplementation in a dose-dependent manner compared to the negative control. The effects were significant ($p<0.01$). A relative improvement from 19.8 % to 28.2 % was demonstrated with dietary inclusion level of phytase of 500 to 2000 U per kg feed compared to the negative control. The phosphorus concentration in excreta was numerically affected by phytase supplementation with a tendency to be significant ($p = 0.120$).

Mineral concentrations in plasma were not significantly affected by the inclusion of the phytase.Tibia strength responded to phytase supplementation in a linear manner ($R^2=0.99$) to 2000 FYT/ kg. Numerical improvement in a range of 12 % to 38 % was recorded. The effects on tibia strength obtained with the phytase were higher than this obtained with the positive control.

Conclusion

The results of the present trial with laying hens demonstrated that the supplementation of RONOZYME® HiPhos to a laying hen diet was effective in improving apparent ileal phosphorus digestibility over the tested dose range from 500 to 2000 FYT/kg feed. A clear dose response as to apparent ileal digestibility was found. Even at the lowest level of the phytase preparation of 500 FYT/kg feed, beneficial effects on P utilization were recorded compared to the P-deficient negative control group.

7.11 Efficacy of IPA phytase (RONOZYME® HiPhos) in turkeys (Spain 2009)

An experiment was conducted at IRTA to evaluate the effect of graduated amounts of RONOZYME® HiPhos on performance, bone mineralization, blood calcium and phosphorus concentration, and apparent calcium and phosphorus retention in turkeys. See Annex 28.

Experimental conditions

216 day-old female turkeys (BUT 9 strain) were placed into 72 cages and randomly assigned one of six experimental diets: the negative control fed the diet based on maize and soybean meal containing 0.27 % non-phytate phosphorus and 1.2 % calcium, the negative control + RONOZYME® HiPhos phytase at 500 FY/kg feed, 1000 FY/kg feed, 2000 FYT/kg feed, 4000 FYT/kg feed, respectively and a positive control fed the diet containing 0.1 % additional non-phytate phosphorus in the form of dicalcium phosphate.

Birds were weighed at 21 days of age and performance was calculated for the respective period. At 21 days excreta were collected quantitatively for 3 days. Blood samples were also obtained at the end, and blood calcium and inorganic phosphorus concentration was determined. The same bird was sacrificed and left tibia was excised for bone ash determination.

Composition and nutrient content of the diets

Main ingredients (%)	Negative control	Positive control
Maize	44.38	43.77
Soybean meal (48% CP)	49.50	49.60
Calculated nutrients (%)		
Crude protein	28.0	28.0
Methionine + Cystine	1.05	1.05
Non-Phytate P	0.27	0.37
Total P	0.54	0.64
Lysine	1.60	1.60
ME (MJ/kg) *	11.7	11.7

* Metabolizable Energy

RONOZYME® HiPhos recoveries in FYT/kg feed and analysed total P in g/kg feed

Treatment Target	1 0	2 500	3 1000	4 2000	5 4000	6 Pos. control 0
Analyzed	59*	522	1040	1966	4397	61*
P (%)	0.52	0.53	0.53	0.53	0.54	0.62

*: does not mean contamination but native activity in feed

Results

Performance between 1 and 21 days of age, apparent P retention, P in plasma and Tibia ash

Treatment No.	Final weight g	Average daily weight gain g/day	FCR	Apparent P retention (% of intake)	Tibia ash (%)
1	368c	13.8c	1.709a	58.2d	37.2e
2	442b	17.3b	1.468bc	68.4c	41.1d
3	464b	18.2b	1.485bc	72.8b	44.6c
4	458b	17.9b	1.492bc	76.2ab	47.7b
5	515a	20.4a	1.419c	78.7a	49.8a
6	438b	17.0b	1.541b	60.7d	43.8c

a,b,c, d Mean values without a common letter indicate significant differences ($p < 0.05$)

Comments
The feed conversion ratio of the negative control was significantly different from that of the other treatments ($P < 0.05$), and no significant differences were found among all treatments containing RONOZYME® HiPhos. However, the positive control resulted in a significantly less efficient feed conversion than the highest inclusion level of IPA phytase ($P<0.05$).

There was a significant quadratic response to RONOZYME® HiPhos supplementation. Interestingly, the positive control resulted in greater tibia ash percentage than the treatment with 500 FY/kg, while in terms of performance the results were very similar in these two treatments, suggesting the response to phytase to be somewhat lower in terms of bone mineralization than in daily weight gain.

Conclusion
It can be concluded that RONOZYME® HiPhos phytase supplementation of the low-P diet in turkeys improved FCR, tibia ash percentage and calcium and phosphorus retention. Blood calcium level also responded to phytase supplementation.

7.12 Efficacy of a novel phytase product (RONOZYME® HiPhos) in young turkeys poults (USA 2009)

An experiment was conducted at University of Missouri to evaluate the effect of RONOZYME® HiPhos on feed conversion, calcium and phosphorus retention, and bone ash in young turkeys. See Annex 29.

Experimental conditions
150 day-old male turkeys (Nicholas 88 strain) were used and randomly assigned to dietary treatments. A completely randomized design was used with 6 replicate pens of 5 poults allotted randomly to dietary treatments from day 1 to day 21.

Dietary treatments included: the negative control corn-soybean meal basal diet (BD) formulated to contain 1.00% calcium (Ca) and 0.30% non phytate phosphorus (npP) diet, the basal diet supplemented with RONOZYME® HiPhos at 250 FYT/kg feed, 500 FYT/kg feed,1000 FYT/kg feed and 2000 FYT/kg feed, respectively. With the exception of Ca and P, all diets met or exceeded the nutrient requirements of turkey poults (NRC, 1994) Ref. 27, and were fed in mash form.Data were analyzed by analysis of variance using the General Linear Models procedures of SAS (1984). Statistical significance was accepted at $P < 0.05$.

Composition and nutrient content of the diets

Main ingredients (%)	**Basal diet**
Maize	45.38
Soybean meal (48%)	49.49
Calculated nutrients (%)	
Crude protein	28.00
Methionine + Cystine	1.05
Available P	0.30
Ca	1.00
Lysine	1.60
ME (Kcal/kg)	2800

RONOZYME® HiPhos recoveries in FYT/kg feed

Treatment Target	N.C. (1) 0	2 250	3 500	4 1000	5 2000
Analyzed	63*	216	448	799	2024

*: does not mean contamination but native activity in feed

Results

Performance between 1 and 21 days of age, Ca and P retention and bone ash

Treatment No.	Body weight gain (g)	FCR	Ca retention (%)	P retention (%)	Bone ash (%)
Negative C.	436b	1.387	44.08c	50.76d	33.83c
2	445b	1.420	59.52b	64.16c	36.89bc
3	510a	1.317	54.99b	64.04c	40.79b
4	526a	1.366	65.53a	71.76b	41.89b
5	542a	1.334	66.81a	74.32a	47.96a

a,b,c Mean values without a common letter indicate significant differences ($p < 0.05$)

Comments

Performance: Body weight gain increased with increasing dietary RONOZYME® HiPhos phytase inclusion with BWG being significantly higher in birds fed 500, 1000 and 2000 FYT/kg diet compared with birds fed the NC diet. There were no significant differences in feed conversion among dietary treatments.

Bone Mineralization: Bone ash increased with increasing dietary RONOZYME® HiPhos phytase inclusion and was significantly higher in birds fed diets supplemented with RONOZYME® HiPhos phytase at 500, 1000, and 2000 FYT/kg compared with birds fed the NC diet.

Calcium and P Retention: Calcium retention increased with increasing dietary RONOZYME® HiPhos phytase concentration and was higher in all diets supplemented with phytase when compared with the NC diet. Phosphorus retention also increased with increasing dietary RONOZYME® HiPhos phytase concentration and was higher in all diets supplemented with phytase when compared with the NC diet.

Conclusion

These data demonstrate conclusively that RONOZYME® HiPhos phytase was effective in improving phytate P utilization.

7.13 Evaluation of IPA phytase (RONOZYME® HiPhos) in turkeys (France 2009)

An experiment was conducted at INRA (France) to evaluate the effect of graduated amounts of RONOZYME® HiPhos on performance, bone mineralization, calcium and phosphorus concentrations in blood serum, and apparent phosphorus utilization in turkeys. See Annex 30.

Experimental conditions

240 day-old male BUT T9 turkeys were used in this 4-week study. From day 9 of the experiment to the end of the study (day 29), the animals were set into cages (2 birds per cage) and assigned to the treatments which are summarized below. At day 29, blood samples were taken from 1 bird per cage. The same bird was slaughtered and left tibia was taken. Prestarter and starter diets were based on maize and soybean meal.

Treatment	Description
R1	Low P basal diet (0.2% available P)
R2	Positive control diet (0.25 % available P)
R3	Positive control diet (0.30 % available P)
R4	Positive control diet (0.35 % available P)
R5	R1 + RONOZYME® HiPhos phytase at 500 FYT/kg diet
R6	R1 + RONOZYME® HiPhos phytase at 1000 FYT/kg diet
R7	R1 + RONOZYME® HiPhos phytase at 2000 FYT/kg diet
R8	R1 + RONOZYME® HiPhos phytase at 4000 FYT/kg diet

Composition and nutrient content of the diets

Main ingredients	%			
Maize	40.4			
Soybean meal (48%)	44.8			
Wheat	5.3-5.8			
Nutrients (calculated) (%)	R1 0.2 av. P	R2 0.25 av. P	R3 0.30 av. P	R4 0.35 av. P
Protein (%)	25.00	25.00	25.00	25.00
Methionine + Cystine	1.15	1.15	1.15	1.15
Available P	0.20	0.25	0.30	0.35
Ca	1.11	1.08	1.07	1.03
Lysine	1.67	1.67	1.67	1.67
ME (Kcal/kg)	2950	2950	2950	2950

RONOZYME® HiPhos recoveries in FYT/kg feed

Treatment Target	R1, R2, R3, R4 0	R5 500	R6 1000	R7 2000	R8 4000
Analyzed	44*, 55*, 44*, 45*	581	919	2327	4075

*: does not mean contamination but native activity in feed

Results

Performance between days 9 and 29, P utilization and bone ash, P in serum, tibia ash

Treatment	Weight (g) Day 29	FCR D9/D29	Apparent utilization of P (% DM)	Tibia ash (% DM)	Inorganic P in serum (mg/l)
R1	767e	1.685a	50.98f	30.13e	32e
R2	860d	1.598bc	52.02f	34.40d	33de
R3	918c	1.616b	52.44ef	38.53c	41d
R4	955bc	1.584bc	54.34e	41.27ab	53c
R5	907cd	1.584bc	60.95d	36.84c	36de
R6	949bc	1.563bcd	66.00c	40.40b	51c
R7	990ab	1.543cd	72.26b	43.37a	63b
R8	1013a	1.519d	76.96a	44.78a	80a
	P< 0.01	P< 0.01	P< 0.01	P< 0.01	P< 0.01

Comments

A positive and significant dose related response to phytase supplementation was noted in terms of live weight and feed conversion. Dietary supplementation with RONOZYME® HiPhos phytase at 500, 1000, 2000 and 4000 FYT/kg significantly improved the P utilization from 51.0% (negative control) to 61.0, 66.0, 72.3 and 77.0%, respectively. As the consequence of this effect, tibia ash percentage significantly increased from 30.1% (negative control) to 36.8, 40.4, 43.4 and 44.8%, respectively.

Conclusion

It can be concluded that the RONOZYME® HiPhos phytase improved the digestibility of P and increased bone mineralization.

8 Human safety

Although this phytase is intended for use in animal feed only, a generally accepted starting point for food safety evaluation of enzymes is based on the concepts initially developed for human food enzymes by Pariza and Foster 1983 (Ref. 4) and further developed by IFBC in 1990 (Ref.28), the EU SCF in 1992, the OECD in 1992 and 1993, ILSI Europe Novel Food Task Force in 1996 and the FAO/WHO in 1996. In 2001 Pariza and Johnson (Ref. 5) published an update of the initial concept of Pariza and Foster in order to take into account questions of genetic engineering further developed by Pariza and Cook in 2010 (Ref. 29).

The primary consideration is the identity and safety of the production organism. Regarding RONOZYME® HiPhos, this assessment is described in detail under section 6 for the microbial production strain, a genetically engineered variant of *Aspergillus oryzae*. The production strain is derived from a safe strain line, which has been used by Novozymes A/S over many years for food and feed enzyme production. Any mycotoxin contamination of RONOZYME® HiPhos formulations arising from the production strain is effectively excluded. In addition, no genetic sequences of concern were added by genetic modification of the production strain. The enzyme is produced by methods and under culture conditions that ensure controlled fermentation. The presence of toxic or undesirable substances as well as the introduction of contaminating micro-organisms is thus prevented.
In addition raw materials of food or feed grades are used during manufacturing. Furthermore, the production strain is absent in the final enzyme preparations. It is the policy of DSM Nutritional Products Ltd. and Novozymes A/S that all enzyme preparations must conform to the purity criteria for enzyme preparations set up by the Food Chemicals Codex (FCC) Ref. 21 and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) Ref. 22. In the d escription of the impurity profile it was shown that these criteria are met. Based on these facts, it is concluded that the resulting enzyme product would be safe for humans even if it was used as an enzyme in food.

This conclusion is corroborated by the toxicological tests (Ames test and micronuclei assay) and the sub chronic toxicity in rats as described previously. None of the studies conducted with RONOZYME® HiPhos produced any adverse effect, which could give rise to concern. In the 13-week sub-chronic oral toxicity study in rats the active ingredient of the product was well tolerated up to the highest dose tested. The No-Observed-Adverse-Effect Level (NOAEL) in this study was determined to be 860 mg Total Organic Solids (TOS)/kg bw/day wich is equal to 523710 FYT/kg bw/day. In one of the target animal species broiler chickens, this NOAEL translates into high safety margins, >1000 fold.

As the production strain is safe and the highest expected TOS exposure of the target animals low, no appreciable or toxic residues in the animal products are expected. The appearance of any substance derived from RONOZYME® HiPhos in animal products, except for minute quantities of amino acids, is highly unlikely.
Therefore, the recommended use of RONOZYME® HiPhos in compound feed for poultry is safe for consumers.

9 Environmental safety

RONOZYME® HiPhos is produced under compliance with the NIH directive for the fermentation of genetically engineered organisms. Therefore there is no release of the live organism into the enviroment. The production organism, *Aspergillus oryzae* is classified by EPA as an exempt organism under TSCA section 5 and its use does not require pre-market clearance.

RONOZYME® HiPhos is a feed enzyme preparation. The phytase it contains will be degraded in the gastrointestinal tract of the animals to amino acids which will be metabolized by the animal and its microflora. Any residual amount of phytase from RONOZYME® ProAct is not expected to have a detectable effect on organic matter in the soil or in the watercourse.

RONOZYME® HiPhos is free of rDNA in the finished product.

10 GRAS EXPERT PANEL OPINION

THE SAFETY AND GENERALY RECOGNIZED AS SAFE (GRAS) STATUS OF THE PROPOSED USES OF RONOZYME®HiPhos, A 6- PHYTASE PREPARATION PRODUCED BY AN *ASPERGILLUS ORZAE* STRAIN EXPRESSING A SYNTHETIC *CITROBACTER braakii* GENE CODING FOR A 6-PHYTASE FOR USE IN POULTRY, SWINE AND FISH RATIONS

Introduction

The undersigned, an independent panel of experts, qualified by their scientific training and national and international experience to evaluate the safety of food and food ingredients (the "Expert Panel"), was specially convened by DSM Nutritional Products, and asked to evaluate the safety and Generally Recognized as Safe (GRAS) status of the proposed uses of RONOZYME® HiPhos, a 6-phytase product for use in poultry, swine and fish feeds, based on scientific procedures as described in Title 21 of the Code of Federal Regulations (21CFR§170.30) (U.S. FDA, 2007). Novozymes A/S and DSM Nutritional Products Ltd. are business partners and co-developed RONOZYME® HiPhos. Novozymes A/S, Copenhagen, Denmark, is responsible for the manufacturing of the product (supplier) while DSM Nutritional Products Ltd. Basel, Switzerland, has market exclusivity for distribution of the product. DSM Nutritional Products, Parsippany, NJ will distribute the product in North America.

RONOZYME® HiPhos is an enzyme preparation that contains a 6-phytase [IUB number 3.1.3.26, CAS number 9001-89-2 (phosphatase, acid)]. The enzyme hydrolyzes bonds between phosphate (P) and myo-inositol in phytic acid and its salt and thus increases the availability of phosphorus from typical plant-based diets. Both the nucleotide sequence of the gene encoding the 6-phytase as well as the resulting primary amino acid sequences of the phytase are known. Efficacy is demonstrated for RONOZYME® HiPhos by significant increases in phosphorus digestibility and utilization in mineral balance studies in the target animal species.

Phytase is a well-established enzyme product used in animal feeds and review papers on the use of phytase in poultry, swine and fish nutrition have been published by Selle & Ravindran 2007; Broz and Ward 2007; Singh 2008; Aureli et *al.* 2011; Vielma et *al.* 2004; Sajjadi et Carter,2004; Dalsgaard et *al.* 2009; Tudkaew et *al.* 2008.

Over the last 10-15 years, DSM Nutritional Products Ltd. and Novozymes A/S have performed several dozen efficacy studies with phytases expressed in *Aspergillus oryzae*. Their efficacy has been substantiated and utility quantified in the target animals. A number of these efficacy and utility studies have been published as noted above.

The addition of the phytase, RONOZYME® HiPhos - as expressed in *Aspergillus oryzae*, to animal diets, which is the subject of the present GRAS evaluation, increases the bioavailability and utilization of phytin-bound phosphorus from plant materials. This in turn allows for a reduction of inorganic phosphorus supplementation in the animal diets without compromising performance. The lower phosphorus content in the diets helps to overcome environmental phosphorus contamination problems arising in areas with high animal production concentration.

A comprehensive search of the scientific literature for safety and toxicity information on RONOZYME® HiPhos was performed by DSM Nutritional Products and included both a general Google literature search, as well as their use of the SciVerse-Scopus abstract and citation

database of peer-reviewed literature and also the internal library sources of both DSM and Novozymes using the following search terms "Aspergillus, Bacillus, Citrobacter, phytases, toxins". The last search was done in late February 2011.The resulting data were compiled into a safety dossier and reviewed by The Tarka Group, Inc., and then subsequently updated by DSM Nutritional Products.

All relevant publications were reviewed, summarized and incorporated into a GRAS dossier, "**THE SAFETY AND GENERALY RECOGNIZED AS SAFE (GRAS) STATUS OF THE PROPOSED USES OF RONOZYME®HiPhos, A 6- PHYTASE PREPARATION PRODUCED BY AN *ASPERGILLUS ORZAE* STRAIN EXPRESSING A SYNTHETIC *CITROBACTER braakii* GENE FOR CODING FOR 6-PHYTASE FOR USE IN POULTRY, SWINE AND FISH RATIONS**," prepared by The Tarka Group, and submitted to the Expert Panel. Copies of the literature were available for review by the Expert Panel.

The Expert Panel evaluated information pertaining to the method of manufacture, product specification, analytical data, intended use levels in specified food formulation rations for the specified avian, porcine and fish species, potential exposure estimates from consumption of foods from all intended uses, safety studies conducted with RONOZYME® HiPhos and other information on safety and tolerance deemed relevant. The members of the Expert Panel were Professor Michael W. Pariza, PhD (Chairman), Professor John A. Thomas, PhD, Fellow, ATS, and Stanley M. Tarka, Jr., PhD, served as a Technical Advisor to the Panel. Following independent and collective critical evaluation of the information summarized in the Dossier, the Expert Panel conferred and unanimously agreed to the decision described herein.

DSM Nutritional Products intends to market RONOZYME® HiPhos as an enzyme for use in significantly liberating phosphorus from plant origin ingredients, making otherwise non-digestible phytate phosphorus bioavailable to the intended species and resulting in a reduction of inorganic phosphorus in the rations as well as a reduction in the amount excreted into the environment. This in turn allows for a reduction of inorganic phosphorus supplementation in the animal diets without compromising performance. The lower phosphorus content in the diets helps to overcome environmental phosphorus contamination problems arising in areas with high animal production concentration. Phytase enzymes derived from *Aspergillus orzyae* variants are permissible as feed ingredients in swine and poultry diets for the purpose of increasing the bioavailability of phytin-bound phosphorus in corn, soybean meal, sunflower meal, hominy, tapioca and plant by-products (Association of American Feed Control Officials, 2011). The product RONOZYME® HiPhos is intended for use in poultry, swine and fish feeds. The recommended use level of RONOZYME® HiPhos is 250 FYT to 4000 FYT/Kg of poultry feed and swine feed and 500 FYT/kg to 2000 FYT/kg of fish feed. One Phytase Unit (FYT) [also denominated U (units) and FYT(B) in reports] is defined as the amount of enzyme that releases 1 µmol of inorganic phosphate from phytate per minute under reaction conditions with a phytate concentration of 5.0 mM at pH 5.5 and temperature 37°C.

As a consequence of the large safety margins, no regulatory maximum dose for RONOZYME® HiPhos in feed is necessary. However, taking into account both cost-benefit and manufacturing considerations, and in order to allow flexibility in feed formulation, the following upper doses are recommended: 4,000 FYT/kg feed for poultry and swine and 2,000 FYT/kg feed for fish feed.

The Expert Panel noted that RONOZYME® HiPhos is a feed enzyme preparation and the phytase it contains will be degraded in the gastrointestinal tract of the animals to amino acids which will be metabolized by the animal and its microflora. Consequently, there are no safety issues with any accumulation of its breakdown products.

The Expert Panel convened via telephone conference call on November 21, 2011, and unanimously concluded that RONOZYME® HiPhos, produced consistent with current good manufacturing practice (cGMP) and meeting appropriate specifications, is safe for its intended uses as listed in paragraph one above and under "Intended Use" below. The Expert Panel further concluded that these intended uses are GRAS based on scientific procedures. It is also the opinion of this Expert Panel that other qualified experts would concur with these conclusions.

The scientific analysis supporting our conclusions is presented below.

Description

The main enzyme activity in RONOZYME® HiPhos is 6-phytase.

Common name: Phytase
Generic name: Phosphoric monoester hydrolase
IUB nomenclature: 6-phytase
IUB number: 3.1.3.26
CAS number: 9001-89-2 (phosphatase, acid)
EINECS No: 232-630-9 (phosphatase, acid)

The 6-phytase is expressed in a genetically engineered *Aspergillus oryzae* production strain The host strain is developed from a strain line which has been used in production at Novozymes A/S for more than 30 years.

The DNA sequence encoding for the mature 6-phytase comprises (b) base pairs (bp). The enzyme hydrolyzes bonds between phosphate (P) and myo-inositol in phytic acid and its salt and thus increases the availability of phosphorus from typical plant-based diets. Both the nucleotide sequence of the gene encoding the 6-phytase as well as the resulting primary amino acid sequences of the phytase are known.

Three product forms of RONOZYME® HiPhos will be available, two dry forms and a liquid form. RONOZYME® HiPhos (GT) is a granulated thermo-tolerant form with a minimum enzyme activity of 10,000 FYT/gram. RONOZYME® HiPhos (M) is a micro granulated form with a minimum enzyme activity of 50,000 FYT/gram. RONOZYME® HiPhos (L) is an aqueous liquid with a minimum enzyme activity of 20,000 FYT/g. Additional forms may be manufactured with feed grade ingredients to satisfy new market needs.

Current Regulatory Approvals for Phytase Uses

Phytase derived from *Aspergillus orzyae* variants are permissible as feed ingredients in swine and poultry diets as noted in Table 1-1 as published in the Manual of the Association of American Feed Control Officials (AAFCO), 2011. The Association of American Feed Control Officials (AAFCO) is a voluntary membership association of local, state and federal agencies charged by law to regulate the sale and distribution of animal feeds and animal drug remedies. Although AAFCO has no regulatory authority, the Association provides a forum for the membership and industry representation to achieve three main goals:ensuring consumer protection, safeguarding the health of animals and humans and providing a level playing field of orderly commerce for the animal feed industry. These goals are achieved by developing and implementing uniform and equitable laws, regulations, standards, definitions and enforcement

policies for regulating the manufacture, distribution and sale of animal feeds - resulting in safe, effective and useful feeds by promoting uniformity amongst member agencies.

Table 1-1. Excerpt from Table 30.1 Enzymes / Source Organisms Acceptable for Use in Animal Feeds AAFCO 2011 Official Publication, pgs 394 & 395

Enzyme Name	Source Enzyme	Typical Substrate	Function	Current Supported Use
Phytase	*Aspergillus niger,* var. *Aspergillus oryzae*, var. *Aspergillus oryzae* expressing the *Peniophora lycii* phytase gene	Corn, soybean meal, sunflower meal, hominy, tapioca, plant by-products	Hydrolyzes phytate	Increases the digestibility of phytin-bound phosphorus in swine and poultry diets

The safety of the enzyme products mentioned in the following table (2-1) have been assessed according to the principles of the European Union's Scientific Committee on Food (SCF) guidelines. These products have been approved in a wide range of countries (e.g. Australia, Canada, Denmark, France, the European Community (6-phytase and xylanase producing strains), or have been the subject of GRAS Notifications accepted by the U.S.FDA or assessed in other reviews in the USA). Table 2 demonstrates that *Aspergillus oryzae* BECh2 was used as the host strain in the construction of Novozymes' production strains for a (b) (4) and the currently globally marketed RONOZYME® P- and RONOZYME® NP *Peniophora lycii* 6-phytase. The latter are approved in the EU as feed additives and marketed under the trade names RONOZYME® P 5000 (CT), RONOZYME® P 20,000 (L) and RONOZYME® NP (CT), RONOZYME® NP (M) and RONOZYME® NP (L); respectively.
(http://ec.europa.eu/food/food/animalnutrition/feedadditives/comm_register_feed_additives_1831-03.pdf)

Table 2-1. (b) (4) food and feed enzymes (b) (4)

(b) (4)

a) At least the following: 13 week acute oral toxicity in rats; Ames test; In vitro human lymphocyte cytogenetic assay

Manufacturing Process

Novozymes A/S and DSM Nutritional Products Ltd. are business partners and co-developed RONOZYME® HiPhos. Novozymes A/S, Copenhagen, Denmark, is responsible for the manufacturing of the product (supplier) while DSM Nutritional Products Ltd. Basel, Switzerland, has market exclusivity for distribution of the product. DSM Nutritional Products, Parsippany, NJ will distribute the product in North America. The manufacturing process is composed of the following steps: fermentation, purification, formulation, and finally, quality control of the finished product.

The phytase in RONOZYME® HiPhos is produced from a non-pathogenic microbial source derived from a safe strain line for which the expression of residual mycotoxin-forming capacity is effectively prevented. It is produced by methods and under culture conditions that ensure controlled fermentation. The introduction of contaminating microorganisms is therefore prevented. Furthermore, the production strain is absent in the final enzyme preparations.

Intended Uses

RONOZYME® HiPhos will be included in animal feeds of poultry, swine and fish for the nutritional purpose of increasing the digestibility of phytate. The recommended use level of RONOZYME® HiPhos is 250 FYT to 4000 FYT/Kg of poultry feed and swine feed and 500 FYT/kg to 2000 FYT/kg of fish feed. One Phytase Unit (FYT) [also denominated U (units) and FYT(B) in reports] is defined as the amount of enzyme that releases 1 µmol of inorganic phosphate from phytate per minute under reaction conditions with a phytate concentration of 5.0 mM at pH 5.5 and temperature 37°C.

As noted above, three product forms of RONOZYME® HiPhos will be available, two dry forms and a liquid form. RONOZYME® HiPhos (GT) is a granulated thermo-tolerant form with a minimum enzyme activity of 10,000 FYT/gram. RONOZYME® HiPhos (M) is a micro granulated form with a minimum enzyme activity of 50,000 FYT/gram. RONOZYME® HiPhos (L) is an aqueous liquid with a minimum enzyme activity of 20,000 FYT/g. Additional forms may be manufactured with feed grade ingredients to satisfy new market needs

Exposure

Novozymes A/S has used *Aspergillus oryzae* production strains for over 40 years. A line of *Aspergillus oryzae* host strains, including (b) (4) have previously been used as host strains for Novozymes' food and feed enzyme products. These production strains were constructed by standard transformation procedures using well-known plasmid vectors and well-characterized DNA sequences that were integrated into the *Aspergillus oryzae* host strain chromosome. Extensive toxicological testing, confirming the safety of enzyme preparations derived from these *Aspergillus oryzae* production strains, has been documented in the scientific literature.. No toxicological effects were observed for any of the test substances produced by strains derived from this *Aspergillus oryzae* lineage of host strains (Greenough et *al.* 1996) Ref 6.

Safety studies, including a 13-week oral toxicity in rats, and genetic toxicity testing including the Ames bacterial mutagenesis test and human lymphocyte cytogenetic assay have been completed on these enzyme preparations. The conclusions from all of these studies were that oral administration to rats of the highest possible dosage level for 13 weeks did not reveal any signs of toxic effects related to treatment. No mutagenic activity was found in any of the test substances by the Ames' test or the human lymphocyte test.

These studies also support the view that strains derived from *Aspergillus oryzae* (b) (4) can be used safely for the production of food and feed enzymes. Accordingly, production strains which are constructed from the host strain, *Aspergillus oryzae* (b) (4) where the genetic modifications are well characterized and specific, utilizing well-known plasmids, and for which the introduced genetic material does not encode for the expression of any known harmful or toxic substances, constitutes a safe strain lineage according to the outline by (b) (4)

The strain designed to produce the HiPhos phytase subject to this application, has been developed from a host strain derived from (b) (4)

It is concluded that the *Aspergillus oryzae* strain used for expression of synthetic *Citrobacter braakii* 6-phytase is a member of the same safe strain (b) (4) as the strains used to express previous approved phytases.

The production strain does not have the capacity to form any aflatoxins nor the mycotoxins (b) (4) that could possibly be linked to the original *Aspergillus* oryzae wild type strain IFO4177.

The EPA performed a risk assessment of *Aspergillus oryzae* in 1997 and updated the report in 2007 as part of the Biotechnology Program under the Toxic Substance Control Act. The EPA noted that industrial strains of A. oryzae lack the ability to produce aflatoxins. (U.S. EPA *Aspergillus oryzae* Final Risk Assessment, 2007) Ref. 20.

Consequently, only (b) (4) remained as substances of some, albeit limited, concern for strain (b) (4) and production strains derived thereof. Tests performed with RONOZYME® HiPhos have shown that those substances are not detected (below limit of quantification).

Additional studies on sequence homology of RONOZYME® HiPhos phytase to known toxins and allergens as recommended by Joint WHO/ FAO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (2001) and showed that the RONOZYME® HiPhos phytase sequence is not a homolog to any toxin of the UNIPROT database. No gene coding for antibiotic production has been added in the construction of the genetically modified production strain *Aspergillus oryzae*. It can therefore be assumed that the *Aspergillus oryzae* production strain is lacking the capability of antibiotic production. No antibiotic resistance markers are present on the expression plasmids. No antibiotic resistance genes have been added to the production strain through them.

All the foregoing data support the safety of exposure to this enzyme.

Safety Data

As part of their safety assessment for the intended uses of RONOZYME® HiPhos Phytase in rations of poultry, swine and fish, the Expert Panel critically evaluated available safety information on RONOZYME® HiPhos Phytase. This included an evaluation of all available and published toxicological tests on mutagenicity, cytogenetic effects and subchronic toxicity, as well as a feeding safety tolerance testing in target animals for RONOZYME® HiPhos Phytase. These are corroborative studies that support the existing published scientific literature on phytases.

In all toxicological studies carried out both *in vitro* and *in vivo*, the enzyme concentrate batch was used. No adverse effects of the test substance were found in these safety tests which are described in detail below and summarized in Table 3. These studies were published in June 2011.

Bacterial Mutation Assay (Ames Test)

RONOZYME® HiPhos was examined for mutagenic activity in four histidine-dependent strains of *Salmonella typhimurium*, strains TA98, TA100, TA1535 and TA1537, and the tryptophan-dependent strain *Escherichia coli* WP2uvrApKM101.The study was conducted in the presence and absence of an activating system derived from rat liver (S9 mix). All tests included solvent (purified water) and positive controls with and without S9 mix. All bacterial strains were tested at concentrations of the test article ranging from 156 to 5000 µg per ml (plate).
The results of the experiments gave no indication of mutagenic activity of RONOZYME® HiPhos in either the absence or presence of S9, when tested under the conditions employed in this study.

Micronucleus Assay

The objective of this study was to evaluate the clastogenic and aneugenic potential of RONOZYME® HiPhos by examining its effects on the frequency of micronuclei in cultured human peripheral blood lymphocytes treated in the absence and presence of S-9.

The test methodology is based on the current version of draft OECD guideline 487 [10] and accepted scientific/regulatory principles described in current guidelines for clastogenicity testing *in vitro*. The study was conducted in compliance with current GLP practices. The highest dose level tested was 5000 μg/ml. (the recommended maximum for *in vitro* chromosome aberration studies according to current regulatory guidelines).

RONOZYME® HiPhos was added at 48 hours following culture initiation (stimulation by PHA). Cells were exposed to the test article for 3 hours in the absence and presence of S-9 (from rats induced with Aroclor). In addition, a continuous 24 hour treatment (equivalent to approximately 1.5 to 2 times the average generation time of cultured lymphocytes from the panel of donors used in this laboratory) in the absence of S-9 was included. All cultures were sampled 24 hours after the beginning of treatment (i.e. 72 hours after culture initiation). Appropriate negative (vehicle) control cultures were included in the test system under each treatment condition.

The proportion of micronucleated binucleate cells (MNBN) in these cultures fell within current historical vehicle control (normal) ranges. Mitomycin C (MMC) and Vinblastine (VIN) were employed as clastogenic and aneugenic positive control chemicals respectively in the absence of rat liver S-9. Cyclophosphamide (CPA) was employed as a clastogenic positive control in the presence of rat liver S-9 microsomes. Cells exposed to these chemicals were sampled in the Main Experiment at 24 hours after the start of treatment; all compounds induced statistically significant increases in the proportion of cells with micronuclei.

The assay system was therefore considered as both sensitive and valid.

Treatment of cells with RONOZYME® HiPhos in the absence and presence of metabolic activation resulted in frequencies of MNBN cells, which were similar to and not significantly ($p \leq 0.05$) different from those observed in concurrent vehicle controls for all concentrations analyzed. The MNBN cell frequency of all RONOZYME® HiPhos treated cultures fell within normal ranges.

Subchronic Oral Toxicity in Rats

The objective of this study was to assess the systemic toxic potential of RONOZYME® HiPhos in the rat when administered daily by oral gavage over a period of 13 weeks.

The study was carried out in accordance with the OECD guideline 408 (1998). It was also conducted in compliance with the requirements of current, international Good Laboratory Practice.

A total of 80 Crl: CD® (SD) rats (40 males and 40 females) no older than 35 days and weighing from 118-145 g for males and 108-135 g for females were used in the study. The animals were allocated into four groups each comprising ten male and ten female rats and administered daily doses of RONOZYME® HiPhos by gavage at doses of 1.0, 3.3 or 10.0 ml/kg/day (10.0 ml/kg/day = 860 mg TOS/kg body weight/day) at a constant dose volume of 10 ml/kg

bodyweight. A similarly constituted control group received the vehicle, purified water, at the same volume dose.

During the study, clinical condition, detailed physical and arena observations, sensory reactivity, grip strength, motor activity, bodyweight, food consumption, ophthalmic examination, hematology, blood chemistry, organ weight, macro pathology and histopathology investigations were undertaken.

At termination, all animals were sacrificed and subjected to a detailed necropsy. There were no deaths during the study and there were no treatment-related findings observed during the routine weekly physical examination, the arena observations or during post- dosing observations. The functional observation battery investigation did not indicate any treatment related effects.

Slightly higher bodyweight gains were observed during the first two weeks of treatment for males receiving 10.0 ml/kg/day of RONOZYME® HiPhos and in the first week of treatment for females receiving 10.0 ml/kg/day. Food consumption was not affected by treatment. There were no treatment-related ophthalmic findings. Hematology and blood chemistry investigations did not indicate any toxicologically significant changes. There were no effects observed from treatment on organ weights and no treatment-related macroscopic or histopathological findings.

It was concluded that oral administration of RONOZYME® HiPhos to CD rats at doses up to 10.0 ml/kg/day for 13 weeks was well tolerated and did not cause any toxicologically significant changes. Consequently, the no-observed-adverse-effect-level (NOAEL) was considered to be 10.0 ml/kg/day, the highest dose level administered, which was equivalent to 860 mg TOS/kg body weight/day.

Table 3. Summary of Safety Studies*

Test	Test Object	Dose	Result	Report
Bacterial Mutation (Ames)	*Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537	156 to 5000 µg /ml (plate) 7 to 225 FYT	No significant increase in the revertant numbers	20088064
	Escherichia coli strain WP2uvrApKM101	156 to 5000 µg /ml (plate) 7 to 225 FYT	No significant increase in the revertant numbers	
Micronucleus Test	Cultured Human Peripheral Blood lymphocyte	3000 to 5000 µg /ml	No significant difference	20086022
13 Week Sub-Chronic Oral Toxicity	Crl: CD® (SD) rats	0, 1, 3.3, 10.0 ml/kg/day or 0; 50,000 ; 165,000 and 500,000 FYT units /Kg/day	no treatment-related macroscopic or histopathological findings	20086016
In vitro Skin Irritation (Episkin)	Episkin Standard Model	10 µl/ tissue or (0.5 FYT/tissue)	Negative	200905296
Acute Eye Irritation	Rabbit	0.11 ml or (5,000 FYT)	Negative	20096001

*** Safety Data published as**

Lichenberg, J, Pedersen, PB, Elvig-Joergensen, SG, Skov, LK, Olsen, CL, Glitsoe, LV.. (2011). Toxicological studies on a novel phytase expressed from synthetic genes in *Aspergillus oryzae*. ***Regulatory Toxicology and Pharmacology* 60 (3): 401-410.**

Effect of RONOZYME® HiPhos Phytase on the Microflora of the Digestive Tract

RONOZYME® HiPhos is a 6-phytase preparation acting on phytate as a substrate. This substrate is typically found in plants and materials derived thereof. As stated earlier, RONOZYME® HiPhos does not contain the production organism and has no antimicrobial activity. This has been further validated on 3 lots of enzyme concentrate.

Furthermore, the product conforms to the JECFA and FCC purity specifications for food enzymes, which stipulate limits for microbiological contaminants. In particular, the absence of contamination by *Salmonella spp.* and enteropathogenic *E.coli* has been established.

Therefore, no direct effects on the microflora of the digestive tract are expected for this product.

Tolerance Studies in Targeted Animal Species

A series of Tolerance studies to ensure safety and lack of adverse effects were conducted in broilers (male and female), laying hens, turkeys (toms), weaned piglets and female gilts, gestating and lactating sows and rainbow trout. These are summarized in Table 4. The poultry studies were published in February 2011 (Aureli et. al. (2011)). The fish study was submitted for publication in October, 2011; see Verlhac-Trichet et. al (2011). The manuscript for the swine studies is Guggenbuhl et. al. (2011).
At 10 times the highest recommended dose, RONOZYME® HiPhos (M) was well tolerated by the broiler chickens and had beneficial effects on phosphorus bioavailability. Dietary administration of RONOZYME® HiPhos (M) resulted in beneficial effects relating to these chickens' performance. The final body weights of birds receiving the phytase at 4000 and 40,000 FYT/kg diet were significantly increased from 1720 g (control) to 1917 and 1925 g; respectively. Due to this increased growth rate in both phytase supplemented groups, the overall feed conversion ratio was numerically improved from 1.913 (control) to 1.838 and 1.796; respectively. No adverse clinical signs were noted and there was no mortality during this study. Furthermore, no pathological changes were observed in birds during the post-mortem necropsy.

Hematological and biochemical examinations did not reveal any obvious changes due to dietary administration of RONOZYME® HiPhos. However, a significantly increased serum concentration of inorganic P was found in both treated groups, and this finding confirms the efficacy of RONOZYME® HiPhos (M) in improving phosphorus bioavailability.

No adverse effects were observed from the use of 40,000 FYT/kg level of phytase on performance, mortality or hematological-biochemical characteristics, or with respect to the maximum recommended level (4000 FYT/kg). The response beyond the 4000 FYT/kg of phytase level was significantly improved for ileal P digestibility.

At 10 times the highest dose recommendation, RONOZYME® HiPhos (M) was well tolerated by the laying hens and had beneficial effects.

The inclusion of RONOZYME® HiPhos Phytase in diets for turkeys up to a level of 40,000 FYT/kg over a period of 42 days (day 0 to 42 of age) had no detrimental effects on performance (feed intake, body weight gain and feed conversion ratio), nor any effect on the levels of total and white blood cells and the concentrations or activity of a number of clinical chemical blood

parameters. The present study did not reveal any signs of intolerance of young turkeys for RONOZYME® HiPhos.

Dietary administration of RONOZYME® HiPhos resulted in beneficial effects on performance of the piglets. The final body weights of the piglets receiving 4000 and 40,000 FYT/kg diet were significantly increased by more than 12% when compared to the negative control. The feed conversion ratio was also significantly improved from 2.75 (control) to 2.18 and 2.20, respectively. No mortality occurred during the study. Furthermore, no pathological changes were observed in piglets during the post-mortem necropsy. No unfavorable effects due to dietary administration of RONOZYME® HiPhos were observed.

The blood biochemistry examination revealed that the values of ALT were increased in all examined animals of all groups, values of CPK were increased in 15 piglets of Group A (control), in 9 piglets of Group B (4000 FYT/kg diet) and 11 piglets of Group C (40,000 FYT/kg diet).

The amount of serum phosphorus was increased above the physiological range in 12 animals of control Group A, in 4 piglets of Group B and 5 piglets of Group C. All other biochemical parameters were within physiological ranges and any deviations were sporadic and not considered to be related to treatment.

The hematological examination revealed that the amount of RBC in animals of all groups was higher (Group A 10, Group B 12 and Group C 8 piglets), the value of HCT was increased in some animals in Groups B and Group C, whereas in animals of Group A it corresponded to the physiological range. The WBC in 1 piglet of Group A and in one piglet of Group C were above the physiological range. All other hematological parameters were either within or below physiological ranges.

In spite of significant differences among treatment groups for some biochemical and haematological parameters, no remarkable or significant differences between the maximum recommended dose (4000 FYT/kg diet)and the highest dose (40,000 FYT/kg diet) of RONOZYME® HiPhos phytase tested were noted for all relevant parameters.

Based on these results, it is concluded that there were no unfavorable effects of the RONOZYME® HiPhos Phytase in weaned piglets when used at the maximum recommended and ten times higher doses.

Long-term supplementation of RONOZYME® HiPhos at the overdose level (40,000 FYT/kg) in sow diets during an overall reproductive cycle, including the successful service after weaning, induced lower estimated body weight losses during the 28-day lactation period and significantly improved body weight gains of these piglets compared to those fed the standard prestarter diet without RONOZYME® HiPhos. Additionally, blood examinations and the weaning to service interval of sows fed with the ten-fold overdose level of RONOZYME® HiPhos showed no negative health or relevant fertility effects.

Results from feeding to salmonid fish demonstrate that RONOZYME HiPhos is well tolerated by salmonid fish fed a diet supplemented as high as 200,000 FYT/kg feed, which represents 100 times the maximum recommended dose for this feed additive.

Table 4. Summary of Target Animal Safety Studies

Target Animal	Number of Animals	Doses in FYT / kg	Study Period In Days	Parameters Compared	Observations	Report
Weaned Piglets Females Gelts	48	0, 4000 & 40,000	42	Pathology Blood Chemistry Body Weight	No Abnormalities No Adverse Effects	00000962
Gestating Sows	36	0, 4000 & 40,000	108	Pathology Blood Chemistry Body Weight	No Abnormalities No Adverse Effects	00003288
Lactating Sows	36	0, 4000 & 40,000	43	Pathology Blood Chemistry Piglet Body Weight	No Abnormalities No Adverse Effects	00003288
Broilers Male/Female	192	0, 4000 & 40,000	35	Pathology Blood Chemistry Body Weight	No Abnormalities No Adverse Effects	00000961
Laying Hens	288	0, 500 to 40,000	56	Pathology Blood Chemistry Egg Production	No Abnormalities No Adverse Effects	00000960
Turkeys-Toms	480	0, 4000 & 40,000	42	Pathology Blood Chemistry Physical Characteristics	No Abnormalities No Adverse Effects	00003289
Rainbow Trout	525	0, 2000, 200,000	58	Pathology Physical Characteristics	No Abnormalities No Adverse Effects	00009074

Targeted Animal Species Safety Margin Calculations

The product RONOZYME® HiPhos is intended for use in poultry, swine and fish feeds. The standard recommended dose range of the product is 500 - 4000 FYT/kg feed.

Based on the NOAEL of 860 mg TOS/kg bw-day derived from the 13 week subchronic study in rats and typical feed intake values as derived from NRC[1] feeding tables, the following safety margins can be calculated for the different categories of animals:

Table 5. Intake estimation and safety margins in target species

Target species	Body weight kg	Typical feed intake kg feed/ day [1] & [2] & [3]	RONOZYME® HiPhos highest use recommendation		Highest expected enzyme intake		Safety margin
			FYT/ kg feed	mg TOS*/ kg feed	FYT/ day	mg TOS/ kg-bw day	(NOAEL / highest intake)
Broiler Chickens, 1st week	0.152	0.019	4000	6.6	76	0.825	1042
Broiler Chickens, 3rd week	0.686	0.070	4000	6.6	280	0.675	1274
Broiler Chickens, 6th week	2.088	0.163	4000	6.6	652	0.515	1670
Laying hens, 30 weeks old	1.50	0.110	4000	6.6	440	0.484	1777
Piglets, 6-7 weeks old	15.0	0.950	4000	6.6	3800	0.418	2057
Growing pigs, 13-14 Weeks old	50.0	3.110	4000	6.6	12440	0.411	2092
Pregnant sows	200	1.9 -2.5	4000	6.6	10,000	0.083	10360
Lactating sows	200	5.3 - 7.0	4000	6.6	28,000	0.231	3723
Fish (salmonids) (carps) (tilapia)	4.5 1.0 0.8	0.07 0.02 0.03	2000 2000 2000	3.3 3.3 3.3	140 40 60	0.051 0.066 0.124	16862 13030 6935

[1] National Research Council, Nutrient Requirements of Poultry. Ninth Revised Edition, National Academy Press, Washington, D.C., 1994.

[2] National Research Council, Nutrient Requirements of Swine. Ninth Revised Edition, National Academy Press, Washington, D.C., 1988.

[3] National Research Council, Nutrient Requirements of Fish, National Academy Press, Washington, D.C., 1993 .
*TOS- Total organic solids

Summary

The substance that is the subject of this GRAS determination is RONOZYME® HiPhos at maximum use levels of 4000 FYT/kg feed for poultry and swine and 2000 FYT/kg feed for fish feed as a digestibility enhancer to facilitate the bioavailability of phosphorus as described in Title 21 of the Code of Federal Regulations (21CFR§170.30) (U.S. FDA, 2007).. The addition of the phytase, RONOZYME® HiPhos - expressed in *Aspergillus oryzae*, to animal diets, which is the subject of the present GRAS evaluation, increases the bioavailability and utilization of phytin-bound phosphorus from plant materials. This in turn allows for a reduction of inorganic phosphorus supplementation in the animal diets without compromising performance. The lower phosphorus content in the diets helps to overcome environmental phosphorus contamination problems arising in areas with high animal production concentration.
RONOZYME® HiPhos brand 6-phytase, is produced consistent with cGMP by Novozymes A/S, and meets appropriate specifications established jointly by Novozymes A/S and DSM Nutritional Products Ltd. The product has excellent stability in animal feeds and a minumum shelf life of 12 months when stored under appropriate conditions. Results from evaluations in

various premixes and feed matrixes gave no indication of any incompatibilities with any of the product forms of RONOZYME® HiPhos and non-phytase feed ingredients.

Animal Safety

Among poultry, broiler chickens are considered as a worst case scenario due to the ratio of typical feed intake versus body weight.

The safety factors as derived from the NOAEL in rats are comfortably large, in excess of three-to-four orders of magnitude.
Safety was confirmed by tolerance studies in broiler chickens, laying hens, turkeys, piglets for fattening, gestating/lactating sows using up to 40,000 FYT/kg feed, which is 10 times the highest recommended dose in FYT. The excessive dose did not produce any adverse effects on body weight gains, reproductive parameters (litter weight), blood cell counts, blood chemistry and gross pathology. Additionally, the safety in fish was also confirmed by tolerance studies in salmonids using up to 20,000 FYT/kg feed, 100 times the highest recommended dose in FYT. The excessive dose did not produce any adverse effects but instead beneficial effects on fish performance.

As a consequence of the large safety margins, no regulatory maximum dose for RONOZYME® HiPhos in feed is necessary. However, taking into account both cost-benefit and manufacturing considerations, and in order to allow flexibility in feed formulation, the following upper dose is recommended: 4000 FYT/kg feed for poultry and swine and 2000 FYT/kg feed for fish feed.

Efficacy has been demonstrated for RONOZYME® HiPhos by the significant increases in phosphorus digestibility and utilization in the mineral balance studies in the target animal species. RONOZYME® HiPhos supplemented diets did not adversely affect the health or performance of the animals. Increased phosphorous availability improved feed utilization and decreases pollution by lowering the phosphorous concentration in the waste stream which lowers the potential for eutrofication of the waterways.

Human Safety
Although RONOZYME® HiPhos phytase is intended for use only in animal feed, a generally accepted starting point for food safety evaluation of enzymes is based on the concepts initially developed for human food enzymes by Pariza and Foster (1983 Ref. 4) and further developed by IFBC in 1990 (Ref. 28), the EU SCF in 1992, the OECD in 1992 and 1993, the ILSI Europe Novel Food Task Force in 1996 and the FAO/WHO in 1996. In 2001, Pariza and Johnson (Ref. 5) published an update of the initial concept of Pariza and Foster, and in order to take into account questions of genetic engineering, this concept was further developed by Pariza and Cook in 2010 (Ref. 29).

The primary consideration is the identity and safety of the production organism. Regarding RONOZYME® HiPhos, this assessment is described in detail under section 6 for the microbial production strain, a genetically engineered variant of *Aspergillus oryzae*. The production strain is derived from a safe strain line, which has been used by Novozymes A/S over many years for food and feed enzyme production. Any mycotoxin contamination of RONOZYME® HiPhos formulations arising from the production strain is effectively excluded. In addition, no genetic sequences of concern were added by genetic modification of the production strain. The enzyme is produced by methods and under culture conditions that ensure controlled fermentation. The presence of toxic or undesirable substances as well as the introduction of contaminating micro-organisms is thus prevented.

In addition, raw materials meeting food or feed grade specifications are used in manufacturing. Furthermore, the production strain is absent in the final enzyme preparations. It is the policy of

DSM Nutritional Products Ltd. and Novozymes A/S that all enzyme preparations must conform to the purity criteria for enzyme preparations established by the Food Chemicals Codex (FCC) Ref. 21 and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) Ref. 22. In the description of the impurity profile, it was shown that these criteria are met. Based on these facts, it is concluded that the resulting enzyme product would be safe for humans if it was used as an enzyme in food.

This conclusion is corroborated by the toxicological tests (Ames test and micronuclei assay) and the subchronic toxicity in rats as described previously. None of the studies conducted with RONOZYME® HiPhos produced any adverse effect which could give rise to concern. In the 13-week subchronic oral toxicity study in rats, the active ingredient of the product was well tolerated up to the highest dose tested. The No-Observed-Adverse-Effect Level (NOAEL) in this study was determined to be 860 mg Total Organic Solids (TOS)/kg bw/day. In one of the target animal species, broiler chickens, this NOAEL would result in a very high safety margin, >1000 fold.

As the production strain is safe and the highest expected TOS exposure of the target animals low, no appreciable or toxic residues in the animal products are expected. The appearance of any substance derived from RONOZYME® HiPhos in animal products, except for minute quantities of amino acids, is highly unlikely.

The data and information provided in the GRAS dossier for the recommended use of all forms of RONOZYME® HiPhos in compounded feed for poultry, swine and fish support the conclusion of an extremely low order of toxicity, lack of genotoxicity, reproductive toxicity, or subchronic toxicity and the weight of the scientific evidence does not support a risk of carcinogenicity from the proposed uses. The scientific evidence presented herein does not indicate that RONOZYME® HiPhos 6-phytase would produce adverse effects in the specified animal species or on human health from consumption of animal food products derived from these species who consumed diets containing RONOZYME® HiPhos. The weight of the evidence summarized above supports the safety of the proposed uses of RONOZYME® HiPhos by DSM.

Conclusion of the Expert Panel

We, the members of the Expert Panel, have independently and collectively, critically evaluated the data and information summarized above and conclude that the proposed uses of RONOZYME® HiPhos for use in feed ingredients for animal feed rations for poultry (laying hens, broilers and breeders, and turkeys), swine, and fish at use levels of 250 FYT to 4000 FYT/Kg of poultry feed and swine feed, and 500 FYT/kg to 2000 FYT/kg of fish feed, manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate feed grade specifications described in this dossier, are safe.

We further conclude that the proposed uses of RONOZYME® HiPhos, manufactured consistent with current Good Manufacturing Practice and meeting appropriate feed grade specifications as described in the dossier, are Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

Michael W. Pariza — 28 Nov 2011

Professor Michael W. Pariza, Ph.D.
Food Research Institute
University of Wisconsin
Panel Chairman

Date

John A. Thomas — 30 NOV. 2011

Professor John A. Thomas, Ph.D.
Fellow, ATS
Indiana University School of Medicine
Panel Member

Date

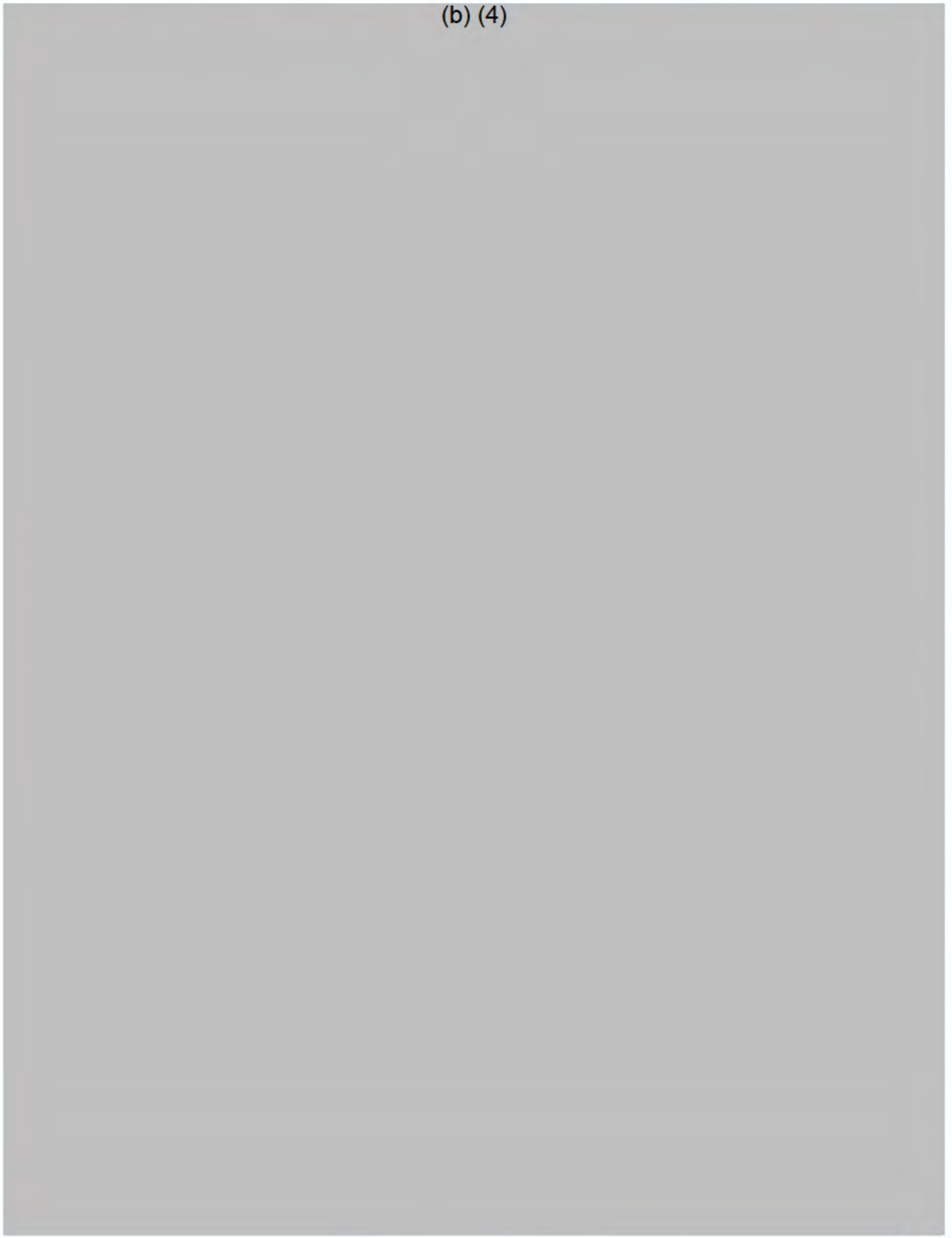
Stanley M. Tarka, Jr. — 01 December, 2011

Stanley M. Tarka, Jr., Ph.D.
President
The Tarka Group, Inc.
Panel Advisor

Date

11 List of Annexes

(b) (4)



(b) (4)

22. Philipps, P. et *al.* (2009). Report No. 00000101: Effect of graded amounts of a microbial phytase (RONOZYME® HiPhos) on growth performance and phosphorus utilization of broiler chickens fed low-phosphorus diet based on maize and soybean meal (BE-15/08). 2009

23. Rodehutscord, M. et *al.* (2009). Report No. 00001790: Dose response study with a new phytase (RONOZYME® HiPhos) in broiler chickens. 2009

24. Philipps, P. et *al.* (2009). Report No. 00001184:Comparison of two formulations of a microbial 6-phytase included at graded levels on growth performance and phosphorus utilization of broiler chickens (BE- 07/09)

25. Francesch, M. et *al.* (2009). Report No. 00000960: Dose response and tolerance study with IPA Mash phytase (RONOZYME® HiPhos) in laying hens fed a maize-based diet. 2009

26. Kwakernaa, K. et *al.* (2009). Report No. 00000959: IPA mash phytase (RONOZYME® HiPhos) improves ileal P and Ca-absorption in laying hens. 2009

27. Aureli, R. et *al.* (2009). Report No. 00000099: Effect of graded levels of bacterial 6- phytase on apparent ileal digestibility of phosphorus in laying hens fed a maize-based diet low in phosphorus content (H-01/09). 2009

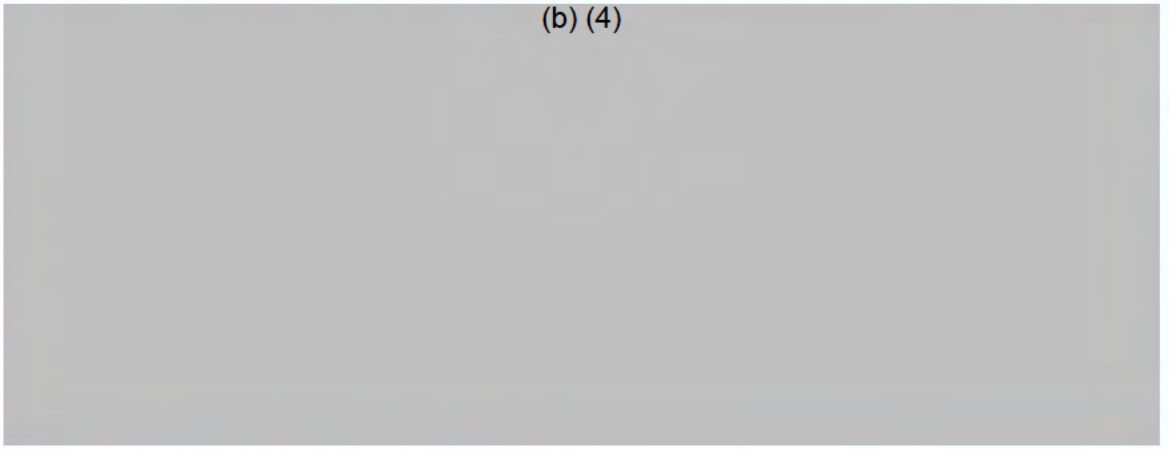
28. Esteve, E. and Broz, J. (2009). Report No. 00001628: Efficacy of IPA PHYTASE (= RONOZYME® HiPhos) in Turkeys. 2009

29. Ledoux, D.R. et *al.* (2009). Report No. 00002585: Efficacy of a novel phytase product (= RONOZYME® HiPhos) in young turkeys poults. 2009

30. Juin, H. and Broz, J. (2009). Report No. 00003287 : Efficacy of IPA phytase (=RONOZYME® HiPhos) in turkeys. 2009

(b) (4)

(b) (4)



12 List of references

1. AAFCO – Official Publication Association of American Feed Control Officials Incorporated (2009): Feed ingredients Definitions. Chapter 5: 363

2. Blumenthal, C.Z. (2004). Production of Toxic Metabolites in *Apergillus niger* , *Apergillus oryzae* and *Trichoderma reesei:* Justification of Mycotoxin Testing in Food Grade Enzyme Preparations Derived from the Three Fungi. Regulatory Toxicology and Pharmacology 39 (2004) 214-228.

3. Tsukuba, E. (2004): Proceedings of the 10th International Congress of Culture Collections (ICCC-10): Traditional Koji mold entering a modern gate

4. Pariza and Foster (1983): Determining the Safety of Enzymes Used in Food Processing, Journal of Food Production, Vol. 46, No.5, pages 453-468

5. Pariza and Johnson (2001): Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century, Regulatory Toxicology and Pharmacology 33; 173-186, 2001

6. Greenough, R.J. et *al.* (1996): Safety Evaluation of a Lipase Expressed in *Aspergillus oryzae*. Fd Chem. Toxic. Vol. 34, 2 (1996) 161 -166

7. Barbesgaard, P., Heldt-Hansen, H.P., and Diderichsen, B. (1992): On The Safety of *Aspergillus oryzae:* A Review. Appl Microbiol Biotechnol 36:569-572.

8. Jorgensen, T.R. (2007): Identification and Toxigenic Potential of the Industrially Important Fungi, *Aspergillus oryzae* and *Aspergillus sojae.* J Food Protection 70:2916-2934.

9. Agency Response Letter GRAS Notice No. GRN 000008

10. Agency Response Letter GRAS Notice No. GRN 000034

11. Agency Response Letter GRAS Notice No. GRN 000043

12. Agency Response Letter GRAS Notice No. GRN 000075

13. Agency Response Letter GRAS Notice No. GRN 000090

14. Agency Response Letter GRAS Notice No. GRN 000103

15. Agency Response Letter GRAS Notice No. GRN 000106

16. Agency Response Letter GRAS Notice No. GRN 000113

17. Agency Response Letter GRAS Notice No. GRN 000122

18. Agency Response Letter GRAS Notice No. GRN 000142

19. Agency Response Letter GRAS Notice No. GRN 000201

20. *Aspergillus oryzae* Final Risk Assessment, Environmental Protection Agency (1997)

21. Enzyme Preparations" Food Chemicals Codex (FCC), 4th edition, 3rd supplement, effective 31 December 2005

22. Compedium of Food Additive Specification, 9th addendum. Joint FAO/ WHO Expert Committee on Food Addtives, 57th session. Rome 2001

23. Selle, P.H. and Ravindran, V. (2007): Microbial Phytase in Poultry Nutrition. Animal Feed Science and Technology 135 (2007) 1 - 41

24. Broz, J. and Ward, N. (2007): The Role of Vitamins and Feed Enzymes in Combating Metabolic Challenges and Disorders. J. Appl. Poultry Res. 16 (2007) 150 – 159

25. Singh, P.K. (2008): Significance of Phytic Acid and Supplemental Phytase in Chicken Nutrition: a review. World's Poultry Science Journal, Vol. 64, 2008

26. Aureli, R. et *al.* (2011): The Efficacy of a Novel Microbial 6-Phytase Expressed in *Aspergillus oryzae* on the Performance and Phosphorus Utilization in Broiler Chickens. International Journal of Poultry Science 10 (2): 160-168,2011

27. National Research Council, Nutrient Requirements of Poultry. Ninth Revised Edition, National Academy Press, Washington, D.C., 1994

28. IFBC (International Food Biotechnology Council). (1990) Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. In: Coulston and Kolbye, Jr. (eds.), Regulatory Toxicology and Pharmacology, 12: S1-S196

29. Pariza, M. and Cook, J. (2010): Determining the Safety of Enzymes Used in Animal Feed. Regulatory Toxicology and Pharmacology 56 (2010) 332–342

30. Lichtenberg, J., Pedersen, P.B., Elvig-Jorgensen, S.G., Skov, L.K., Olsem, C.L., and Glistoe, L.V. (2011): Toxicological Studies On A Novel Phytase Expressed From Synthetic Genes in *Aspergillus oryzae.* Regulatory Toxicology and Pharmacology 60 (2011) 401–410.

31. Pontoppidan, K., Glitsoe, V., Guggenbuhl, P., Quintanna, A. P., Nunes, C. S., Petterson, D. and Sandberg, A. S. (2012): *In vitro* and *in vivo* degradation of *myo*-inositol hexakisphosphate by a phytase from *Citrobacter braakii.* Archives of Animal Nutrition (*manuscript accepted but not yet published)*

32. Mullaney, E. J. and Ullah, A.H.J. (2003): The Term Phytase Comprises Several Different classes of Enzymes. Biochemical & Biophysical Research Communications 312 (2003) 179-184.

33. Lipase Enzyme Preparation from *Rhizopus nivens*; affirmation of GRAS Status as a Direct Food Ingredient.Fed. Reg. 63 (85) 24416-24419.

34. Statement of Policy: Foods Derived From New Plant Varieties. Fed. Reg. 57 (104) 22984 – 23005.

35. Hidvegi, M. and Laszitity, R. (2002): Pytic Acid Content of Cereals and Legumes and Interaction with Proteins. Periodica Polytechnica Ser. Chem. Eng. Vol. 46, NO. 1–2, PP. 59–64 (2002).

36. Bridgewater, J. (1976): Fundamental Powder Mixing Mechanisms. Powder Technology 15 (1976) 215-236.

37. Chowhan, Z. T. and Linn, E. E. (1979(: Mixing of Pharmaceutical Solids. I. Effect of Particle Size on Mixing in Cylindricl Shear and V-Shaped Tumbling Mixers. Powder Technology 24 (1979) 237-224.

38. W. Larrabee Editor *A Guide to Mixing Microingredients in Feed' 3rd Ed.*,,(Merck Sharp & Dohme, Rhaway, NJ, 1976)

39. Goodband, B., Groesbeck, C., Tokach, M, Dritz, S., DeRouchey, J. and Nelssen, J. (2006): Effects of Diet Particle Size on Pig Growth Performance, Diet Flow Ability, and Mixing Characteristics. Proceedings of the 2006 Manitoba Swine Seminar. www.gov.mb.ca/agriculture/livestock/pork/pdf/bab20s09.pdf

40. Industry Technical Bulletin: 'Importance of the Feed Particle Size', ISA, A Hendrix Genetics Company. www.isapoultry.com/en/Information/Technical%20Bulletins/~/media/Files/ISA/Information/Technical%20Bulletins/Nutrition/Feed%20presentation%20for%20commercial%20layers.ashx

41. Amornthewaphat, N., Behnke, K.C and Hancock, J.D.(1998): Effects of particle size and mixing time on uniformity and segregation in pig diets. Conference Presentation at Swine Day, Manhattan, KS, November 19, 1998. http://hdl.handle.net/2097/2749

THIS PAGE INTENTIONALLY BLANK

ANNEXES

SUBMISSION

CONTINUED

IN

NEXT

VOLUME

SUBMISSION

CONTINUED

FROM

PREVIOUS

VOLUME

TAB

ANNEX I

Pages 119-502 withheld in their entirety under (B)(4)

TAB

ANNEX 22

Annex 22

Philipps, P. et *al.* (2009). Report No. 00000101: Effect of graded amounts of a microbial phytase (RONOZYME® HiPhos) on growth performance and phosphorus utilization of broiler chickens fed low-phosphorus diet based on maize and soybean meal (BE-15/08). 2009

REPORT No. 00000101
Regulatory Document

DSM

Document Date: 09-June-2009

Author(s): Philipps P and Aureli R

NRD/CA, DSM Nutritional Products France

Title: Effect of graded amounts of a microbial phytase on growth performance and phosphorus utilization of broiler chickens fed low-phosphorus diet based on maize and soybean meal (BE-15/08).

Project No. 6106

Compound No.

Summary

The effect of a liquid preparation of a microbial phytase on growth performance and phosphorus utilization of broiler chickens was studied in a short term trial from day 8 to 22 of life. The birds were fed low-phosphorus diets based on maize and soybean meal. The pelleted diet contained 3.9 g total phosphorus and 5.2 g calcium per kg feed.

To verify dose-response effects of the enzyme, animals received feed supplemented with 250, 500, 1000, 2000, 4000 and 8000 U phytase per kg diet.

The supplementation of low P diet with the microbial phytase significantly improved weight gain and feed conversion ratio in male broiler chickens at 22 days of age. Utilization of phosphorus was significantly increased and consequently the amount of P excreted in the faeces was reduced. P-utilization was dependent on level of phytase and could be described by an exponential function. The phytase was as well efficacious in improving tibia ash and tibia strength. At levels above 250 U per kg feed the bone strength was improved more than 2 folds compared to the negative control. With increasing levels of phytase, improvements in tibia ash ranging between 15 % and 32 % were noted compared to the results obtained with the low P basal diet.

In most measured parameters, even at low dosages, the treatments supplemented with phytase performed equally or even outperformed those supplemented with additional mineral P (positive controls).

This report consists of pages 1-16

Distribution

Dr. J. Broz, NRD/CA
Dr. M. Eggersdorfer, NRD
Dr. J. Pheiffer, NRD/PA
Dr J-F. Hecquet, NBD/RA
Dr. J.-P. Ruckebusch, ANH/PM

Approved

Name	Signature	Date
Main Author Dr. P. Philipps, NRD/CA	Pedro Philipps	09/06/09
Competence Mgr Dr. C. Simões-Nunes, NRD/CA		09.06.09
Research Center Head Dr. A.-M. Kluenter, NRD/CA	A.-M. Kluenter	10 6 2009
Project Manager Dr. F. Fru, NRD/PA		15.06.09

Nomenclature and Structural Formula

IPA phytase, PPQ 27987 with analysed phytase activity of 24850 U/g

Author(s): Petra Philipps and Raffaella Aureli

Department(s) and Adress(es) : NRD/CA, DSM Nutritional Products France

Title: Effect of graded amounts of a microbial phytase on growth performance and phosphorus utilization of broiler chickens fed low-phosphorus diet based on maize and soybean meal (BE-15/08).

Abstract

The effect of a liquid preparation of a microbial phytase on growth performance and phosphorus utilization of broiler chickens was studied in a short term trial from day 8 to 22 of life. The birds were fed low-phosphorus diets based on maize and soybean meal. The pelleted diet contained 3.9 g total phosphorus and 5.2 g calcium per kg feed.
To verify dose-response effects of the enzyme, animals received feed supplemented with 250, 500, 1000, 2000, 4000 and 8000 U phytase per kg diet.
The supplementation of low P diet with the microbial phytase significantly improved weight gain and feed conversion ratio in male broiler chickens at 22 days of age. Utilization of phosphorus was significantly increased and consequently the amount of P excreted in the faeces was reduced. P-utilization was dependent on level of phytase and could be described by an exponential function. The phytase was as well efficacious in improving tibia ash and tibia strength. At levels above 250 U per kg feed the bone strength was improved more than 2 folds compared to the negative control. With increasing levels of phytase, improvements in tibia ash ranging between 15 % and 32 % were noted compared to the results obtained with the low P basal diet.
In most measured parameters, even at low dosages, the treatments supplemented with phytase performed equally or even outperformed those supplemented with additional mineral P (positive controls).

INTRODUCTION

A greater proportion of the total phosphorus in plant is present in phytate form. Phosphorus in phytate form is poorly available for poultry because they lack phytase, the enzyme that hydrolyzes phytate into inorganic phosphorus and inositol. P in animal excreta originates either from feed or from endogenous secretions. The amount of P in animal excreta is continuously on the rise due to geometric intensification of animal farming. This has encouraged the interest to use phytases to enhance the use of endogenous P in plants and thereby reduce the need for inorganic phosphorus supplementation, included in animal feed to satisfy the animal's physiological need for phosphorus.
The aim of the present trial was to study the effects of a microbial phytase on growth performance and phosphorus utilization of broiler chickens fed low-phosphorus diets. Due to the low amount and activity of native phytases in maize and soybean meal, they were the ingredients of choice for the basal diets. To verify dose-response effects, the animals were fed diets supplemented, per kg feed, with 250, 500, 1000, 2000, 4000 and 8000 U of a liquid preparation of the phytase.
In addition, three treatments supplemented with additional DCP were included in the trial to have 4.9, 5.3 and 5.7 g phosphorus per kg feed.
Phosphorus utilization, considered to be the most sensitive parameter for measuring the efficiency of phytase, was determined based on quantitative measurements of P consumption and excretion.

MATERIALS AND METHODS

The trial (BE-15/08) was performed from May to June 2008 at the Research Center for Animal Nutrition and Health (DSM Nutritional Products France, F-68305 Village-Neuf) according to the official French norms for experiments with live animals. Day-old male broiler chickens (ROSS "PM3"), were supplied by a commercial hatchery (Joseph Grelier S.A., Elevage avicole de la Bohadière, F-49290 Saint-Laurent de la Plaine, France). The chickens were housed in wire-floored battery cages, which were kept in an environmentally controlled room. The room temperature was adapted according to the requirements of the chickens. Feed and tap water were available for *ad libitum* consumption. The chickens were fed with a low phosphorus basal diet supplemented with 50 µg vitamin D_3 / kg (corresponding to 2000 IU per kg feed) until day 8, when the trial started. On day 8, the chickens were divided by weight into groups, each comprising of 8 birds, which were allocated to one of the different treatments. Each treatment was replicated with 6 groups. The groups were weighed on days 8, 15, and 22. Feed consumption for the intermediate periods was determined and body weight gain (WG) and feed conversion ratio (FCR) were calculated.

The basal diet was supplemented with 12.5 µg/kg vitamin D_3 corresponding to 500 IU/kg to fulfil the recommendation for chickens of that age (GfE 1999). The basal diet was based on maize and soybean meal as main ingredients and had a content of about 217 g crude protein, 12.7 MJ ME_N, 3.9 g total phosphorus (P), and 5.2 g calcium (Ca) per kg feed. All other nutrients met the requirements of growing broilers in accordance to their age. The analyses of the nutrient content in the feed samples (Table 1) were performed according to standard methods (VDLUFA, 1976).The detailed composition of the basal diet, the analysed nutrient contents and the ME (calculated on the basis of analysed nutrients using EC-equation, EEC, 1986), are shown in **Table 1**.

Apart from the control treatment without phytase supplementation, all other treatments were supplemented with phytase batch N° PPQ 27987 (with analysed phytase activity of 24850 U/g) at one of the following doses: 250, 500, 1000, 2000, 4000 and 8000 U /kg feed, and with additional DCP to contain 4.9, 5.3 and 5.7 g total P per kg feed, that means 0.8 g, 1.2 g and 1.6 g P more than in the negative control diet, per kg feed.

Feed was pelleted (3 x 25 mm) at about 75°C. Then appropriate amounts of the liquid preparation of phytase were diluted with 400 ml water. The solutions were sprayed onto the pellet feed to get the final concentrations in the feed corresponding to the different treatments. For procedural balance of all treatments 400 ml of water were also sprayed onto the pellets of the negative control and the three positive controls.
Feed samples were analyzed for phytase activities. The determination of the phytase activity in the product and in the experimental diets was performed by BIOPRACT GmbH, D-12489 Berlin (Germany) on behalf of DSM Nutritional Products. One unit (U) of phytase is defined as the activity that releases 1µmol inorganic phosphate from 5.0 mM phytate per minute at pH 5.5 and 37 °C. Phytate in feed was determined colorimetrically as released P after extraction, elution and wet digestion with HNO_3/H_2SO_4 (AOAC, 1990).

Excreta were collected from day 14 to day 17 by a total collection method. During this period the excreta from 4 selected groups of male chickens per treatment were quantitatively collected once per day. The excreta from the four days were pooled per group and were stored frozen (at -20°C), each day directly after collection. After thawing, the total excreta of each group were homogenized, representative samples were taken and the percentage of dry matter and ash, as the concentration of phosphorus and calcium were determined. Ca and total P were determined by Induction Coupled Plasma according to DIN EN ISO 11885:1997 (DIN EN ISO 1998) after mineralization with H_2SO_4 / Na_2SO_4.

On day 22, blood samples from 4 male chickens randomly chosen from each group were taken from the *Vena jugularis*. The concentrations of inorganic phosphate (Pi) and Ca in the plasma were determined according to the method described by Henry (1974) and Gindler and King (1972), using Roche Diagnostic kits PHOS 03183793 122 and Ca 20763128 322 with a HITACHI 912 automatic analyzer.

The chickens were sacrificed by cervical dislocation at 23 days of age and the right tibias were taken from 4 chickens randomly selected from each group. Tibias were kept frozen at -20°C until analysis of ash content and breaking strength.
A segment of the central portion of the bone shaft (about 2 cm long) was prepared for determination of bone strength. A LR10K compression machine with a XLC/10K/A1 force captor and a compression device TH23-196/AL (Lloyd Instruments, Fareham, UK) was used to determine the force (in Newton) necessary to break the bone. Broken bones were pooled per cage, defatted with ethanol and ether, dried and incinerated at 550°C to determine the ash content.

For the statistical evaluation of all data a one-factorial (treatment: phytase/P level) analysis of variance was carried out, using the software "Stat Box Pro", version 5.0 (Grimmer soft 1995) in which differences in treatment means with $p < 0.05$ were considered as significant. Newman-Keuls test was used as post hoc to compare treatment means. Non-linear regression analyses were performed with the program Origin 7.0. An exponential model of the following type was fitted to the data:

$$y = a+b\,(1-\exp(-kx))$$

with a: response (y-value) at zero phytase supplementation
b: maximum response to supplemented phytase (a+b = upper asymptote)
k: parameter describing the steepness of the curve
x: supplemented phytase (U/kg)
y: response

RESULTS AND DISCUSSION

Proximate analyzes in the negative control diet were close to the calculated values. P content was almost as expected and the difference among P content in positive and negative controls was respectively 1.1 g P, 1.3 g P and 1.7 g P per kg feed **(Table 2)**. Analyzed Ca contents were approximately 8 % less than calculated. The analyzed content of non-phytic acid phosphorus in the basal diet was at 1.6 g per kg feed, calculated as the difference between total phosphorus content and content of phytic phosphorus per kg feed
The analysed phytase activities in the experimental feed are listed in **Table 3**. Phytase activities were generally in accordance to the target dosages.

The results of the growth performance from day 8 to day 22 are shown in **Table 4**. Adding dicalcium phosphate (DCP) to the negative control diet resulted in significant improvements of weight gain (WG) and feed conversion ration (FCR), clearly indicating that the negative control diet was P-deficient. At a supplementation level of + 1.1 g (analyzed) DCP per kg feed, WG and FCR were improved by 62 % and 13.3 %, respectively, compared to the negative control diet.
Phytase supplementation resulted in a significant improvement of the weight gain and the feed conversion ratio compared to the negative control diet. The lowest phytase inclusion level of 250 $U.kg^{-1}$ already resulted in a significantly higher WG (+ 61.2%) and better FCR (- 11%). Comparable improvements of weight gain to those obtained with the positive controls were already noted for the two lowest inclusion levels of phytase. Increased phytase supplementation from 250 to 8000 $U.kg^{-1}$ resulted in a significant improvement of the WG and the FCR with significant differences among the supplemented treatments. Weight gain and feed conversion ratio were improved in a logarithmic dose response manner with increasing doses of phytase. The response of weight gain and feed conversion ratio to the addition of phytase to the diet could be described by non-linear regressions **(Figure 1 and 2)**.

The mortality observed throughout the trial was higher for the control treatment (12.5 %) than the other treatments, but was within an acceptable range.

The results of apparent utilization of phosphorus and calcium are presented in **Table 5.** The apparent utilization of phosphorus was significantly improved by the action of the phytase. The apparent P-utilization increased with increasing dietary levels of the phytase. Compared to the negative control diet, an improvement in a range of 17 % to 51 % was obtained with graded amount of phytase. The effect of the phytase supplementation on P utilization for all supplementary levels was further confirmed by a significant reduction in P excretion, in which reductions of about 44.6 % and 48.2 % were respectively obtained at 1000 and 2000 U per kg feed supplementations **(Figure 4).** The utilization of P in the negative control diet was 51.8 %, and it was increased to an estimated asymptotic value of 78.2 % with phytase supplementation (**Figure 3**).
The improvement of P-utilization in the phytase treatments indicated that phytate-P was liberated due to the action of the phytase. P-utilization of the positive control groups was lower than of all phytase groups indicating that phytate phosphorus was less utilized by the broiler chickens, which was confirmed by the increasing phosphorus content in excreta.

The apparent Ca-utilization **(Table 5)** was as well significantly improved in all treatments compared to the negative control diet. Similar to the P-utilization, the effect was dose-dependent with significant differences among the dosages. Utilization of Ca in the negative control diet was 39.8%, and it was increased up to an estimated asymptotic value of 72.6% by including graded amounts of phytase.

Results of plasma concentration of inorganic phosphorus (Pi) and Ca are presented in **Table 6.** The P_i-concentration in the plasma was significantly increased in all treatments compared to the negative control diet. With increasing dietary inclusion level of phytase the P_i-concentration in the plasma increased in a dose-dependent manner **(Figure 5).** The Ca-concentration in the plasma was decreased when phytase was added to the basal diet.

Table 7 shows the effect of phytase supplementation on parameters of bone mineralization. Supplementing phytase, irrespective of the dose, significantly improved tibia strength compared to the negative control diet. Tibia strength values increased in a pattern corresponding to supplementation levels. At levels above 250 U the bone strength was improved more than 2 folds.
The effects of phytase supplementation on tibia ash, a parameter that indicates the extent of bone mineralization, were significant for all treatments compared to the negative control diet. With increasing levels of phytase, improvements ranging between 15 % and 32 % were noted. Again, comparable response as in the positive controls including additional DCP was found already for low inclusion levels of the phytase, confirming its efficacy. An exponential dose-dependent relationship was found for the tibia ash (**Figure 6**), in which the slope rose very fast with increasing phytase in the diet, and levels out above 2000 U per kg diet.

The results of this current study demonstrate that the supplementation of low P diets with the new microbial phytase significantly improved the weight gain and the feed conversion ratio of male broiler chickens at 22 days of age. The utilization of phosphorus was significantly increased and consequently the amount of P excreted in the faeces was reduced. P-utilization was improved dependent on level of phytase and could be described by an exponential function with P utilization approaching an asymptote at 78%. The phytase is efficacious in releasing phytate-P. In most cases, even at low dosages, the treatments supplemented with phytase performed equally or even outperformed those supplemented with additional mineral P (positive controls).

REFERENCES

AOAC (1990)
Official Methods of Analysis of the AOAC [Association of Official Analytical Chemists] Arlington, Virginia 22201 USA

DIN EN ISO (1998)
Bestimmung von 33 Elementen durch induktiv gekoppelte Plasma-Atom-Emissionsspektrometrie (ISO 11885:1997; E22, 1998-04; Deutsche Fassung EN ISO 11885:1997), Beuth Verlag GmbH

EEC (1986)
Directive de la Commission du 9 avril 1986 fixant la méthode de calcul de la valeur énergétique des aliments composés destinés à la volaille, Journal Officiel des Communautés Européennes, L 130, 53-54

GRIMMERSOFT (1995)
StatBoxPro, Version 5.0, Manuel d'utilisation

GfE (Gesellschaft für Ernährungsphysiologie), 1999. Empfehlung zur Energie – und Nährstoffversorgung der Legehennen und Masthühner (Broiler), DLG-Verlag, Frankfurt am Main

GINDLER, E.M. and J.D. KING (1972)
Determination of calcium concentration in human serum.AM.J.Clin.Pathol.58, 376-382

HENRY R. (Editor) (1974) Clinical Chemistry:
Principles and Techniques, 2nd Edition New York, NY: Harper & Row, 723

ORIGINLAB CORPORATION (2002)
Origin Version 7, Programming Guide

VDLUFA (1976)
[Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten,Hrsg Handbuch der landwirtschaftlichen Versuchs- und Untersuchungsmethodik (Methodenbuch). Band III: Die chemische Untersuchung von Futtermitteln, 3. Auflage 1976 mit 1. Erg. Lief.1983, 2. Erg. Lief. 1993, 3. Erg. Lief. 1997, VDLUFA-Verlag, Darmstadt

Table 1: Composition of the basal diet

Ingredients (%)	
Maize	60.20
Soybean meal (50 % CP)	35.50
Soybean oil	2.00
DL-Methionine	0.20
L-Lysin	0.05
DCP	0.25
$CaCO_3$	0.58
Sand	0.32
NaCl	0.15
Premix [1] without Vitamin D_3	1.00
Calculated content:	
ME_N (MJ/kg)[2]	12.6
Crude protein (g/kg)	216
Calcium (g/kg)	6.0
Total P (g/kg)	4.1
Lysine (%)	1.22
Methionine + Cystine (%)	0.90
Analyzed content:	
ME_N(MJ/kg)[3]	12.7
Crude protein (g/kg)	217
Calcium (g/kg)	5.2
Total P (g/kg)	3.9
Phytate P (g/kg)	2.3
Non Phytate-P (g/kg)	1.6

[1] Including Avatec
[2] Calculated with EC-equation based on values from nutritional tables
[3] Calculated with EC-equation based on analysed crude nutrients

Table 2: Analyzed P and Ca concentration in samples of the experimental diets

Treatment	Product	Total P ($g.kg^{-1}$ feed)		Ca ($g.kg^{-1}$ feed)	
		expected	measured	expected	measured
A	Negative control	4.1	3.9	6.0	5.2
P	Positive control	4.9	5.0	6.0	5.6
Q	Positive control	5.3	5.2	6.0	5.7
R	Positive control	5.7	5.6	6.0	5.6

Table 3: Analyzed product activity in samples of the experimental diets

Treatment	Product	Dose ($U.kg^{-1}$)	Phytase content ($U.kg^{-1}$ feed)
A	Negative control	-	78
B	Microbial phytase	250	255
C	Microbial phytase	500	505
D	Microbial phytase	1000	1035
E	Microbial phytase	2000	1878
F	Microbial phytase	4000	3605
G	Microbial phytase	8000	8019

Table 4: Performance of broiler chickens (day 8 to day 22) fed different supplemental levels of microbial phytase, mean ± stdev

Product	Negative control	Microbial phytase						Positive controls		
Treatment	A	B	C	D	E	F	G	P	Q	R
Dose (U.kg^{-1})	-	250	500	1000	2000	4000	8000	4.9 g.kg^{-1}	5.3 g.kg^{-1}	5.7 g.kg^{-1}
cages x birds	6 x 8	6 x 8	6 x 8	6 x 8	5 x 8	6 x 8	6 x 8	6 x 8	6 x 8	6 x 8
Weight gain (g/bird)	**451^{E}**	**727^{D}**	**778^{BCD}**	**809^{BC}**	**880^{A}**	**837^{AB}**	**806^{BC}**	**730^{D}**	**753^{CD}**	**756^{CD}**
	± 63	± 41	± 25	± 38	± 68	± 26	± 47	± 32	± 29	± 29
%	*100.0*	*161.2*	*172.4*	*179.3*	*195.2*	*185.5*	*178.7*	*162.0*	*167.1*	*167.6*
Feed intake (g/bird)	**708^{D}**	**1018^{BC}**	**1033^{BC}**	**1080^{ABC}**	**1114^{AB}**	**1156^{A}**	**1101^{ABC}**	**998^{C}**	**1037^{BC}**	**1028^{BC}**
	± 80	± 46	± 33	± 37	± 20	± 130	± 44	± 47	± 32	± 32
%	*100.0*	*143.7*	*145.8*	*152.5*	*157.2*	*163.2*	*155.4*	*140.9*	*146.4*	*145.2*
Feed conversion (g feed/g gain)	**1.576^{A}**	**1.402^{B}**	**1.329^{BC}**	**1.339^{BC}**	**1.270^{C}**	**1.382^{BC}**	**1.368^{BC}**	**1.366^{BC}**	**1.378^{BC}**	**1.361^{BC}**
	± 0.049	± 0.026	± 0.020	± 0.085	± 0.075	± 0.153	± 0.042	± 0.010	± 0.018	± 0.016
%	*100.0*	*89.0*	*84.3*	*84.9*	*80.6*	*87.7*	*86.8*	*86.7*	*87.4*	*86.4*
Mortality (%)	12.5	2.1	2.1	0.0	4.2	0.0	6.3	6.3	2.1	4.2

Newman-Keuls test: Means within a row, not sharing a common superscript, are significantly different ($p<0.05$).

Table 5: Apparent utilization of phosphorus and calcium in male broiler chickens fed different supplemental levels of microbial phytase, mean ± stdev.

Product	Negative control	Microbial phytase						Positive controls		
Treatment	A	B	C	D	E	F	G	P	Q	R
Dose (U.kg^{-1})	-	250	500	1000	2000	4000	8000	4.9 g.kg^{-1}	5.3 g.kg^{-1}	5.7 g.kg^{-1}
cages x birds	4 x 8	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4
Dry matter intake (g / bird / day)	46.2 ^{D} ± 5.7	60.5 ^{C} ± 4.1	63.1 ^{ABC} ± 2.7	67.4 ^{ABC} ± 3.2	69.9 ^{A} ± 1.5	68.2 ^{AB} ± 2.4	67.2 ^{ABC} ± 4.2	61.2 ^{BC} ± 2.7	65.2 ^{ABC} ± 0.9	64.1 ^{ABC} ± 2.7
Apparent P utilization (% of intake)	**51.8** ^{F} ± 1.3	**60.8** ^{D} ± 2.8	**69.0** ^{C} ± 0.8	**73.6** ^{B} ± 1.4	**74.9** ^{AB} ± 2.6	**77.3** ^{AB} ± 2.7	**78.2** ^{A} ± 2.8	**57.2** ^{DE} ± 2.8	**57.6** ^{DE} ± 3.5	**54.6** ^{EF} ± 0.8
%	*100.0*	*117.4*	*133.2*	*142.1*	*144.6*	*149.2*	*151.0*	*110.4*	*111.1*	*105.4*
P in excreta (g/kg DM faeces)	**8.3** ^{C} ± 0.2	**6.9** ^{D} ± 0.4	**5.5** ^{E} ± 0.2	**4.6** ^{F} ± 0.2	**4.3** ^{F} ± 0.4	**4.0** ^{F} ± 0.4	**3.9** ^{F} ± 0.5	**10.0** ^{B} ± 0.7	**9.9** ^{B} ± 0.8	**11.2** ^{A} ± 0.2
%	*100.0*	*83.1*	*66.3*	*55.4*	*51.8*	*48.2*	*47.0*	*120.5*	*119.3*	*134.9*
Apparent Ca utilization (% of intake)	**39.8** ^{G} ± 2.4	**52.1** ^{F} ± 2.5	**61.2** ^{CD} ± 1.0	**65.2** ^{BC} ± 2.8	**67.8** ^{AB} ± 2.6	**70.2** ^{AB} ± 3.4	**72.6** ^{A} ± 3.3	**55.5** ^{EF} ± 2.9	**60.3** ^{CDE} ± 4.5	**59.0** ^{DE} ± 2.1
%	*100.0*	*130.9*	*153.8*	*163.8*	*170.4*	*176.4*	*182.4*	*139.4*	*151.5*	*148.2*

Newman-Keuls test: Means within a row, not sharing a common superscript, are significantly different ($p<0.05$)

Table 6: Concentrations of inorganic phosphorus (P_i) and Ca in the plasma of male broiler chickens fed different supplemental levels microbial phytase, mean ± stdev.

Product	Negative control	Microbial phytase						Positive controls		
Treatment	A	B	C	D	E	F	G	P	Q	R
Dose ($U.kg^{-1}$)	-	250	500	1000	2000	4000	8000	4.9 $g.kg^{-1}$	5.3 $g.kg^{-1}$	5.7 $g.kg^{-1}$
cages x birds	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4
P_i (mmol/L)	**1.16** ^{D} ± 0.15	**1.45** ^{CD} ± 0.37	**1.88** ^{B} ± 0.18	**2.49** ^{A} ± 0.10	**2.61** ^{A} ± 0.12	**2.53** ^{A} ± 0.15	**2.78** ^{A} ± 0.11	**1.74** ^{BC} ± 0.42	**2.32** ^{A} ± 0.08	**2.52** ^{A} ± 0.11
%	*100.0*	*125.0*	*162.1*	*214.7*	*225.0*	*218.1*	*239.7*	*150.0*	*200.0*	*217.2*
Ca (mmol/L)	**3.07** ^{A} ± 0.15	**2.94** ^{AB} ± 0.05	**2.93** ^{AB} ± 0.05	**2.88** ^{AB} ± 0.05	**2.81** ^{B} ± 0.04	**2.84** ^{B} ± 0.04	**2.94** ^{AB} ± 0.10	**2.88** ^{AB} ± 0.12	**2.90** ^{AB} ± 0.06	**2.97** ^{AB} ± 0.09
%	*100.0*	*95.8*	*95.4*	*93.8*	*91.5*	*92.5*	*95.8*	*93.8*	*95.5*	*96.7*

Newman Keuls test: Means within a row, not sharing a common superscript, are significantly different ($p<0.05$)

Table 7: Tibia ash and tibia strength of male broiler chickens fed different supplemental levels of microbial phytase, mean ± stdev.

Product	Negative control	Microbial phytase						Positive controls		
Treatment	A	B	C	D	E	F	G	P	Q	R
Dose (U.kg^{-1})	-	250	500	1000	2000	4000	8000	4.9 g.kg^{-1}	5.3 g.kg^{-1}	5.7 g.kg^{-1}
cages x birds	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4
Tibia strength (N)	**76** ^{C} ± 39.4	**167** ^{AB} ± 32.6	**234** ^{A} ± 43.7	**214** ^{A} ± 38.7	**243** ^{A} ± 76.9	**234** ^{A} ± 47.3	**229** ^{A} ± 36.7	**128** ^{BC} ± 10.6	**169** ^{AB} ± 24.9	**182** ^{AB} ± 35.1
%	*100*	*218.2*	*306.5*	*279.6*	*318.5*	*306.1*	*300.1*	*167.2*	*221.9*	*238.6*
Tibia ash (%)	**40.7** ^{C} ± 1.60	**46.8** ^{B} ± 1.62	**50.2** ^{AB} ± 3.87	**51.2** ^{A} ± 0.84	**51.9** ^{A} ± 0.53	**52.0** ^{A} ± 0.74	**53.6** ^{A} ± 3.91	**46.2** ^{B} ± 0.90	**49.7** ^{AB} ± 0.87	**49.8** ^{AB} ± 0.95
%	*100*	*115.1*	*123.4*	*125.8*	*127.7*	*127.8*	*131.7*	*113.6*	*122.1*	*122.5*

Newman-Keuls test: Means within a row, not sharing a common superscript, are significantly different ($p<0.05$).

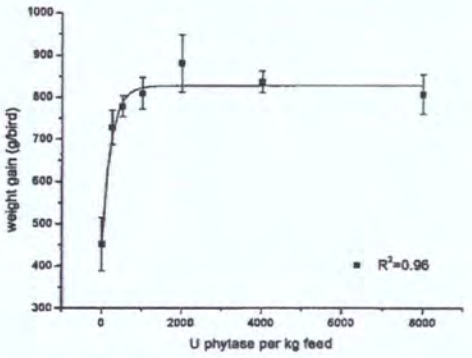


Figure 1: Effect of supplementation of phytase on weight gain

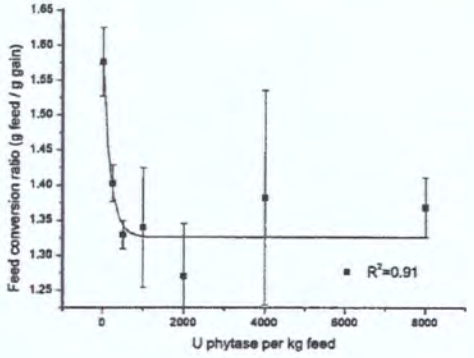


Figure 2: Effect of supplementation of phytase on feed conversion ratio

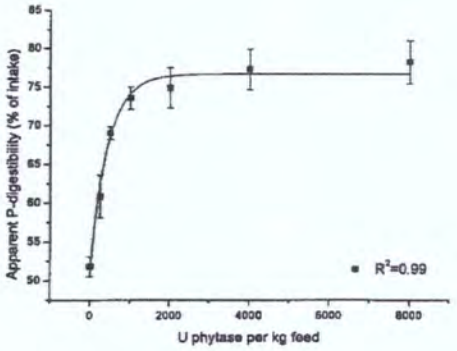


Figure 3: Effect of supplementation of phytase on apparent phosphorus utilization

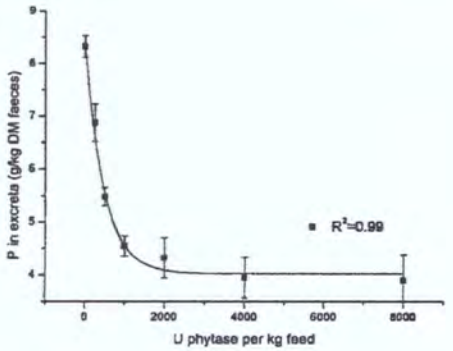


Figure 4: Effect of supplementation of phytase on P in excreta

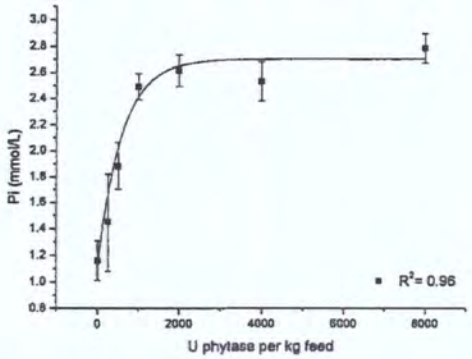


Figure 5: Effect of supplementation of phytase on P_i concentration in plasma

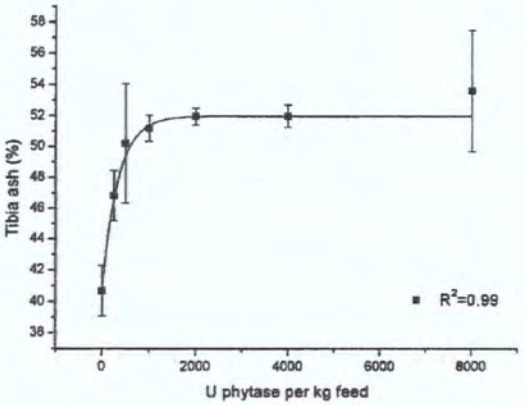


Figure 6: Effect of supplementation of phytase on tibia ash

Table 7: Non linear regressions describing the effects of supplementation with the microbial phytase on various parameters

	a	b	k	R^2
Weight gain (g/bird)	459.1	368.2	0.0045	0.96
Feed conversion ratio (g feed/g gain)	1.6	-0.3	0.0055	0.91
Apparent P utilization (%)	51.7	25.0	0.0023	0.99
P in excreta (g/kg DM faeces)	8.4	-4.3	0.0021	0.99
Tibia ash (%)	40.7	11.2	0.0030	0.99
Pi in plasma (mmol/L)	1.1	1.6	0.0017	0.96

efsa
European Food Safety Authority

FEEDAP UNIT

ANNEX [illegible]

TRIAL PROTOCOL DATA SHEET: FOR TERRESTRIAL ANIMALS

Identification of the additive: **IPA Phytase**	Batch number: **PPQ 27987**
Trial ID: **BE-15/08**	Location: **DSM Nutritional Products France; Research Centre for Animal Nutrion & Health, F-68128 Village-Neuf**
Start date and exact duration of the study: **May-27-2008 to June-18-2008, 2 weekd (1 week pre-trial period)**	
Number of treatment groups (+ control(s)): **6 (+4)**	Replicates per group: **6**
Total number of animals: **480**	Animals per replicate: **8**
Dose(s) of the additive/active substance(s)/agent(s) (mg/Units of activity/CFU kg^{-1} complete feed/L^{-1} water)	
Intended: **0/250/500/1000/2000/8000 U.kg-1**	Analysed: **78/255/505/1035/1878/3605/8019 U.kg-1**
†	
Substances used for comparative purposes:	
Intended dose:	Analysed:
Animal species/category: **Broiler**	
Breed: **ROSS PM3**	Identification procedure: **per cage number**
Sex: **Males** Age at start: **8 days**	Body weight at start: **171 g**
Physiological stage: **Growing**	General health: **Normal (P-deficient basal diet)**
Additional information for field trials:	
Location and size of herd or flock:	
Feeding and rearing conditions:	
Method of feeding:	
Diets (type(s)): **low phosphorus basal diet**	
Presentation of the diet: Mash ☐ Pellet ☒ Extruded ☐ Other	
Composition (main feedingstuffs): **60.2 % maize / 35.50 % corn**	
Nutrient content (relevant nutrients and energy content)	
Intended values: **per kg: 12.6 MJ ME, 216 g crude protein, 4.1 g tot. P, 1.6 g non phytate P, 6.0 g Ca**	
Analysed values: **per kg: 12.7 MJ ME, 217 g crude protein, 3.9 g tot. P, 5.2 g Ca**	
Date and nature of the examinations performed: **Growth performance, app. P utilisation, bone quality, plasma**	
Method(s) of statistical evaluation used: **one-factoral analysis of variance (factor: treatment), Newman-Keuls-test, non-linear regression analysis**	
Therapeutic/preventive treatments (reason, timing, kind, duration): **Nothing to report**	
Timing and prevalence of any undesirable consequences of treatment: **Nothing to report**	

[1] Please submit this form using a common word processing format (e.g. MS Word).

FEEDAP UNIT

Date **18-Feb-2010**	Signature Study Director
	Petra Pluijms

† In case the concentration of the additive in complete feed/water may reflect insufficient accuracy, the dose of the additive can be given per animal day^{-1} or mg kg^{-1} body weight or as concentration in complementary feed.

Raw data of Trial BE-15/08

I. INTRODUCTION

The following documentation summarizes supplementary raw data concerning the trial BE-15/08 performed 27-May-2008 to 18-June 2008 at the Research Center for Animal Nutrition (NRD/CA, DSM Nutritional Products France, F-68128 Village-Neuf). This trial was reported under the following title: Effect of graded amounts of a microbial phytase on growth performance and phosphorus utilization of broiler chickens fed low-phosphorus diets based on maize and soybean meal (BE-15/08).(Philipps et al.2009)

REFERENCES

P. PHILIPPS and AURELI, R. (2009):
Effect of graded amounts of a microbial phytase on growth performance and phosphorus utilization of broiler chickens fed low-phosphorus diets based on maize and soybean meal (BE-15/08). (DSM Report No 00000101, Regulatory Document, 09-June-2009

II. Raw data of Trial BE-15/08

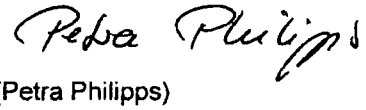
Petra Philipps and Raffaella Aureli,

Effect of graded amounts of a microbial phytase on growth performance and phosphorus utilization of broiler chickens fed low-phosphorus diets based on maize and soybean meal (BE-15/08).(DSM Report No. 00000101 , Regulatory Document, 09-June-2009

RDR 00000101

Analytical data on feed
Animal performance data
Data on apparent utilization of phosphorus
Data on calcium and inorganic phosphorus in plasma
Data on tibia strength and tibia ash

09-June-2009



(Petra Philipps)

DSM Nutritional Products
B.P.170
F-68305 Saint-Louis cedex
France

2.1 Analytical data on feed

(see also tables 1,2 and 3 of report 00000101)

2.1.1 Nutrient content in feed

2.1.2 Ca/P/TiO_2

2.1.3 Phytate in feed

2.1.4 Phytase activity in feed

Service Volaille
BE-15/08 : Traitement A

Analyses d'aliments									
Echantillons	Matière sèche = MS en %	Cendres en %		Fibres en %		Graisses en %		Protéines en %	
			100% MS		100% MS		100% MS		100% MS
A	88.20	5.15	5.84	2.71	3.07	5.49	6.22	21.674	24.574

Echantillons	Matière sèche = MS en %	Amidon en %		Sucre en %	
		VD-LUFA	100% MS	VD-LUFA	100% MS
A	88.20	40.90	46.37	5.10	5.78

25/07/2008

SERVICE VOLAILLE
BE-15/08 : ALIMENTS

CALCIUM - PHOSPHORE ICP					
Echantillon	**gCa/100g MS**	**gCa/100g MF**	**gP/100g MS**	**gP/100g MF**	**MS%**
A	0.60	0.52	0.44	0.39	87.95
B	0.57	0.50	0.44	0.39	88.06
C	0.59	0.52	0.44	0.38	88.03
D	0.60	0.53	0.46	0.41	88.02
E	0.62	0.54	0.46	0.41	87.98
F	0.62	0.55	0.43	0.38	87.96
G	0.57	0.50	0.41	0.36	87.99
P	0.64	0.56	0.57	0.50	88.23
Q	0.64	0.57	0.60	0.52	88.08
R	0.63	0.56	0.64	0.56	87.90

DSM NUTRITINAL PRODUCTS
PHYTATE-P/NON PHYTATE-P
BE-15/08

BE-15/08	Composition de l'aliment	% P Phytique	g P Brut analysé	% P Phytique x g P Brut analysé	x % dans l'aliment
mais	0.602	**0.66**	2.3	1.518	**0.9138**
soja	0.355	**0.6**	6.31	3.786	**1.34403**
P bicalcique		**0**		**0**	**0**
g/kg Phytate P					2.257866
µg/g Phytate P					2257.866
mg/g Phytate					**8.007**

Valeurs théoriques

Phosphore total	Phytate-P (mg/g)	Non Phytate-P (mg/g)
3.9	2.26	1.64

BIOPRACT GmbH

Report of Analysis

30. Mai. 08

DSM Nutritional Products France
Dr. Petra Philipps
CRNA - BP170
F-68305 Saint-Louis Cedex
France

Parameter: Phytase
Request N°: 6a
Product:
Theme N°: 6106
Batch used: PPQ 27987

Registration date: 28.05.2008 **Customer/Manufacturer:** NRD/CA

Sample Number	Sample Label	Declaration U/kg	Found U/kg	Average	STDEV	CV
01	BE-15/08 Treatment	0	41			
	A - P		81	**61**	28	46%
01 rep.	BE-15/08 Treatment	0	77			
	A - P		79	**78**	1	2%
02	BE-15/08 Treatment	250	253			
	B - P		256	**255**	2	1%
03	BE-15/08 Treatment	500	539			
	C - P		471	**505**	48	10%
04	BE-15/08 Treatment	1000	1032			
	D - P		1037	**1035**	4	0%
05	BE-15/08 Treatment	2000	1936			
	E - P		1819	**1878**	83	4%
06	BE-15/08 Treatment	4000	3812			
	F - P		3397	**3605**	293	8%
07	BE-15/08 Treatment	8000	7977			
	G - P		8060	**8019**	59	1%
08	BE-15/08 Treatment	0				
	P - P			**LOQ**		
09	BE-15/08 Treatment	0				
	Q - P			**LOD**		
10	BE-15/08 Treatment	0				
	R - P			**LOD**		

M - mash
P - pellet
C - crumb
PM - premix
E - expanded
F - flour
TQ -Tel Quel

Page 1 of 1

LOD - Limit of Detection
LOQ - Limit of Quantification

Responsible Analyst
J. König

2.2 Animal performance data

(see also table 4 of report 00000101)

2.2.1 Raw data on growth performance and feed consumption on a weekly base

2 pages

TRAITEMENTS	N° cage	Sexe	Nombre d'animaux à J15	Nombre d'animaux à J22	Poids des morts de J8 à J15 en g	Poids des morts de J15 à J22 en g	Poids des animaux à J8 en g	Poids des animaux à J15 en g	Poids des animaux à J22 en g	Poids brut d'aliment à J8 en g	Poids brut d'aliment à J15 en g	Poids brut d'aliment à J22 en g
TT	-	S	N2	N3	M1	M2	TG1	TG2	TG3	FB1	FB2	FB3
A	1	Mâles	8	8			1302	2970	4459	18489	16220	13476
B	2	Mâles	8	8			1309	3403	6971	18524	15743	10641
C	3	Mâles	8	8			1309	3605	7335	18245	15363	10288
D	4	Mâles	8	8			1300	3876	7981	18384	15263	9607
E	5	Mâles	8	8			1308	3985	8320	18586	15455	9620
F	6	Mâles	8	8			1301	3815	8271	18385	15332	9386
G	7	Mâles	8	8			1315	3789	7816	18263	15064	9200
P	14	Mâles	8	8			1301	3444	7105	18578	15745	10648
Q	15	Mâles	8	8			1305	3595	7349	18378	15326	9934
R	16	Mâles	8	8			1316	3466	7419	18385	15564	10090
A	17	Mâles	7	6	202	398	1321	2774	3686	18274	15948	13515
B	18	Mâles	7	7	211		1306	3350	6275	18407	15722	11148
C	19	Mâles	8	8			1281	3661	7599	18245	15381	10056
D	20	Mâles	8	8			1296	3832	7568	18330	15323	10208
E	21	Mâles	8	8			1311	3853	8285	18106	15028	9169
F	22	Mâles	8	8			1307	3710	8049	18723	15673	9753
G	23	Mâles	8	8			1312	3856	8100	18389	15348	9483
P	30	Mâles	8	8			1293	3538	7045	18321	15487	10532
Q	31	Mâles	8	8			1322	3608	7274	18531	15569	10328
R	32	Mâles	7	7	166		1297	3093	6331	18342	15791	11229
A	33	Mâles	6	6	422		1304	2405	3922	18393	16294	13767
B	34	Mâles	8	8			1302	3461	6826	18335	15354	10342
C	35	Mâles	8	8			1305	3671	7609	18176	15182	9727
D	36	Mâles	8	8			1302	3927	7780	18191	15102	9594
E	37	Mâles	8	8			1302	3643	7749	18289	15311	9644
F	38	Mâles	8	8			1299	3838	7966	18059	14835	9146
G	39	Mâles	7	7	193		1288	3278	6951	18194	15365	10255
P	46	Mâles	7	7	291		1300	2948	5880	18358	15846	11711
Q	47	Mâles	8	8			1301	3537	7150	18392	15525	10401
R	48	Mâles	8	8			1312	3362	7001	18144	15445	10380
A	49	Mâles	8	7		467	1305	2993	3913	18260	15964	13344
B	50	Mâles	8	8			1293	3488	6736	18170	15220	10505
C	51	Mâles	8	8			1314	3571	7619	18619	15746	10277
D	52	Mâles	8	8			1294	3687	7992	18589	15650	9980
E	53	Mâles	8	7		883	1287	3888	7277	18248	15119	9415
F	54	Mâles	8	8			1297	3871	8024	18127	14986	9409
G	55	Mâles	8	8			1288	3658	7579	18564	15557	10082
P	62	Mâles	8	7		648	1291	3562	6276	18256	15331	10568
Q	63	Mâles	8	8			1300	3702	7768	18636	15550	9917
R	64	Mâles	8	8			1300	3546	7293	18377	15465	10090
A	65	Mâles	8	7		408	1288	3077	4059	18356	15905	13166
B	66	Mâles	8	8			1292	3667	7417	18510	15417	9966
C	67	Mâles	8	8			1284	3478	7214	18240	15403	10228
D	68	Mâles	8	8			1296	3841	8021	18508	15462	9764
E	69	Mâles	8	8			1283	3880	8006	18259	15155	9449
F	70	Mâles	8	8			1275	3688	7611	18335	15331	9793
G	71	Mâles	8	7		411	1293	3576	6168	18339	15384	10750
P	78	Mâles	8	8			1285	3652	7444	18394	15296	9999
Q	79	Mâles	7	7	354		1301	3103	6391	18268	15477	10739
R	80	Mâles	8	8			1299	3570	7489	18111	15136	9697
A	81	Mâles	8	8			1293	3358	5740	18243	15410	11527
B	82	Mâles	8	8			1296	3646	7555	18517	15504	9950
C	83	Mâles	7	7	318		1303	3209	6771	18249	15444	10488
D	84	Mâles	8	8			1301	3913	7257	18049	14909	9056
E	85	Mâles	7	7	157		1284	3316	8173	18628	15844	10706
F	86	Mâles	8	8			1284	3758	7992	18698	15619	7360
G	87	Mâles	8	7		670	1289	3639	6975	18214	15126	9551
P	94	Mâles	7	7	148		1293	3236	6410	18024	15309	10729
Q	95	Mâles	8	8			1290	3634	7130	18386	15393	10250
R	96	Mâles	8	7		645	1280	3586	6684	18197	15276	10135

TRAITEMENTS	N° cage	Sexe	Gain de poids de J8 à J15 en g par animal	Gain de poids de J15 à J22 en g par animal	Gain de poids de J8 à J22 en g par animal	Consommation d'aliment de J8 à J15 par animal	Consommation d'aliment de J15 à J22 par animal	Consommation d'aliment de J8 à J22 par animal	Indice de consommation de J8 à J15	Indice de consommation de J15 à J22	Indice de consommation de J8 à J22
TT	-	S	WG1	WG2	WGT	AL1	AL2	ALT	IC1	IC2	ICT
A	1	Mâles	206.5	186.1	394.6	283.6	343.0	626.6	1.360	1.843	1.588
A	17	Mâles	231.2	218.0	449.2	324.9	405.0	721.0	1.405	1.857	1.605
A	33	Mâles	237.8	252.8	490.7	327.8	421.2	746.7	1.378	1.666	1.522
A	49	Mâles	211.0	184.9	395.9	287.0	349.2	632.9	1.360	1.889	1.599
A	65	Mâles	223.6	195.2	418.9	306.4	384.7	683.8	1.370	1.971	1.633
A	81	Mâles	258.1	297.8	555.9	354.1	485.4	839.5	1.372	1.630	1.510
B	2	Mâles	261.8	446.0	707.8	347.6	637.8	985.4	1.328	1.430	1.392
B	18	Mâles	315.3	417.9	733.2	375.4	653.4	1027.4	1.191	1.564	1.401
B	34	Mâles	269.9	420.6	690.5	372.6	626.5	999.1	1.381	1.489	1.447
B	50	Mâles	274.4	406.0	680.4	368.8	589.4	958.1	1.344	1.452	1.408
B	66	Mâles	296.9	468.8	765.6	386.6	681.4	1068.0	1.302	1.454	1.395
B	82	Mâles	293.8	488.6	782.4	376.6	694.3	1070.9	1.282	1.421	1.369
C	3	Mâles	287.0	466.3	753.3	360.3	634.4	994.6	1.255	1.361	1.320
C	19	Mâles	297.5	492.3	789.8	358.0	665.6	1023.6	1.203	1.352	1.296
C	35	Mâles	295.8	492.3	788.0	374.3	681.9	1056.1	1.265	1.385	1.340
C	51	Mâles	282.1	506.0	788.1	359.1	683.6	1042.8	1.273	1.351	1.323
C	67	Mâles	274.3	467.0	741.3	354.6	646.9	1001.5	1.293	1.385	1.351
C	83	Mâles	295.6	508.9	804.4	372.8	708.0	1079.0	1.261	1.391	1.341
D	4	Mâles	322.0	513.1	835.1	390.1	707.0	1097.1	1.212	1.378	1.314
D	20	Mâles	317.0	467.0	784.0	375.9	639.4	1015.3	1.186	1.369	1.295
D	36	Mâles	328.1	481.6	809.8	386.1	688.5	1074.6	1.177	1.430	1.327
D	52	Mâles	299.1	538.1	837.3	367.4	708.8	1076.1	1.228	1.317	1.285
D	68	Mâles	318.1	522.5	840.6	380.8	712.3	1093.0	1.197	1.363	1.300
D	84	Mâles	326.5	418.0	744.5	392.5	731.6	1124.1	1.202	1.750	1.510
E	5	Mâles	334.6	541.9	876.5	391.4	729.4	1120.8	1.170	1.346	1.279
E	21	Mâles	317.8	554.0	871.8	384.8	732.4	1117.1	1.211	1.322	1.281
E	37	Mâles	292.6	513.3	805.9	372.3	708.4	1080.6	1.272	1.380	1.341
E	53	Mâles	325.1	553.6	878.7	391.1	739.1	1129.3	1.203	1.335	1.285
E	69	Mâles	324.6	515.8	840.4	388.0	713.3	1101.3	1.195	1.383	1.310
E	85	Mâles	313.2	693.9	1007.1	398.4	734.0	1132.3	1.272	1.058	1.124
F	6	Mâles	314.3	557.0	871.3	381.6	743.3	1124.9	1.214	1.334	1.291
F	22	Mâles	300.4	542.4	842.8	381.3	740.0	1121.3	1.269	1.364	1.330
F	38	Mâles	317.4	516.0	833.4	403.0	711.1	1114.1	1.270	1.378	1.337
F	54	Mâles	321.8	519.1	840.9	392.6	697.1	1089.8	1.220	1.343	1.296
F	70	Mâles	301.6	490.4	792.0	375.5	692.3	1067.8	1.245	1.412	1.348
F	86	Mâles	309.3	529.3	838.5	384.9	1032.4	1417.3	1.245	1.951	1.690
G	7	Mâles	309.3	503.4	812.6	399.9	733.0	1132.9	1.293	1.456	1.394
G	23	Mâles	318.0	530.5	848.5	380.1	733.1	1113.3	1.195	1.382	1.312
G	39	Mâles	307.3	524.7	832.0	398.2	730.0	1127.9	1.296	1.391	1.356
G	55	Mâles	296.3	490.1	786.4	375.9	684.4	1060.3	1.269	1.396	1.348
G	71	Mâles	285.4	434.1	719.5	369.4	669.9	1033.0	1.294	1.543	1.436
G	87	Mâles	293.8	541.6	835.3	386.0	753.7	1138.5	1.314	1.392	1.363
P	14	Mâles	267.9	457.6	725.5	354.1	637.1	991.3	1.322	1.392	1.366
P	30	Mâles	280.6	438.4	719.0	354.3	619.4	973.6	1.262	1.413	1.354
P	46	Mâles	258.6	418.9	677.5	335.1	590.7	924.5	1.296	1.410	1.365
P	62	Mâles	283.9	451.3	735.2	365.6	639.4	1003.4	1.288	1.417	1.365
P	78	Mâles	295.9	474.0	769.9	387.3	662.1	1049.4	1.309	1.397	1.363
P	94	Mâles	300.7	453.4	754.1	390.4	654.3	1044.8	1.298	1.443	1.386
Q	15	Mâles	286.3	469.3	755.5	381.5	674.0	1055.5	1.333	1.436	1.397
Q	31	Mâles	285.8	458.3	744.0	370.3	655.1	1025.4	1.296	1.430	1.378
Q	47	Mâles	279.5	451.6	731.1	358.4	640.5	998.9	1.282	1.418	1.366
Q	63	Mâles	300.3	508.3	808.5	385.8	704.1	1089.9	1.285	1.385	1.348
Q	79	Mâles	280.7	469.7	750.4	363.3	676.9	1037.8	1.295	1.441	1.383
Q	95	Mâles	293.0	437.0	730.0	374.1	642.9	1017.0	1.277	1.471	1.393
R	16	Mâles	268.8	494.1	762.9	352.6	684.3	1036.9	1.312	1.385	1.359
R	32	Mâles	279.7	462.6	742.3	363.7	651.7	1015.4	1.300	1.409	1.368
R	48	Mâles	256.3	454.9	711.1	337.4	633.1	970.5	1.317	1.392	1.365
R	64	Mâles	280.8	468.4	749.1	364.0	671.9	1035.9	1.297	1.434	1.383
R	80	Mâles	283.9	489.9	773.8	371.9	679.9	1051.8	1.310	1.388	1.359
R	96	Mâles	288.3	506.6	794.9	365.1	695.8	1059.4	1.267	1.373	1.333

2.3 Data on apparent utilization of phosphorus

(see also table 5 of report 00000101)

4 pages

TRAITEMENTS	N° éch	TARE	Aliment brut J1	Aliment brut J5	Matière sèche aliment en %	Taux P en % de MS aliment	Taux Ca en % de MS aliment	Quantité de fèces produite en 4 jours en g	MS de fèces frais en %	Taux P en % de MS fèces	Taux Ca en % de MS fèces	Poids des animaux à J1 en g	Poids des animaux à J5 en g	Nombre d'animaux à J1	Nombre d'animaux à J5	Nombre d'animaux X nombre de jours	Poids des morts
TT	-	T	FED1	FED5	DMFE	PHOFE	CAFE	FA4	DMFA	PHOFA	CAFA	W1	W5	N1	N5	FT	M
A1	1	307	16990	15484	87.93	0.44	0.60	1524	26.82	0.86	1.44	2380	3400	8	8	32	
A17	2	307	16718	15310	87.93	0.44	0.60	1404	29.72	0.83	1.43	2257	3070	7	7	28	
A65	3	306	16771	15135	87.93	0.44	0.60	1571	29.22	0.83	1.44	2455	3497	8	8	32	
A81	4	307	16443	14462	87.93	0.44	0.60	1970	26.50	0.81	1.36	2606	3949	8	8	32	
B2	5	454	16765	14583	87.93	0.44	0.60	2549	23.50	0.68	1.13	2614	4229	8	8	32	
B18	6	448	16676	14712	87.93	0.44	0.60	2161	25.44	0.72	1.18	2636	3809	8	7	31	211
b50	7	450	16323	14116	87.93	0.44	0.60	2488	23.92	0.71	1.18	2711	4256	8	8	32	
B82	8	451	16613	14220	87.93	0.44	0.60	2899	20.76	0.64	1.10	2790	4605	8	8	32	
C3	9	450	16428	14168	87.93	0.44	0.60	2607	23.01	0.53	0.90	2739	4528	8	8	32	
C19	10	455	16457	14187	87.93	0.44	0.60	3022	19.35	0.54	0.96	2746	4542	8	8	32	
C67	11	460	16469	14253	87.93	0.44	0.60	2418	24.49	0.57	0.95	2673	4361	8	8	32	
C83	12	355	16474	14342	87.93	0.44	0.60	2554	21.43	0.55	0.93	2358	4017	7	7	28	
D4	13	299	16452	13991	87.93	0.44	0.60	2822	22.08	0.47	0.82	2878	4831	8	8	32	
D20	14	297	16452	14171	87.93	0.44	0.60	2560	22.20	0.45	0.80	2872	4677	8	8	32	
D68	15	302	16667	14136	87.93	0.44	0.60	3008	21.33	0.47	0.89	2790	4832	8	8	32	
D84	16	299	16110	13578	87.93	0.44	0.60	2860	22.02	0.43	0.76	2886	4896	8	8	32	
E5	17	456	16650	14150	87.93	0.44	0.60	2947	22.14	0.39	0.71	2910	4967	8	8	32	
E21	18	454	16214	13687	87.93	0.44	0.60	3166	21.40	0.48	0.82	2827	4890	8	8	32	
E53	19	452	16322	13790	87.93	0.44	0.60	3183	20.59	0.42	0.73	2841	4906	8	8	32	
E85	20	453	16966	14672	87.93	0.44	0.60	2697	22.91	0.44	0.77	2451	4238	7	7	28	
F6	21	454	16558	13999	87.93	0.44	0.60	3013	22.44	0.35	0.67	2763	4850	8	8	32	
F22	22	456	16885	14387	87.93	0.44	0.60	2967	23.12	0.44	0.79	2708	4658	8	8	32	
F70	23	454	16484	14123	87.93	0.44	0.60	2660	22.85	0.41	0.71	2747	4582	8	8	32	
F86	24	455	16799	14282	87.93	0.44	0.60	2978	21.80	0.38	0.66	2810	4804	8	8	32	
G7	25	299	16274	13751	87.93	0.44	0.60	2776	22.33	0.36	0.62	2803	4767	8	8	32	
G23	26	298	16510	14025	87.93	0.44	0.60	2788	21.66	0.34	0.57	2851	4834	8	8	32	
G71	27	301	16507	14284	87.93	0.44	0.60	2322	24.17	0.45	0.73	2732	4319	8	8	32	
G87	28	477	16344	13789	87.93	0.44	0.60	3259	19.07	0.41	0.76	2737	4703	8	8	32	
P14	53	307	16793	14621	88.23	0.57	0.64	2089	27.46	0.93	1.08	2645	4284	8	8	32	
P30	54	299	16556	14386	88.23	0.57	0.64	2174	26.86	0.94	1.06	2667	4298	8	8	32	
P62	55	315	16385	14218	88.23	0.57	0.64	1942	25.45	1.05	1.26	2736	4312	8	8	32	
P94	56	298	16321	14253	88.23	0.57	0.64	2006	26.67	1.06	1.25	2448	3979	7	7	28	
Q15	57	352	16521	14132	88.08	0.60	0.64	2683	23.35	0.95	0.90	2693	4420	8	8	32	
Q31	58	408	16724	14381	88.08	0.60	0.64	2762	22.82	1.07	1.06	2634	4424	8	8	32	
Q79	59	452	16464	14420	88.08	0.60	0.64	2199	26.70	0.89	0.87	2322	3834	7	7	28	
Q95	60	459	16570	14166	88.08	0.60	0.64	2663	24.57	1.03	1.10	2697	4498	8	8	32	
R16	61	451	16654	14380	87.90	0.64	0.63	2498	25.90	1.12	1.02	2580	4302	8	8	32	
R32	62	448	16744	14782	87.90	0.64	0.63	2385	23.50	1.10	0.93	2332	3787	7	7	28	
R80	63	349	16260	13913	87.90	0.64	0.63	2756	22.14	1.10	0.96	2663	4498	8	8	32	
R96	64	349	16454	13987	87.90	0.64	0.63	2792	22.51	1.15	1.06	2601	4552	8	8	32	

SERVICE VOLAILLE
BE-15/08 : FECES FRAICHES

CALCIUM -PHOSPHORE						
Feces	gCa/100g MS	gCa/100g MF	gP/100g MS	gP/100g MF	C%	MS%
1	1.44	0.39	0.86	0.23	4.15	26.82
2	1.43	0.43	0.83	0.25	4.32	29.72
3	1.44	0.42	0.83	0.24	4.37	29.22
4	1.36	0.36	0.81	0.21	3.94	26.50
5	1.13	0.27	0.68	0.16	3.25	23.50
6	1.18	0.30	0.72	0.18	3.58	25.44
7	1.18	0.28	0.71	0.17	3.31	23.92
8	1.10	0.23	0.64	0.13	2.89	20.76
9	0.90	0.21	0.53	0.12	3.10	23.01
10	0.96	0.19	0.54	0.10	2.63	19.35
11	0.95	0.23	0.57	0.14	3.30	24.49
12	0.93	0.20	0.55	0.12	2.86	21.43
13	0.82	0.18	0.47	0.10	2.94	22.08
14	0.80	0.18	0.45	0.10	2.97	22.20
15	0.89	0.19	0.47	0.10	2.81	21.33
16	0.76	0.17	0.43	0.09	2.88	22.02
17	0.71	0.16	0.39	0.09	2.86	22.14
18	0.82	0.18	0.48	0.10	2.80	21.40
19	0.73	0.15	0.42	0.09	2.65	20.59
20	0.77	0.18	0.44	0.10	2.85	22.91
21	0.67	0.15	0.35	0.08	2.87	22.44
22	0.79	0.18	0.44	0.10	2.91	23.12
23	0.71	0.16	0.41	0.09	2.97	22.85
24	0.66	0.14	0.38	0.08	2.76	21.80
25	0.62	0.14	0.36	0.08	2.77	22.33
26	0.57	0.12	0.34	0.07	2.67	21.66
27	0.73	0.18	0.45	0.11	3.07	24.17
28	0.76	0.15	0.41	0.08	2.53	19.07
53	1.08	0.30	0.93	0.26	3.81	27.46
54	1.06	0.28	0.94	0.25	3.69	26.86
55	1.26	0.32	1.05	0.27	3.60	25.45
56	1.25	0.33	1.06	0.28	3.86	26.67
57	0.90	0.21	0.95	0.22	2.99	23.35
58	1.06	0.24	1.07	0.24	3.06	22.82
59	0.87	0.23	0.89	0.24	3.48	26.70
60	1.10	0.27	1.03	0.25	3.32	24.57
61	1.02	0.26	1.12	0.29	3.33	25.90
62	0.93	0.22	1.10	0.26	3.03	23.50
63	0.96	0.21	1.10	0.24	2.86	22.14
64	1.06	0.24	1.15	0.26	2.99	22.51

TRAITEMENTS	N° éch	Quantité d'aliment ingéré en g de MS par jour et animal	Rétention du P en g	Coefficient d'utilisation apparente du P en %	Rétention du Ca en g	Coefficient d'utilisation apparente du Ca en %	Gain de poids de J1 à J5 en g par animal et jour	Indice de consomation	Phosphore dans l'excrétion (g/kg de MS)	Calcium dans l'excrétion (g/kg de MS)
TT	-	DMI	RPHO	RPHOP	RCA	RCAP	WG1-5	IC1-5		
A1	1	41.382	0.094	51.829	0.101	40.851	31.9	1.476	8.6	14.4
A17	2	44.216	0.098	50.331	0.099	37.245	29.0	1.732	8.3	14.3
A65	3	44.954	0.102	51.524	0.103	38.324	32.6	1.570	8.3	14.4
A81	4	54.434	0.128	53.429	0.139	42.658	42.0	1.475	8.1	13.6
B2	5	59.957	0.159	60.347	0.186	51.677	50.5	1.351	6.8	11.3
B18	6	55.708	0.144	58.703	0.168	50.368	53.7	1.419	7.2	11.8
b50	7	60.644	0.159	59.471	0.184	50.605	48.3	1.428	7.1	11.8
B82	8	65.755	0.188	64.863	0.220	55.713	56.7	1.318	6.4	11
C3	9	62.101	0.191	69.911	0.233	62.531	55.9	1.263	5.3	9
C19	10	62.375	0.191	69.453	0.225	60.176	56.1	1.264	5.4	9.6
C67	11	60.892	0.183	68.119	0.223	61.034	52.8	1.313	5.7	9.5
C83	12	66.952	0.202	68.578	0.245	61.037	59.3	1.285	5.5	9.3
D4	13	67.624	0.216	72.500	0.263	64.815	61.0	1.260	4.7	8.2
D20	14	62.678	0.205	74.384	0.250	66.604	56.4	1.264	4.5	8
D68	15	69.547	0.221	72.292	0.257	61.523	63.8	1.239	4.7	8.9
D84	16	69.575	0.230	75.243	0.283	67.912	62.8	1.260	4.3	7.6
E5	17	68.695	0.235	77.766	0.290	70.317	64.3	1.215	3.9	7.1
E21	18	69.437	0.218	71.511	0.268	64.310	64.5	1.225	4.8	8.2
E53	19	69.575	0.232	75.888	0.289	69.267	64.5	1.226	4.2	7.3
E85	20	72.040	0.236	74.517	0.291	67.297	63.8	1.284	4.4	7.7
F6	21	70.317	0.247	79.700	0.302	71.503	65.2	1.226	3.5	6.7
F22	22	68.640	0.222	73.574	0.269	65.205	60.9	1.281	4.4	7.9
F70	23	64.876	0.221	77.375	0.277	71.269	57.3	1.287	4.1	7.1
F86	24	69.162	0.239	78.536	0.302	72.662	62.3	1.262	3.8	6.6
G7	25	69.327	0.243	79.597	0.309	74.232	61.4	1.285	3.6	6.2
G23	26	68.283	0.243	80.926	0.314	76.550	62.0	1.253	3.4	5.7
G71	27	61.084	0.200	74.447	0.255	69.601	49.6	1.401	4.5	7.3
G87	28	70.207	0.241	77.994	0.295	70.086	61.4	1.300	4.1	7.6

base be1508_raw data

27/04/2009

BE-15/08
Bilan Fécès

TRAITEMENTS	N° éch	Quantité d'aliment ingéré en g de MS par jour et animal	Rétention du P en g	Coefficient d'utilisation apparente du P en %	Rétention du Ca en g	Coefficient d'utilisation apparente du Ca en %	Gain de poids de J1 à J5 en g par animal et jour	Indice de consomation	Phosphore dans l'excrétion (g/kg de MS)	Calcium dans l'excrétion (g/kg de MS)
P14	53	59.886	0.199	58.337	0.218	56.909	51.2	1.325	9.3	10.8
P30	54	59.831	0.193	56.617	0.216	56.430	51.0	1.330	9.4	10.6
P62	55	59.748	0.205	60.098	0.219	57.355	49.3	1.375	10.5	12.6
P94	56	65.164	0.199	53.574	0.214	51.241	54.7	1.351	10.6	12.5
Q15	57	65.757	0.233	59.051	0.268	63.631	54.0	1.383	9.5	9
Q31	58	64.491	0.207	53.580	0.235	56.888	55.9	1.309	10.7	10.6
Q79	59	64.298	0.238	61.569	0.267	64.781	54.0	1.352	8.9	8.7
Q95	60	66.170	0.223	56.097	0.237	56.043	56.3	1.335	10.3	11
R16	61	62.464	0.214	53.592	0.225	57.064	53.8	1.321	11.2	10.2
R32	62	61.593	0.215	54.641	0.237	61.042	52.0	1.348	11	9.3
R80	63	64.469	0.229	55.609	0.246	60.644	57.3	1.279	11	9.6
R96	64	67.765	0.236	54.442	0.245	57.340	61.0	1.264	11.5	10.6

		Quantité d'aliment ingéré en g de MS par jour et animal	Rétention du P en g	Coefficient d'utilisation apparente du P en %	Rétention du Ca en g	Coefficient d'utilisation apparente du Ca en %	Gain de poids de J1 à J5 en g par animal et jour	Indice de consomation	Phosphore dans l'excrétion (g/kg de MS)	Calcium dans l'excrétion (g/kg de MS)
A	**Mean**	**46.2**	**0.11**	**51.8**	**0.11**	**39.8**	**33.9**	**1.563**	**8.3**	**14.2**
	Stdv	5.7	0.015	1.3	0.019	2.4	5.6	0.121	0.2	0.4
B	**Mean**	**60.5**	**0.16**	**60.8**	**0.19**	**52.1**	**52.3**	**1.379**	**6.9**	**11.5**
	Stdv	4.1	0.018	2.8	0.022	2.5	3.7	0.053	0.4	0.4
C	**Mean**	**63.1**	**0.19**	**69.0**	**0.23**	**61.2**	**56.0**	**1.281**	**5.5**	**9.4**
	Stdv	2.7	0.008	0.8	0.010	1.0	2.7	0.023	0.2	0.3
D	**Mean**	**67.4**	**0.22**	**73.6**	**0.26**	**65.2**	**61.0**	**1.256**	**4.6**	**8.2**
	Stdv	3.2	0.011	1.4	0.014	2.8	3.3	0.011	0.2	0.5
E	**Mean**	**69.9**	**0.23**	**74.9**	**0.28**	**67.8**	**64.3**	**1.238**	**4.3**	**7.6**
	Stdv	1.5	0.008	2.6	0.011	2.6	0.3	0.031	0.4	0.5
F	**Mean**	**68.2**	**0.23**	**77.3**	**0.29**	**70.2**	**61.5**	**1.264**	**4.0**	**7.1**
	Stdv	2.4	0.013	2.7	0.017	3.4	3.3	0.027	0.4	0.6
G	**Mean**	**67.2**	**0.23**	**78.2**	**0.29**	**72.6**	**58.6**	**1.310**	**3.9**	**6.7**
	Stdv	4.2	0.021	2.8	0.027	3.3	6.0	0.064	0.5	0.9
P	**Mean**	**61.2**	**0.20**	**57.2**	**0.22**	**55.5**	**51.5**	**1.345**	**10.0**	**11.6**
	Stdv	2.7	0.005	2.8	0.002	2.9	2.3	0.023	0.7	1.1
Q	**Mean**	**65.2**	**0.23**	**57.6**	**0.25**	**60.3**	**55.0**	**1.345**	**9.9**	**9.8**
	Stdv	0.9	0.013	3.5	0.018	4.5	1.2	0.031	0.8	1.1
R	**Mean**	**64.1**	**0.22**	**54.6**	**0.24**	**59.0**	**56.0**	**1.303**	**11.2**	**9.9**
	Stdv	2.7	0.011	0.8	0.010	2.1	4.0	0.038	0.2	0.6

base be1508_raw data

27/04/2009

BE-15/08
Bilan Fécès

2.4 Data on calcium and inorganic phosphorus in plasma

(see also table 6 of report 00000101)

4 pages

TRAITEMENTS	Taux de P plasmatique en mg/dl	Taux de Ca plasmatique en mg/dl	Taux de P plasmatique en mmol/L	Taux de Ca plasmatique en mmol/L
A	3.70	11.80	1.19	2.94
A	4.20	13.10	1.36	3.27
A	3.30	11.90	1.07	2.97
A	3.20	12.40	1.03	3.09
B	4.50	12.00	1.45	2.99
B	4.30	11.80	1.39	2.94
B	3.20	11.50	1.03	2.87
B	6.00	11.90	1.94	2.97
C	6.40	12.00	2.07	2.99
C	5.80	11.50	1.87	2.87
C	5.10	11.80	1.65	2.94
C	6.00	11.70	1.94	2.92
D	7.60	11.80	2.45	2.94
D	7.30	11.30	2.36	2.82
D	8.00	11.60	2.58	2.89
D	7.90	11.50	2.55	2.87
E	8.50	11.30	2.74	2.82
E	7.60	11.10	2.45	2.77
E	8.00	11.20	2.58	2.79
E	8.20	11.50	2.65	2.87
F	7.80	11.20	2.52	2.79
F	8.20	11.60	2.65	2.89
F	7.20	11.30	2.32	2.82
F	8.20	11.40	2.65	2.84
G	8.90	12.10	2.87	3.02
G	8.60	11.40	2.78	2.84
G	8.80	12.20	2.84	3.04
G	8.10	11.50	2.62	2.87
P	5.50	11.10	1.78	2.77
P	5.20	11.80	1.68	2.94
P	3.80	12.10	1.23	3.02
P	7.00	11.20	2.26	2.79
Q	7.20	11.80	2.32	2.94
Q	7.50	11.80	2.42	2.94
Q	7.20	11.30	2.32	2.82
Q	6.90	11.60	2.23	2.89
R	7.50	11.60	2.42	2.89
R	7.70	11.60	2.49	2.89
R	7.70	12.30	2.49	3.07
R	8.30	12.10	2.68	3.02

TRAITEMENTS	Taux de P plasmatique en mg/dl		Taux de Ca plasmatique en mg/dl		Taux de P plasmatique en mmol/L		Taux de Ca plasmatique en mmol/L	
A	**3.60**	*0.45*	**12.30**	*0.59*	**1.16**	*0.15*	**3.07**	*0.15*
B	**4.50**	*1.15*	**11.80**	*0.22*	**1.45**	*0.37*	**2.94**	*0.05*
C	**5.83**	*0.54*	**11.75**	*0.21*	**1.88**	*0.18*	**2.93**	*0.05*
D	**7.70**	*0.32*	**11.55**	*0.21*	**2.49**	*0.10*	**2.88**	*0.05*
E	**8.08**	*0.38*	**11.28**	*0.17*	**2.61**	*0.12*	**2.81**	*0.04*
F	**7.85**	*0.47*	**11.38**	*0.17*	**2.53**	*0.15*	**2.84**	*0.04*
G	**8.60**	*0.36*	**11.80**	*0.41*	**2.78**	*0.11*	**2.94**	*0.10*
P	**5.38**	*1.31*	**11.55**	*0.48*	**1.74**	*0.42*	**2.88**	*0.12*
Q	**7.20**	*0.24*	**11.63**	*0.24*	**2.32**	*0.08*	**2.90**	*0.06*
R	**7.80**	*0.35*	**11.90**	*0.36*	**2.52**	*0.11*	**2.97**	*0.09*

N° éch.	Phosphore mg/dl Moyenne	Calcium (mg/dl) Moyenne
1	3.34	11.86
2	3.87	11.96
3	2.98	12.35
4	4.81	10.86
5	4.55	13.72
6	3.48	12.29
7	4.48	13.33
8	4.21	12.98
9	2.98	11.28
10	3.22	12.24
11	3.78	12.50
12	3.24	11.76
13	3.64	12.70
14	2.56	12.24
15	3.70	12.74
16	2.89	12.01
17	5.11	11.97
18	5.74	12.16
19	3.26	12.28
20	3.83	11.45
21	4.69	12.05
22	3.86	11.37
23	3.84	11.49
24	4.77	12.24
25	3.67	11.15
26	2.52	12.03
27	3.60	11.25
28	2.98	11.67
29	7.67	12.02
30	4.57	11.85
31	6.21	11.05
32	5.53	12.68
33	8.33	12.25
34	6.90	12.17
35	5.50	11.02
36	4.70	12.57
37	6.09	11.32
38	7.67	12.04
39	4.71	11.36
40	4.62	11.38
41	4.13	13.20
42	5.93	11.36
43	4.87	12.02
44	5.59	10.56
45	6.99	11.61
46	7.08	11.58
47	5.35	11.61
48	4.38	12.07
49	6.81	11.83
50	7.22	11.91
51	8.09	10.98
52	8.33	12.54
53	8.17	12.21
54	5.69	11.17
55	7.91	10.25
56	7.62	11.73
57	7.80	11.21

58	7.25	**11.72**
59	7.14	**11.36**
60	9.66	**11.99**
61	7.63	**11.71**
62	7.09	**10.78**
63	8.35	**11.08**
64	8.40	**12.58**
65	8.55	**10.97**
66	8.04	**11.80**
67	9.25	**11.49**
68	8.03	**11.05**
69	8.25	**11.33**
70	6.92	**10.83**
71	8.15	**11.46**
72	7.27	**10.71**
73	8.37	**11.11**
74	7.97	**12.13**
75	7.32	**10.34**
76	8.27	**11.23**
77	7.31	**11.03**
78	7.33	**10.97**
79	9.69	**11.55**
80	8.47	**12.31**
81	7.71	**11.77**
82	7.77	**11.53**
83	8.41	**10.77**
84	7.41	**10.82**
85	8.72	**11.77**
86	8.03	**11.79**
87	8.27	**10.99**
88	7.97	**11.82**
89	7.53	**12.31**
90	7.46	**11.36**
91	7.08	**11.32**
92	6.87	**10.29**
93	7.77	**11.81**
94	7.99	**11.29**
95	9.37	**11.35**
96	7.50	**10.99**
97	8.91	**11.70**
98	9.13	**12.52**
99	8.75	**12.33**
100	8.68	**12.04**
101	8.97	**11.17**
102	8.81	**11.55**
103	8.74	**11.02**
104	7.83	**11.93**
105	8.51	**14.04**
106	9.20	**11.63**
107	8.25	**12.01**
108	9.26	**11.29**
109	8.45	**12.03**
110	8.44	**11.66**
111	7.64	**10.65**
112	7.98	**11.57**
209	4.94	**10.86**
210	6.88	**11.00**
211	3.47	**11.87**
212	6.86	**10.54**

213	4.34	**12.43**
214	3.19	**12.43**
215	5.82	**11.28**
216	7.30	**11.12**
217	3.41	**12.62**
218	4.01	**11.98**
219	4.03	**11.76**
220	3.83	**12.17**
221	9.03	**9.80**
222	5.99	**11.48**
223	4.62	**11.50**
224	8.38	**12.17**
225	8.05	**12.24**
226	7.35	**12.52**
227	6.36	**10.83**
228	6.95	**11.69**
229	8.00	**12.06**
230	6.73	**12.41**
231	7.47	**11.30**
232	7.72	**11.43**
233	7.99	**11.09**
234	7.53	**12.86**
235	7.40	**10.72**
236	5.80	**10.62**
237	7.21	**11.40**
238	6.56	**11.43**
239	6.82	**11.83**
240	6.83	**11.67**
241	8.19	**12.01**
242	7.13	**10.61**
243	7.99	**12.20**
244	6.82	**11.57**
245	7.40	**12.23**
246	8.23	**12.23**
247	7.57	**10.87**
248	7.49	**10.96**
249	8.04	**11.39**
250	8.43	**11.75**
251	6.88	**12.31**
252	7.40	**13.69**
253	8.86	**11.03**
254	7.91	**11.76**
255	8.32	**12.07**
256	8.17	**13.39**

2.5 Data on tibia strength and tibia ash

(see also table 7 of report 00000101)

TRAITEMENTS	Résistance osseuse en N	Taux de cendres en %
A	47.50	38.65
A	37.80	40.16
A	104.80	42.09
A	115.30	41.80
B	138.80	47.26
B	184.80	47.16
B	139.50	44.51
B	203.20	48.29
C	288.70	50.11
C	202.90	48.42
C	194.90	46.64
C	249.40	55.59
D	190.50	50.64
D	172.50	50.36
D	234.40	52.23
D	256.50	51.45
E	161.90	51.72
E	215.70	51.38
E	250.20	52.63
E	344.80	52.04
F	238.90	50.89
F	294.60	52.28
F	180.70	52.59
F	220.60	52.07
G	177.30	51.42
G	229.90	51.88
G	250.20	51.55
G	259.20	59.42

TRAITEMENTS	Résistance osseuse en N	Taux de cendres en %
P	134.90	47.18
P	138.30	45.49
P	116.50	45.38
P	120.80	46.71
Q	200.90	50.66
Q	159.80	49.83
Q	142.10	49.66
Q	174.90	48.54
R	185.00	50.11
R	197.20	50.72
R	132.70	48.48
R	213.90	50.03

TRAITEMENTS	Résistance osseuse en N	Taux de cendres en %
A	**76.35**	**40.67**
	39.35	*1.60*
B	**166.58**	**46.80**
	32.55	*1.62*
C	**233.98**	**50.19**
	43.69	*3.87*
D	**213.48**	**51.17**
	38.71	*0.84*
E	**243.15**	**51.94**
	76.89	*0.53*
F	**233.70**	**51.96**
	47.32	*0.74*
G	**229.15**	**53.57**
	36.67	*3.91*

TRAITEMENTS	Taux de cendres en %	Résistance osseuse en N
P	**127.63**	**46.19**
	10.60	*0.90*
Q	**169.43**	**49.67**
	24.90	*0.87*
R	**182.20**	**49.84**
	35.06	*0.95*

III. Trial Protocol Data Sheet

Trial Protocol Data Sheet

According to EFSA Journal (2008) 778, 5-13 Technical guidance: Tolerance and efficacy studies in target animals
Data sheet to be filled out by the applicant and signed by the study director and then added to each trial report concerning safety and efficacy of the additive for the target animal

For terrestrial animals

Identification of the additive: IPA phytase	Batch number: PPQ 27987
Trial ID: BE-15/08	
Location: Research Center for Animal Nutrition (DSM Nutritional Products France, F-68128 Village-Neuf)	
Start date and exact duration of the study:May-27-2008 to June-18-2008, 2 weeks (1 week pre-trial period)	
Number of treatment groups (+ control(s)): 6 (+4)	Replicates per group: 6
Total number of animals: 480	Animals per replicate: 8
Dose(s) of the additive/active substance(s)/agent(s) (mg/Units of activity/CFU kg^{-1} complete feed/L^{-1} water)	
Intended:0/250/500/1000/2000/8000 $U.kg^{-1}$	Analysed: 78/255/505/1035/1878/3605/8019 $U.kg^{-1}$
Substances used for comparative purposes:	
Intended dose:	Analysed:
Animal species/category: Broiler	
Breed: Ross PM3	Identification procedure: per cage number
Sex: Males Age at start:8 days	Body weight at start: 171 g
Physiological stage: Growing	General health: normal (P-deficient basal diet)
Additional Information for field trials:	
Location and size of herd or flock:	
Feeding and rearing conditions:	
Method of feeding:	
Diets (type(s)): low phosphorus basal diet	
Presentation of the diet: Mash ☐ Pellet ☒ Extruded ☐ Other	
Composition (main feedingstuffs): 60.20% maize / 35.50% SBM	
Nutrient content (relevant nutrients and energy content)	
Intended values: per kg: 12.6 MJ/ME, 216 g Crude protein, 4.1 g total P, 6.0 g Calcium, 1.6 g Non Phytate P	
Analysed values: per kg: 12.7 MJ/ME, 217 g Crude protein, 3.9 g total P, 5.2 g Calcium	
Date and nature of the examinations performed: growth performance, apparent phosphorus utilization, bone quality, plasma	
Method(s) of statistical evaluation used: one-factorial analysis of variance (factor: treatment), Newman-Keuls test, non-linear regression analysis	
Therapeutic/preventive treatments (reason, timing, kind, duration): nothing to report	
Timing and prevalence of any undesirable consequences of treatment: nothing to report	

Date 09-June-2009	Signature Study Director Reba Pluijms

TAB

ANNEX 23

Annex 23

Rodehutscord, M. et *al*. (2009). Report No. 00001790: Dose response study with a new phytase (RONOZYME® HiPhos) in broiler chickens. 2009

REPORT No. 00001790
Regulatory Document

DSM

Document Date:	26 October, 2009
Author(s):	M. Rodehutscord[1], J. Boguhn[1] and J. Broz[2]
	[1] Institute of Animal Nutrition, University Hohenheim, Stuttgart (Germany) [2] Animal Nutrition and Health R&D, DSM Nutritional Products Ltd, Basel
Title:	**Dose response study with a new phytase (IPA Mash Phytase) in broiler chickens**
Project No.	6106

Summary

The efficacy of a new bacterial 6-phytase (IPA Mash Phytase) was tested in 3 to 4-week old broiler chickens. The basal low-P diet contained maize and soybean meal as the main feed ingredients and total P and Ca concentrations were 4.7 and 10.8 g/kg dry matter, respectively. The diet was supplemented with IPA Phytase at levels of 500, 1000, 2000 and 4000 U/kg or remained unsupplemented. Excretions of P and Ca were determined in a balance trial with 10 individual birds per dietary treatment and tibiae were obtained after the birds received their respective diets for 12 days. Another 500 chickens (10 pens with 10 birds per treatment) were fed for 7 days until slaughtering and removal of digesta for the determination of the precaecal P and Ca digestibility. The effect of phytase on the utilization of both minerals was highly significant. The utilization of P and Ca was improved by phytase supplementation from 47 and 28% in the basal diet (negative control) to a maximum of 75 and 48% at the highest level of supplementation, respectively. Tibia contents of ash, P and Ca were improved by phytase supplementation as well. The precaecal digestibility of P was significantly increased from 32% to 73% with increasing phytase supplementation. It is concluded that this new phytase product is efficient in broiler chickens. By supplementing this phytase in combination with a reduced use of inorganic P in the diet beneficial environmental effects can be achieved by reduced P excretion.

This report consists of Pages I – II and 1 – 12 & Annex C

Distribution

Dr. M. Eggersdorfer, NRD
Dr. F. Fru, NRD/PA
Mr. J.-F. Hecquet, NBD/RG
Dr. A.-M. Klünter, NRD/CA
Dr. J. Pheiffer, NRD/PA
Dr. P. Philipps, NRD/CA
Mr. J.-P. Ruckebusch, ANH/GM

Approved

Name	Signature	Date
Main Author Dr. J. Broz, NRD/CA	signed by J. Broz	27.10.2009
Principal Scientist / Competence Mgr Dr. J. Broz, NRD/CA	signed by J. Broz	27.10.2009
Research Center Head Dr. A.-M. Klünter, NRD/CA	signed by A.-M. Klünter	28.10.2009
Project Manager Dr. F. Fru, NRD/PA	signed by F. Fru	29.10.2009

Nomenclature and Structural Formula

IPA phytase (M), enzyme product containing bacterial 6-phytase (EC 3.1.3.26), produced by submerged fermentation of a genetically modified *Aspergillus oryzae* strain. Lot PPQ 28683 was used in this study, manufactured by Novozymes A/S, Bagsvaerd, Denmark.

UNIVERSITÄT HOHENHEIM
INSTITUT FÜR TIERERNÄHRUNG
Professor Dr. Markus Rodehutscord

Dose response study with a new phytase (IPA Mash Phytase) in broiler chickens

Report to DSM Nutritional Products, Basel

Emil-Wolff-Str. 8-10 • 70599 Stuttgart • Telefon: 0711-459 22420 • Telefax: 0711-459 22421 • E-Mail: markus.rodehutscord@uni-hohenheim.de

Introduction

The major proportion of phosphorus (P) in plant feed ingredients is found in the form of phytate, which is largely unavailable to monogastric animals. Microbial phytase can improve the utilisation of phytate P, which has extensively been shown in different poultry species.
Phytase efficacy is not entirely predictable, however. The benefit achieved is known to depend upon several factors, including the raw materials used, the source of phytase, the age of the animals, dietary contents of calcium, phosphorus and vitamin D_3, and the level of phytase activity present in the ingredients used. Different phytase products also may be different in their efficacy depending on origin.

It was the objective of the present experiment to study the effects of a new phytase product on the precaecal (pc) digestibility and utilisation of P in broiler chickens. Tibia responses were also studied.

Material and methods

Diets

From day 1 to 13 post hatch the birds received a starter diet based on maize and soybean meal that was calculated to be adequate in ME and all nutrients including P according to the recommendations of GfE[1]. Analysed nutrient concentrations are shown in Table 1. Due to a technical failure the sodium content was too low during the first 7 days, which explains that the growth of the birds in the pre-treatment phase was low.

Table 1: Intended and analysed phytase activity and analysed concentrations of crude nutrients, P, and Ca in the diets

Diet	Phytase activity (U/kg)		Ash	Crude protein	Crude fibre	Ether extract	P	Ca
	Intended	Analysed	(g/kg dry matter)					
Starter			68	260	37	62	7.6	13.1
A	0	< 50	62	275	37	60	4.7	10.8
B	500	466	63	247	36	64	4.6	9.8
C	1000	1012	64	247	32	63	4.7	12.1
D	2000	1939	63	250	34	63	4.3	10.2
E	4000	3644	62	243	36	63	4.8	10.4

The experimental basal diet also was based on maize (541 g/kg) and solvent-extracted soybean meal from dehulled seed (400 g/kg), but without a mineral P supplementation in order to achieve a sufficiently low basal P level. In addition, soybean oil (20 g/kg), calcium carbonate

[1] GfE. 1999. Empfehlungen zur Energie- und Nährstoffversorgung der Legehennen und Masthühner (Broiler). DLG Verlags GmbH, Frankfurt/Main.

(21 g/kg), and a P-free vitamin and mineral mix[2] (13 g/kg) were included. The diet contained TiO_2 as indigestible marker (5 g/kg) and was not pelleted.

Diet preparation was done in the certified feed mill facilities of Hohenheim University, Research Station for Animal Husbandry, Animal Breeding and Small Animal Breeding in 72800 Eningen, Germany. The total amount of feed needed for the experiment was mixed in one lot and subsequently divided into 5 equal parts. Phytase was then supplemented to achieve activities as detailed in Table 1, and the diets were mixed again. The basal diet remained without the supplement. Intended activities as well as P and Ca concentrations were confirmed by analyses (Table 1).

The test product was a bacterial 6-phytase (IPA Mash Phytase) and was supplied by DSM Nutritional Products, Basel, Switzerland. It was expressed in a genetically modified strain of *Aspergillus oryzae*. The lot number was PPQ 28683 and the product was provided in a powder form containing 58,753 U/g.

Animals, housing and sampling

The experiment was conducted in the Research Station for Animal Husbandry, Animal Breeding and Small Animal Breeding of the University Hohenheim, 72800 Eningen, Germany. Six hundred day-old broiler chickens (Ross 308) were allocated to 50 pens of 12 birds each on a wood shavings bedding. Birds underwent routine vaccination against Coccidiosis, Newcastle disease, and Infectious bursal disease on d 3, 10, and 14, respectively. Diets did not contain a coccidiostat. On d 11, all birds got a vitamin preparation via the drinking water.

On d 14 of the experiment 50 birds (the one with the highest BW from each pen, 10 per treatment) were housed individually in balance cages. From d 14 to 24 the respective experimental diet was fed slightly restricted (50 g per bird and day) in order to avoid feed refusals. On d 25 the experimental diet was offered for *ad libitum* intake. From d 19 to 24 excreta were quantitatively collected, pooled for each bird and stored at -20°C. Later the excreta were mixed and oven-dried at 65°C for 72 h prior to analysis. Broilers were weighed at the beginning and the end of the collection period.
On d 26 the birds were killed, the tibia bones removed and stored at -20°C until further handling.

A total of 500 broilers remained in their pens for determination of precaecal (pc) digestibility of P and Ca. Ten pens were allocated to each of the five experimental diets in a way that an equal distribution of all treatments over the animal house was given. The respective experimental diet was offered for *ad libitum* intake for 7 days until slaughtering by carbon dioxide exposure on d 21. The medial and terminal section between Meckel's diverticulum and 2 cm anterior to the ileo-caeco-colonic junction was isolated. The digesta was flushed out with double-distilled water, pooled per pen, and immediately frozen at -20°C until freeze-drying.

[2] Premix contained per kg: NaCl 77 g; Cholinchloride 154 g; Sodium bicarbonate 231 g; Vitamin A 6,000,000 I.E.; Vitamin D3 1,500,000 I.E.; Vitamin E 15 g; Vitamin B1 1.5 g ; Vitamin B2 3 g; Vitamin B6 3 g; Vitamin B12 15 mg; Vitamin K3 1.2 g; Nicotinic acid 25 g; Pantothenic acid 7 g; Biotin 50 mg; Folic acid 500 mg; Fe 90 g; Mn 120 g; Zn 80 g; Cu 15 g; I 1.7 g; Se 0.5 g; Co 0.6 g

Body weight gain, feed consumption, and gain to feed ratio were determined per pen for the period between d 14 and 21.

Analyses and data evaluation

Concentrations of dry matter and crude nutrients were determined according to VDLUFA standard methods[3]. Samples of feed, excreta and digesta were ground through a sieve with 1-mm pore size and treated in the institute's laboratory for analyses of P, Ca, and Ti according to Boguhn et al. (2009)[4].

Adhesive tissues on the tibiae were removed by incubating the bones for 24 h at 58°C in a solution that mainly consisted of water, fatty acid alcohol, protease and alpha-amylase (Biozym SE, SPINRAD®, interTee Handels GmbH, 22848 Norderstedt, Germany). Bones were then cleaned in distilled water and remaining soft tissues removed. Bones were dried for 4 h at 65°C. Cleaned air dry bones were later incinerated at 550°C and the remaining ash was analysed as mentioned above for the other samples.

Measurements of P, Ca, and Ti were made using an inductively coupled plasma spectrometer (ICP-OES). Phytase activity in the feed was determined according to Engelen et al. (1994)[5] by Biopract GmbH, Berlin, Germany.

'Utilisation' was calculated as the difference between measured intake and measured excretion relative to intake. Precaecal digestibility (y) was calculated based on the ratio of the nutrient under study and TiO_2 in diet and digesta according to the generally accepted equation:

$$y\ (\%) = 100 - 100 \times \frac{TiO_2 \text{ in diet (g/kg)}}{TiO_2 \text{ in digesta (g/kg)}} \times \frac{\text{Nutrient in digesta (g/kg)}}{\text{Nutrient in diet (g/kg)}}$$

Data were subjected to *glm* procedure using the software package SAS for Windows 9.2. In case of a significant treatment effect means were compared using t-test. The Dunnett test was used to detect effects of a supplementation of phytase to the control without phytase.

Non-linear regression analysis was performed with the program GraphPad Prism 5.02. An exponential model of the following type was fitted to the data:

$$y = a \times (1 - e^{(-b \times (x-c))})$$

with a: upper y asymptote (estimated maximum)
b: parameter describing the steepness of the curve
c: estimated x intercept
y: response criterion
x: supplemented phytase (U/kg).

[3] Naumann, C. and R. Bassler. 1976. VDLUFA-Methodenbuch, Vol. III. Die chemische Untersuchung von Futtermitteln with supplements 1983, 1988, 1993, 1997, 2004, and 2006. VDLUFA-Verlag, Darmstadt.
[4] Boguhn, J., T. Baumgärtel, A. Dieckmann, and M. Rodehutscord. 2009. Determination of titanium dioxide supplements in different matrices using two methods involving photometer and inductively coupled plasma optical emission spectrometer measurements. Archives of Animal Nutrition 63, 337-342.
[5] Engelen, A. J., F.C. van der Heeft, P.H. Randsdorp, and E.L.C. Smit. 1994. Simple and rapid determination of phytase activity. Journal of AOAC International 77, 760-764.

Results

The study on the pc digestibility could be finished without any problems. Body weight gain, feed consumption, and the gain to feed ratio were significantly increased by phytase supplementation (Table 2).

Table 2: Body weight (BW), BW gain, feed consumption and gain to feed ratio of broiler chickens in the 7-day experimental period (Means and SD, 10 replicates per treatment)

Phytase	Treatment	Initial BW	Final BW	BW gain	Feed consumption	Gain to feed ratio
U/kg		g	g	g	g	g/g
0	A	167	315[a]	149[a]	254[a]	0.59[a]
		12	27	16	21	0.05
500	B	162	328[a]	165[b]	268[ab]	0.62[a]
		6	16	12	16	0.05
1000	C	161	331[a]	171[*b]	276[b]	0.62[a]
		11	28	18	28	0.05
2000	D	164	356[*b]	192[*c]	288[*bc]	0.67[*b]
		12	30	19	29	0.04
4000	E	164	373[*b]	209[*c]	304[*c]	0.69[*b]
		9	23	18	27	0.06
P		0.74	<0.001	<0.001	<0.001	<0.001

[*] Means are significantly different from the unsupplemented treatment A according to Dunnett test.
[abc] Values without a common superscript are significantly different according to t-test ($p \leq 0.05$)

The pc digestibility of P increased from 32% to 73% with increasing phytase supplementation (Table 3). Each level of phytase supplementation led to a significant increase in pc P digestibility. The pc digestibility of Ca was 49% in the unsupplemented basal diet. Phytase supplementation also led to a significant increase in pc Ca digestibility.

The broiler chickens in the balance trial weighed 210 g on d 14 and 300 g at the end of the trial. Growth was not significantly affected, which can be explained by the feed restriction that was employed. The excretion of P was significantly reduced ($P < 0.001$) by phytase supplementation (Table 3). Correspondingly, the effect of phytase on the utilisation of P also was highly significant. A distinct plateau in P utilisation was not achieved within the level of supplementation studied (Figure 1).

The excreted amounts of Ca were significantly lower at the two highest levels of supplementation compared to the control. The utilisation of Ca increased from 28 to 48 % with increasing phytase supplementation.

Table 3: Precaecal (pc) digestibility, excreted amounts and utilisation of P and Ca of broiler chickens (Means and SD, n = 10 birds per treatment)

Phytase	Treatment	pc digestibility (%)		Utilisation (%)		Excreted amounts (mg/d)	
U/kg		P	Ca	P	Ca	P	Ca
0	A	32[a]	49[a]	47[a]	28[a]	97[a]	311[a]
		5.5	4.0	12.2	17.2	21.8	88
500	B	43[*b]	52[ab]	57[*b]	38[b]	86[ab]	292[ab]
		6.5	4.3	6.2	7.2	9.9	27
1000	C	53[*c]	55[*bd]	62[*bc]	40[*bc]	78[*b]	283[abc]
		5.9	5.1	6.7	9.0	13.8	43
2000	D	60[*d]	51[a]	68[*c]	43[*bc]	66[*c]	266[bc]
		4.9	4.7	4.7	4.1	8.2	20
4000	E	73[*e]	56[*cd]	75[*d]	48[*c]	51[*d]	248[*c]
		4.2	1.8	3.2	4.2	6.6	20
P		<0.001	0.002	<0.001	<0.001	<0.001	0.05

[*] Means are significantly different from the unsupplemented treatment A according to Dunnett test.

[abcde] Values without a common superscript are significantly different according to t-test ($p \leq 0.05$)

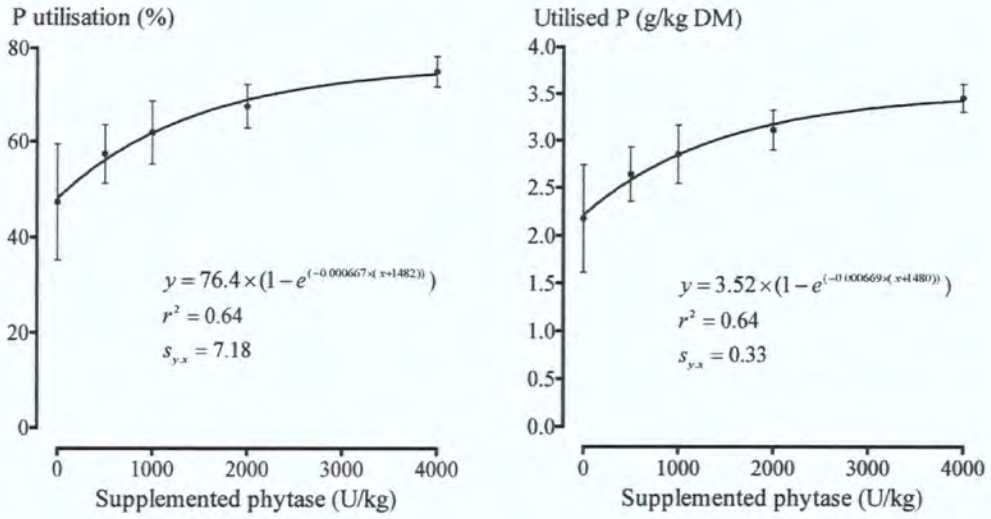


Figure 1: Effect of phytase supplementation on P utilisation (left) and content of utilised P in the diet (right) (Means and SD, n=10 birds per treatment)

The values for pc P digestibility followed the same principle but were different in the level from data for P utilisation. This may have different reasons. The most likely can be seen in the differences of the methodical approaches. While the utilisation data are based on total collection of excreta for 5 days, pc digestibility is based on the digesta spot sampled in the moment of slaughter. Phosphate absorption in the postileal section has not been described so far. But perhaps phosphate absorption in the intestine was not yet completed in the section that was sampled *post mortem*.

Tibia contents of ash, P and Ca were significantly improved by phytase supplementation (Table 4). The ash concentration of the tibiae from the birds fed the unsupplemented control diet was 42.5% (on dry matter basis), and it was increased up to an estimated value of 50.7%, respectively (Figure 2). Concentrations of Ca and P in tibia ash were only slightly affected by phytase supplementation (Table 4). P concentration in tibia ash increased from 172 to 177 g/kg ash. The Ca to P ratio was significantly lower at the two highest levels of supplementation compared to the other treatments.

Table 4: Content of crude ash (g/kg DM), P and Ca of the tibia (Means and SD, n = 10 birds per treatment)

Phytase U/kg	Treatment	Ash	g/kg ash		mg		Ca : P
			Ca	P	Ca	P	
0	A	425^{d}	370^{ab}	172^{b}	96^{d}	45^{d}	2.15^{a}
		29.1	7.6	4.2	23.2	10.7	0.04
500	B	452^{c}	371^{ab}	173^{b}	126^{*c}	59^{*c}	2.14^{a}
		35.2	8.7	3.6	20.4	9.5	0.03
1000	C	473^{*bc}	376^{a}	175^{ab}	133^{*bc}	62^{*bc}	2.15^{a}
		28.2	8.0	3.9	17.8	8.2	0.02
2000	D	486^{*ab}	368^{b}	175^{ab}	147^{*b}	70^{*b}	2.11^{*b}
		23.9	8.9	3.5	22.3	10.1	0.03
4000	E	505^{*a}	372^{ab}	177^{*a}	170^{*a}	81^{*a}	2.10^{*b}
		14.7	7.0	3.9	15.3	7.1	0.03
$P =$		<0.001	0.34	0.09	<0.001	<0.001	<0.01

* Means are significantly different from the unsupplemented treatment A according to Dunnett test.

^{abcd} Values without a common superscript are significantly different according to t-test ($p \leq 0.05$)

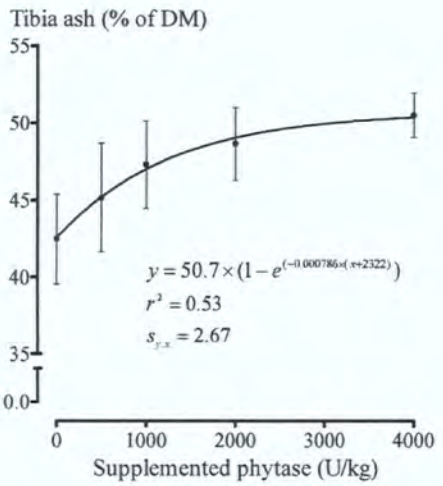


Figure 2: Effect of phytase supplementation on the concentration of ash in tibia dry matter (Means and SD, n=10 birds per treatment)

Summary and conclusions

A new bacterial 6-phytase (IPA Mash Phytase) was tested in 3 to 4-week old broiler chickens. The basal diet was mainly based on maize and soybean meal and had P and Ca concentrations of 4.7 and 10.8 g/kg dry matter, respectively. The diet was supplemented at levels of 500, 1000, 2000 and 4000 U/kg or remained unsupplemented. Excretions were determined in a balance trial with 10 individual birds per diet and tibiae were obtained after the birds had received their respective diets for 12 days. Another 500 chickens (10 pens with 10 birds per treatment) were fed for 7 days until slaughtering and removal of digesta for the determination of the precaecal digestibility.

The effect of phytase on the utilisation of P and Ca was highly significant. The utilisation of P and Ca was improved by phytase supplementation from 47 and 28% in the basal diet to a maximum of 75 and 48% at the highest level of supplementation, respectively. Tibia contents of ash, P and Ca were improved by phytase supplementation. The precaecal digestibility of P was significantly increased from 32% to 73% with increasing phytase supplementation.

Significant effects were found in P retention, precaecal P digestibility and tibia P. It is therefore concluded that the new phytase product is efficient in broiler chickens. By supplementing this phytase in combination with a reduced use of inorganic P in the diet beneficial environmental effects can be achieved by reduced P excretion.

Stuttgart, October 26, 2009

Prof. Dr. M. Rodehutscord Dr. J. Boguhn

Annex tables 1 to 4 are part of this report.

Annex table 1: Initial body weight (BW), BW gain, feed intake, gain to feed ratio and calculated precaecal (pc) digestibility of P and Ca (pen basis)

Pen	Diet	Initial BW (g/bird)	BW gain (g/bird)	Feed intake (g/bird)	Gain to feed ratio	pc digestibility P %	pc digestibility Ca %
1	0	186	163	275	0.59	42.3	44.8
2	500	158	171	251	0.68	42.5	54.0
3	1000	162	174	284	0.61	47.1	52.9
4	2000	173	206	301	0.68	52.2	46.1
5	4000	176	212	332	0.64	72.9	54.9
6	0	168	140	229	0.61	30.4	50.5
7	500	163	146	281	0.52	43.3	59.2
8	1000	182	201	314	0.64	47.9	53.7
9	2000	164	176	263	0.67	64.2	54.4
10	4000	168	210	291	0.72	73.5	57.8
11	0	151	124	212	0.58	31.2	47.7
12	500	160	168	250	0.67	39.1	47.7
13	1000	153	140	251	0.56	56.7	53.6
14	2000	171	212	305	0.69	59.2	49.2
15	4000	156	199	297	0.67	68.8	55.2
16	0	182	160	260	0.62	31.6	49.9
17	500	159	161	296	0.54	36.0	44.9
18	1000	144	163	244	0.67	50.1	48.1
19	2000	190	232	343	0.68	62.5	55.8
20	4000	174	208	357	0.58	68.1	56.9
21	0	177	170	271	0.63	32.7	47.3
22	500	170	177	286	0.62	44.4	51.4
23	1000	166	189	275	0.69	53.6	51.6
24	2000	155	188	267	0.71	61.2	44.3
25	4000	170	225	303	0.74	74.9	57.2
26	0	168	159	257	0.62	35.9	58.2
27	500	167	170	262	0.65	39.3	50.3
28	1000	171	187	327	0.57	58.0	60.6
29	2000	162	182	318	0.57	52.7	50.9
30	4000	175	237	331	0.71	66.7	55.7
31	0	158	162	273	0.59	29.9	49.7
32	500	171	175	274	0.64	35.8	51.8
33	1000	153	160	243	0.66	41.4	47.1
34	2000	156	175	260	0.67	62.6	55.4
35	4000	159	172	279	0.61	69.6	56.1
36	0	159	144	251	0.57	26.8	45.7
37	500	151	146	250	0.58	40.6	55.2
38	1000	154	159	290	0.55	59.1	61.8
39	2000	157	176	265	0.66	58.2	44.4
40	4000	156	225	297	0.76	79.8	57.9
41	0	150	124	266	0.47	21.3	44.4
42	500	162	179	268	0.67	47.3	51.8
43	1000	161	178	274	0.65	55.1	60.0
44	2000	162	191	300	0.64	65.2	56.8
45	4000	155	203	273	0.75	73.3	58.8
46	0	168	141	244	0.58	32.8	50.0
47	500	161	162	260	0.62	58.1	57.5
48	1000	160	158	264	0.60	58.5	56.6
49	2000	149	184	260	0.71	66.4	51.2
50	4000	152	203	283	0.72	76.8	52.9

Annex table 2: Initial body weight (BW), BW gain, feed intake, excreted amounts and utilisation of P and Ca (individual data)

Bird	Diet	Initial BW	BW gain	Feed intake	Excretion		(intake-excretion) / intake * 100	
					P	Ca	P	Ca
		(g)	(g/d)	(g/d)	(mg/d)	(mg/d)	%	%
1	0	338	25.4	48.8	111.6	393.7	44.2	14.9
2	500	300	32.4	49.7	84.4	318.4	58.7	32.6
3	1000	311	28.0	50.0	78.4	320.3	61.9	32.6
4	2000	336	30.3	50.0	64.0	293.7	68.8	38.1
5	4000	303	32.4	50.0	43.0	234.2	79.1	50.6
6	0	335	17.2	36.1	93.2	263.6	37.1	23.0
7	500	306	28.5	50.0	81.6	298.0	60.2	37.1
8	1000	320	31.6	50.0	70.7	278.8	65.6	41.3
9	2000	323	28.4	50.0	65.1	275.0	68.4	42.2
10	4000	317	29.1	50.0	48.7	247.0	76.3	48.0
11	0	293	24.9	50.0	80.5	238.9	60.7	49.6
12	500	268	26.0	50.0	83.7	259.6	59.3	45.4
13	1000	293	27.9	50.0	73.6	273.6	64.2	42.5
14	2000	294	29.2	50.0	54.4	239.5	73.5	49.5
15	4000	317	29.3	50.0	54.5	253.7	73.5	46.5
16	0	350	27.7	50.0	100.4	339.4	51.1	28.4
17	500	312	28.9	50.0	77.0	281.5	62.5	40.7
18	1000	259	23.8	50.0	53.0	179.6	74.2	62.2
19	2000	339	30.2	50.0	57.3	272.7	72.1	42.5
20	4000	298	32.2	50.0	55.5	276.5	72.9	41.7
21	0	342	31.2	50.0	124.5	439.8	39.3	7.2
22	500	321	31.2	50.0	90.6	324.1	55.9	31.8
23	1000	298	28.2	50.0	85.1	286.3	58.6	39.8
24	2000	274	22.7	50.0	65.5	260.5	68.1	45.1
25	4000	310	30.6	50.0	50.0	237.3	75.6	50.0
26	0	245	25.3	49.7	56.4	172.4	72.3	63.4
27	500	303	26.2	50.0	69.0	263.3	66.4	44.6
28	1000	293	30.6	50.0	76.7	276.0	62.7	42.0
29	2000	300	32.5	50.0	82.4	289.3	59.9	39.0
30	4000	311	32.2	50.0	45.6	258.5	77.8	45.5
31	0	280	31.8	50.0	100.9	380.6	50.8	19.7
32	500	313	24.3	50.0	94.1	279.4	54.2	41.2
33	1000	310	30.3	50.0	70.2	281.6	65.9	40.8
34	2000	296	25.0	45.0	75.4	250.4	59.2	41.4
35	4000	311	27.6	50.0	55.1	233.6	73.2	50.8
36	0	309	30.6	50.0	120.1	357.8	41.5	24.5
37	500	294	28.3	49.3	90.1	258.1	55.5	44.9
38	1000	257	45.3	50.0	76.3	280.8	62.9	40.9
39	2000	283	31.0	50.0	66.5	282.1	67.6	40.6
40	4000	296	32.6	50.0	65.6	277.8	68.0	41.5
41	0	234	12.7	32.5	72.9	199.8	45.2	35.1
42	500	293	30.2	50.0	86.4	307.7	58.0	35.2
43	1000	293	29.6	50.0	99.4	336.8	51.7	29.2
44	2000	290	32.8	50.0	67.2	263.3	67.3	44.5
45	4000	285	33.9	50.0	46.5	244.1	77.3	48.6
46	0	296	19.6	39.5	112.7	320.9	30.4	14.3
47	500	301	28.8	45.0	105.7	330.7	42.9	22.7
48	1000	288	28.7	50.0	99.2	318.8	51.8	33.0
49	2000	248	31.8	50.0	59.9	236.8	70.8	50.1
50	4000	303	24.6	50.0	48.0	212.7	76.6	55.2

Annex table 3: Ash, P and Ca content of the tibiae (individual data)

Bird	Diet	Ash g/kg DM	Ca g/kg ash	P g/kg ash	Ca mg	P mg
1	0	400	356	169	80.5	38.3
2	500	471	361	173	119.7	57.2
3	1000	493	370	169	127.5	58.3
4	2000	524	370	175	174.0	82.3
5	4000	514	358	173	159.0	76.8
6	0	422	370	177	77.3	37.0
7	500	496	380	173	151.5	69.0
8	1000	501	384	179	139.3	64.8
9	2000	511	378	176	175.5	82.0
10	4000	520	382	181	183.0	86.8
11	0	475	380	174	68.3	31.2
12	500	504	371	169	97.0	44.3
13	1000	435	393	181	148.2	68.2
14	2000	446	378	178	152.5	71.7
15	4000	521	379	180	173.3	82.3
16	0	452	373	174	136.7	63.8
17	500	486	368	174	141.2	66.8
18	1000	474	378	176	129.5	60.2
19	2000	478	367	176	165.2	79.3
20	4000	519	378	183	151.7	73.5
21	0	439	363	169	131.5	61.3
22	500	434	390	182	145.5	67.7
23	1000	487	369	173	122.0	57.3
24	2000	479	366	173	128.8	60.7
25	4000	482	369	175	196.2	93.0
26	0	417	370	169	105.3	48.0
27	500	440	361	171	137.5	65.0
28	1000	436	379	178	154.0	72.2
29	2000	453	365	171	152.8	71.7
30	4000	503	366	176	171.0	82.0
31	0	411	369	168	97.5	44.5
32	500	427	366	169	123.5	57.0
33	1000	458	373	173	164.5	76.5
34	2000	496	364	177	108.7	52.8
35	4000	481	372	176	181.5	85.8
36	0	387	370	168	101.3	46.0
37	500	413	370	173	108.3	50.5
38	1000	445	373	176	106.5	50.2
39	2000	496	349	167	125.5	60.0
40	4000	498	371	178	151.3	72.8
41	0	392	383	178	76.5	35.5
42	500	439	371	174	142.0	66.5
43	1000	484	366	170	117.7	54.7
44	2000	495	379	178	155.0	72.8
45	4000	504	376	178	175.3	83.3
46	0	453	370	178	88.0	42.3
47	500	405	368	174	96.3	45.5
48	1000	516	373	173	125.0	58.0
49	2000	486	368	176	132.0	63.0
50	4000	510	370	170	153.3	70.5

Annex table 4: Statistical analysis

Dependent variable	Mean squares	R square	CV	Root MSE	F
Final body weight	5476	0.43	7.43	25.3	8.6
BW gain	5626	0.64	9.48	16.8	19.9
Feed consumption	3718	0.35	8.91	24.8	6.1
Gain to feed ratio	0.017	0.37	8.03	0.05	6.7
pc digestibility of P	2494	0.88	10.5	5.45	84.0
pc digestibility of Ca	88.5	0.31	7.88	4.15	5.1
P excretion (mg/d)	3205	0.62	17.5	13.3	18.3
Ca excretion (mg/d)	5847	0.19	16.9	47.3	2.6
Non-excreted P (%)	1099	0.65	11.7	7.27	20.8
Non-excreted Ca (%)	551	0.35	24.4	9.63	5.9
Tibia ash	9712	0.54	5.79	27.1	13.2
Tibia P (g/kg ash)	31.1	0.16	2.20	3.8	2.12
Tibia Ca (g/kg ash)	76.2	0.09	2.18	8.1	1.16
Tibia P (mg)	1763	0.65	14.6	9.2	20.7
Tibia Ca (mg)	7287	0.62	14.9	20.0	18.2
Tibia Ca : Tibia P	0.005	0.30	1.5	0.03	4.9

efsa
European Food Safety Authority

FEEDAP UNIT

ANNEX C [1]

TRIAL PROTOCOL DATA SHEET: FOR TERRESTRIAL ANIMALS

Identification of the additive: **IPA Mash Phytase**	Batch number: **PPQ 28683**
Trial ID: **IPA-04/09**	Location: **Hohenheim University**
Start date and exact duration of the study: **April 6, 2009; 26 days**	
Number of treatment groups (+ control(s)): **5**	Replicates per group: **10**
Total number of animals: **550**	Animals per replicate: **10 and 100**
Dose(s) of the additive/active substance(s)/agent(s) (mg/Units of activity/CFU kg^{-1} complete feed/L^{-1} water)	
Intended: **500, 1000, 2000, 4000 U/kg**	Analysed: **466, 1012, 1939, 3644 U/kg**
[†]	
Substances used for comparative purposes: **none**	
Intended dose:	Analysed:
Animal species/category: **Broiler chickens**	
Breed: **Ross 308**	Identification procedure: **birds in balance cages: rings**
Sex: **mixed sex** Age at start: **14 d**	Body weight at start: **164 or 210 g (bal. trial)**
Physiological stage: **normal**	General health: **good**
Additional information for field trials:	
Location and size of herd or flock:	
Feeding and rearing conditions:	
Method of feeding:	
Diets (type(s)): **Maize/soybean meal-based, low-P**	
Presentation of the diet: Mash ☒ Pellet ☐ Extruded ☐ Other	
Composition (main feedingstuffs): **Maize (54%), soybean meal (40%), soybean oil (2%)**	
Nutrient content (relevant nutrients and energy content)	
Intended values: **24 % CP in DM, 0.45 % P in DM, 1.0 % Ca in DM**	
Analysed values: **25.2 % CP in DM, 0.46 % P in DM, 1.07 % Ca in DM**	
Date and nature of the examinations performed: **excreta collection d 19-24, tibia d 26, ileal digesta d 21**	
Method(s) of statistical evaluation used: **Standard analytical methods, ANOVA, Regression analysis**	
Therapeutic/preventive treatments (reason, timing, kind, duration): **Routine vaccination against Coccidiosis, Newcastle disease, Infectious bursal disease on d 3, 10, 14**	
Timing and prevalence of any undesirable consequences of treatment:	
Date **October 26, 2009**	Signature Study Director

[†] In case the concentration of the additive in complete feed/water may reflect insufficient accuracy, the dose of the additive can be given per animal day^{-1} or mg kg^{-1} body weight or as concentration in complementary feed.

[1] Please submit this form using a common word processing format (e.g. MS Word).

TAB

ANNEX 24

Annex 24

Philipps, P. et *al*. (2009). Report No. 00001184:Comparison of two formulations of a microbial 6-phytase included at graded levels on growth performance and phosphorus utilization of broiler chickens (BE- 07/09)

REPORT No. 00001184
Research Project Document

DSM

Document Date: 09-October-2009

Author(s): Philipps P, Aureli R and Nasir Z

NRD/CA, DSM Nutritional Products France

Title: Comparison of two formulations of a microbial 6-phytase included at graded levels on growth performance and phosphorus utilization of broiler chickens (BE-07/09).

Project No. 6562

Compound No.

Summary
The effects of a liquid preparation and a new salt coated preparation of the IPA phytase on growth performance and phosphorus utilization of broiler chickens were studied in a short term trial from day 8 to day 22 of life. The birds were fed low-phosphorus diets based on maize and soybean meal. The pelleted diet contained 3.8 g total phosphorus and 5.6 g calcium per kg feed. The two forms of the bacterial 6-phytase were included at 500, 1000 and 2000 U /kg feed, respectively. The results of this current study demonstrated that the supplementation of low P diet with the IPA phytase in liquid or in salt coated form significantly improved the weight gain and the feed conversion ratio of male broiler chickens at 22 days of age. The utilization of phosphorus was significantly increased and consequently the amount of P excreted in the faeces was reduced. P-utilization was improved dependent on level of phytase and could be described by an exponential function. The IPA phytase was effective in releasing phytate-P according to the effects obtained on tibia and toe ash. The efficiency of IPA Phytase (s. coated) recorded in this trial was comparable to that of IPA Phytase (L) for growth parameters, bone parameters and for utilization of phosphorus and calcium. In most parameters, the treatments supplemented with higher dosages of IPA phytase performed equally or even outperformed the treatment supplemented with additional mineral P (positive control). In the present study a first batch of the salt coated form of IPA phytase was tested. It is suggested to confirm the efficiency of the salt coated formulation in an additional study.
This report consists of 17 pages

Distribution

Dr. JBroz, NRD/CA
Dr. M. Eggersdorfer, NRD
Dr. JPheiffer, NRD/PA
Dr JF. Hecquet, NBD/RA
Dr. J-P. Ruckebusch, ANIMPM
Dr. G. Weber, NRD/SC
Scientists NRD/CA

Approved

Name	Signature	Date
Main Author Dr. P. Philipps, NRD/CA	Signed by Dr. P. Philipps	09.10.09
Competence Mgr Dr. C.Simões Nunes, NRD/CA	Si gned by Dr. C.Simões Nunes	15.10.09
Research Center Head (optional) Dr. A.-M. Hünter, NRD/CA	Signed by Dr. A.-M. Hünter	09.10.09

Nomenclature and Structural Formula

A liquid preparation of bacterial 6-phytase (IPA Phytase (L) Hiphos), batch PPQ28432 was used in this study, manufactured by Novozymes A/S, Bagsvaerd, Denmark

A salt coated preparation of bacterial 6-phytase (IPA Phytase, Hiphos), batch PPQ29773 was used in this study, manufactured by Novozymes A/S, Bagsvaerd, Denmark

Author(s): Petra Philipps, Raffaella Aureli and Zahid Nasir

Department(s) and Adress(es) : NRD/CA, DSM Nutritional Products France

Title: Comparison of two formulations of a microbial 6-phytase included at graded levels on growth performance and phosphorus utilization of broiler chickens (BE-07/09).

Abstract

The effects of a liquid preparation and a new salt coated preparation of the IPA phytase on growth performance and phosphorus utilization of broiler chickens were studied in a short term trial from day 8 to day 22 of life. The birds were fed low-phosphorus diets based on maize and soybean meal. The pelleted diet contained 3.8 g total phosphorus and 5.6 g calcium per kg feed. The two forms of the bacterial 6-phytase were included at 500, 1000 and 2000 U /kg feed, respectively.

The results of this current study demonstrated that the supplementation of low P diet with the IPA phytase in liquid or in salt coated form significantly improved the weight gain and the feed conversion ratio of male broiler chickens at 22 days of age.
The utilization of phosphorus was significantly increased and consequently the amount of P excreted in the faeces was reduced. P-utilization was improved dependent on level of phytase and could be described by an exponential function. The IPA phytase was effective in releasing phytate-P according to the effects obtained on tibia and toe ash.
The efficiency of IPA Phytase (s. coated) recorded in this trial was comparable to that of IPA Phytase (L) for growth parameters, bone parameters and for utilization of phosphorus and calcium.
In most parameters, the treatments supplemented with higher dosages of IPA phytase performed equally or even outperformed the treatment supplemented with additional mineral P (positive control).

In the present study a first batch of the salt coated form of IPA phytase was tested.
It is suggested to confirm the efficiency of the salt coated formulation in an additional study.

INTRODUCTION

Phosphorus is one of the main essential elements involved in the formation of mineralized tissues. Almost two third of total phosphorus in poultry diets are in the form of phytate phosphorus, which is poorly available to the poultry due to the low activity of the endogenous phytase present in their digestive tract.The inability of poultry to utilize phytate-P necessitates the addition of organic phosphate sources in diet formulations and also results in the excretion of large amounts of P in the litter and increases the cost of formulations. Phytase supplementation to the diets is one of main nutritional approaches to improve dietary phytate P bioavailability.

The aim of the present trial was to compare the effects of two forms of a microbial 6- phytase, IPA Phytase liquid and IPA Phytase salt coated on growth performance and phosphorus utilization of broiler chickens fed a low-phosphorus diet. Due to the low amount and activity of native phytases in maize and soybean meal, they were the ingredients of choice for the basal diets. The animals were fed diets supplemented, per kg feed, with 500, 1000, and 2000 U of each form of the phytase. In addition, one treatment supplemented with additional DCP was included in the trial to have 5.6 g total phosphorus per kg feed. Phosphorus utilisation, considered to be the most sensitive parameter for measuring the efficiency of phytase, was determined based on quantitative measurements of P consumption and excretion.

MATERIALS AND METHODS

The trial (BE-07/09) was performed at the Research Center for Animal Nutrition and Health (DSM Nutritional Products France, F-68305 Village-Neuf) according to the official French norms for experiments with live animals. Day-old male broiler chickens (ROSS PM3), were supplied by a commercial hatchery (Joseph Grelier S.A., Elevage avicole de la Bohadière, F-49290 Saint-Laurent de la Plaine, France). The chickens were housed in wire-floored battery cages, which were kept in an environmentally controlled room. The room temperature was adapted according to the requirements of the chickens. Feed and tap water were available for *ad libitum* consumption. The chickens were fed with a low phosphorus basal diet supplemented with 37.5 µg vitamin $D_3.kg^{-1}$ (corresponding to 1500 IU per kg feed) until day 8, when the trial started. On day 8, the chickens were divided by weight into groups, each comprising of 8 birds, which were allocated to one of the different treatments. Each treatment was replicated with 12 groups. The groups were weighed on days 8, 15, and 22. Feed consumption for the intermediate periods was determined and body weight gain (WG) and feed conversion ratio (FCR) were calculated.

The basal diet was supplemented with 12.5 µg.kg^{-1} vitamin D_3 corresponding to 500 IU.kg^{-1} to fulfil the recommendation for chickens of that age (GfE 1999). The basal diet was based on maize and soybean meal as main ingredients and had a content of 222 g crude protein, 12.6 MJ ME_N, 3.8 g total phosphorus (P), and 5.6 g calcium (Ca) per kg feed. All other nutrients except for phosphorus met the requirements of growing broilers in accordance to their age. The analyses of the nutrient content in the feed samples (Table 1) were performed according to standard methods (VDLUFA, 1976, 1997). The detailed composition of the basal diet, the analysed nutrient contents and the ME (calculated on the basis of analysed nutrients using EC-equation, EEC, 1986), are shown in **Table 1**.

Beside the control treatment without enzyme supplementation, graded levels of phytase were added to a phosphorus deficient basal diet. The unsupplemented P-deficient basal diet was also fed as such (negative control diet) and was supplemented with 1.5 g P from dicalcium phosphate (DCP) as positive control diet. The diet was supplemented with the bacterial 6-phytase in liquid form (lot PPQ28432 with analysed phytase activity of 26000 U.g^{-1}) and with

the bacterial 6-phytase in salt coated form (lot PPQ29773 with analysed phytase activity of 13300 $U.g^{-1}$). The two forms of the bacterial 6-phytase were included at one of the following doses: 500, 1000, 2000 $U.kg^{-1}$ feed.

The added amount of the test product was based on the analysed phytase activity. Appropriate amounts of the salt coated product were mixed with a small quantity of the basal feed as a premix which was then added to the feed to get the final concentration, according to the treatment. After mixing, the feed was pelleted (3 x 25 mm) at about 70°C.
Appropriate amounts of the liquid preparation of the phytase product were diluted with 600 ml water and sprayed onto the pellet feed to get the final concentrations in the feed corresponding to the different treatments. For procedural balance of all treatments, 600 ml of water were also sprayed onto the pellets of the negative and the positive control diets as well as onto the diets supplemented with the salt coated form of the phytase
Feed samples were taken for analysis of the phytase activities. The determination of the phytase activity in the experimental diets was performed by BIOPRACT GmbH, D-12489 Berlin (Germany) on behalf of DSM Nutritional Products. One unit (U) of phytase is defined as the activity that releases 1 µmol inorganic phosphate from 5.0 mM phytate per minute at pH 5.5 and 37 °C.
Phytate in feed was determined colorimetrically as released P after extraction, elution and wet digestion with HNO_3/H_2SO_4 (AOAC, 1990).

Excreta were collected from birds from day 14 to day 17 by a total collection method. During this period the excreta from 4 selected groups of male chickens per treatment were quantitatively collected once per day. The excreta from the four days were pooled per group and were stored frozen (at -20°C), each day directly after collection. After thawing the total excreta of each group were homogenized, representative samples were taken and the percentage of dry matter and ash, as the concentration of phosphorus and calcium were determined. Ca and total P were determined by Induction Coupled Plasma according to DIN EN ISO 11885:1997 (DIN EN ISO 1998) after mineralization with H_2SO_4 / Na_2SO_4

On day 22, blood samples from 4 male chickens randomly chosen from each group were collected from the *Vena jugularis*. The concentrations of inorganic phosphate (Pi) and calcium (Ca) in the plasma were determined with a Cobas® 6000 module C 501 automatic analyzer according to the method described by Fahrney (1974) and Gindler and King (1972), using Roche Diagnostic kits PHOS 03183793 122 and Ca 20763128 322.

The chickens were euthanized by cervical dislocation at 23 day of age and the right tibias were taken from 4 chicken randomly chosen from each group. Tibiae were defleshed, and cartilaginous caps were removed after collection. They were kept frozen in plastics bags at -20°C until analysis of ash content and breaking strength.
A segment of the central portion of the bone shaft (about 2 cm long) was prepared for use in determining bone strength. A LR10K compression machine with a KC/10KA1 force captor and a compression device TP3-196/AL (Lloyd Instruments, Fareham, UK) was used to determine the force (in Newton) necessary to break the bone. Broken bones were pooled per cage, defatted with ethanol and ether, dried and incinerated at 550°C to determine the percentage ash.
In addition, toe samples were obtained by severing the left middle toe through the joint between the second and the third tarsal bones from the distal end. The toes of the four chickens within a cage were pooled. The composite samples were dried and then ashed in a muffle furnace at 550°C to determine toe ash as a percentage of dry weight.

For the statistical evaluation of all data a one-factorial (treatment: phytase level) analysis of variance was carried out, using the software "Stat Box Pro", version 5.0 (Grimmer soft 1995) in which differences in treatment means with $p < 0.05$ were considered as significant. Newman-Keuls test was used as post hoc to compare treatment means. Non-linear regression analyses were performed with the program Origin 7.0. An exponential model of the following type was fitted to the data:

$$y = a+b\ (1-\exp\ (-kx))$$

with a: response (y-value) at zero phytase supplementation
b: maximum response to supplemented phytase (a+b = upper asymptote)
k: parameter describing the steepness of the curve
x: supplemented phytase (U/kg)
y: response (P utilization or utilized P concentration in the diet)

RESULTS AND DISCUSSION

Proximate analyses in the negative control diet were closed to the calculated values. P content was lower as expected and the difference between P content in the positive and the negative control was 1.1 g P **(Table 2)**. Analysed Ca content was in the both diets approximatively 7 % less than calculated. The content of non-phytic acid phosphorus in the basal diet was 0.8 g per kg feed, calculated as the difference between total phosphorus content and content of phytic phosphorus per kg feed.

The analysed phytase activities in the experimental feed are listed in **Table 3**. As intended, the native phytase activity in the basal diet was under the limit of quantification (LOQ).
The phytase activities measured in the treatments B, C and D supplemented with the phytase in liquid form were in good agreement with the target dosage.
For the treatment E, an activity of 599 $U.kg^{-1}$ was found in the feed sample which is about 20 % more than the target dose of 500 $U.kg^{-1}$. After pelleting this treatment, the enzyme dropped to 531 $U.kg^{-1}$, meaning that about 27 % of its activity was lost through pelleting. For the treatment F, the activity measured in the pellet feed was higher than this measured in the mash feed. An activity of 2493 $U.kg^{-1}$ was found for the treatment G which is about 24 % more than the target dose of 2000 $U.kg^{-1}$. On pelleting, this activity was reduced by 24 % to 1900 U kg^{-1}. Even if the pelleting conditions led to a decrease of the salt coated phytase activity in the pellet feed compared to the mash feed, the activities were in accordance with the target dosage.

The results of the growth performance from day 8 to day 22 are presented in **Table 4.**
The mortality observed throughout the present trial was very high for the negative control (44.3 %). Even for the positive control diet still a mortality rate of 11.5 % was recorded, indicating that due to the lower phosphorus content in the basal diets the deficiency conditions were stronger than intended. Therefore the results on growth performance have to be interpreted with care.

Adding dicalcium phosphate (DCP) to the negative control diet resulted in a significant improvement of the weight gain (WG) and the feed conversion ratio (FCR), clearly indicating that the negative control diet was P-deficient. At a supplementation level of + 1.5 g (calculated) DCP per kg feed, the WG and the FCR were improved by 106 % and 26.4 % respectively compared to the negative control diet.
Increased phytase supplementation from 500 to 2000 $U.kg^{-1}$ resulted in a significant improvement of the WG and the FCR ratio compared to the negative control diet. The two forms

of the IPA phytase tested at the lowest inclusion level already resulted in a significantly higher WG and better FCR compared to the negative control diet. The WG was improved by 97 %and 84 %respectively with the phytase in liquid form and in salt coated form. The FCR was improved by 26.5 %and 24.9 %respectively . The WG and the FCR were improved in a logarithmic dose response manner with increasing phytase. The response of weight gain and feed conversion ratio to the addition of phytase to the diet can be described by non-linear regressions (**Figure 1 and 2**). The effects on WG and FCR of liquid IPA phytase were numerically higher than this obtained with salt coated IPA phytase at the same dosage but not in a significant manner. Inclusion of 2000 U of liquid IPA phytase resulted in a significantly higher weight gain compared to the positive control.

The results of apparent utilization of phosphorus and calcium are presented in **Table 5**. The apparent utilization of phosphorus was significantly improved with increasing dietary levels of the phytase.
Compared to the negative control diet, an improvement in a range of 45 %o 75 %and 40 %o 60 %was obtained with graded level of the IPA phytase (L) and the IPA phytase (s. coated), respectively (**Figure 3**). The effects on P-utilization were significantly comparable between the two forms of IPA Phytase included at 500 and 1000 $U.kg^{-1}$ .For the highest inclusion level of 2000 $U.kg^{-1}$, the supplementation with the liquid form resulted in a higher P-utilisation than with the salt coated form.
The effect of the phytase supplementation on P utilization for all supplementary levels was further confirmed by a significant reduction in P excretion **(Figure 4)** over the negative control diet. The concentration of phosphorus in excreta recorded for the two phytases included at 500 and 1000 U.kg-1 was significantly comparable.
The P-utilization of the positive control groups was lower than of all other groups clearly indicating that most of the additional mineral phosphorus was utilized by the broiler chickens whereas the phytate-P was still excreted, which was confirmed by the increasing phosphorus content in excreta.

The apparent Ca-utilization **(Table 5)** was significantly improved in all treatments compared to the negative control diet. Similar to the P-utilization, the effect was dose-dependent with significant differences among the dosages. Utilization of Ca in the negative control diet was 32.8 %and it was increased up to an estimated asymptotic value of 66.7 %and 67.1 %by including different levels of IPA phytase (L) and IPA phytase (s. coated) respectively. The results indicate that in addition to P release there is an additional availability of calcium caused by the supplementation with phytase.

Results of plasma concentrations of inorganic phosphorus (Pi) and Ca are presented in **Table 6**. The Pi-concentration in the plasma was significantly increased by all treatments compared to the negative control diet. The Pi concentration in the plasma increased with increasing dietary inclusion level of either IPA phytase (L) or IPA phytase (s. coated). The effects on Pi concentration in the plasma were comparable between both forms of phytase at the inclusion of 500 and 2000 $U.kg^{-1}$. At 1000 $U.kg^{-1}$ the effects obtained with the liquid form of the phytase was significantly higher than this obtained with the salt coated form. The Ca-concentration in the plasma was decreased when phytase was added to the basal diet.

Table 7 shows the effects of phytase supplementation on parameters of bone mineralization. Supplementing phytase, irrespective of the dose, significantly improved tibia strength compared to the negative control diet. Tibia strength values increased in a pattern corresponding to supplementation levels. The effects of phytase supplementation on tibia ash, a parameter that indicates the extent of bone mineralisation, were significant for all treatments compared to the negative control diet. With increasing levels of phytase, important improvements ranging

between 25 % 37 % and 23 % 36 % were noted respectively with IPA phytase (L) and with IPA phytase (s. coated). An exponential dose-dependent relationship was found for the tibia ash (**Figure 6**), in which the slope rose very fast with increasing levels of phytase in the diet. The inclusion of either IPA phytase (L) or IPA phytase (s. coated) resulted in a comparable improvement of the tibia ash and tibia strength.

Toe ash measurements are shown in **Table 7 and Figure 7**. In this experiment, phytase supplementation was effective in improving toe ash compared to the negative control diet. The effects on toe ash were comparable between both forms of the IPA phytase. In the present study, no significant difference between dosages were noted.

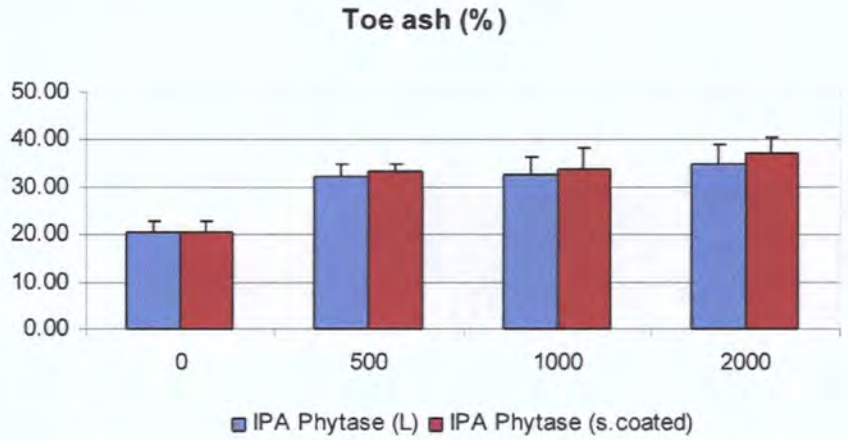


Figure 7: Toe ash content in male broiler chickens

The results of this current study demonstrate that the supplementation of low P diet with the IPA phytase in liquid or in salt coated form significantly improved the weight gain and the feed conversion ratio of male broiler chickens at 22 days of age.
The utilization of phosphorus was significantly increased and consequently the amount of P excreted in the faeces was reduced. P-utilization was improved dependent on level of phytase and could be described by an exponential function. The IPA phytase was effective in releasing phytate-P according to the effects obtained on tibia and toe ash.
The efficiency of IPA Phytase (s. coated) recorded in this trial was comparable to that of IPA Phytase (L) for growth parameters, bone parameters and for mineral utilization.
In most parameters, the treatments supplemented with higher dosages of IPA phytase performed equally or even outperformed the treatment supplemented with additional mineral P (positive control).
In the present study a first batch of the salt coated form of IPA phytase was tested.
It is suggested to confirm the efficiency of the salt coated formulation in an additional study.

REFERENCES

AOAC (1990)
Official Methods of Analysis of the AOAC [A ssociation of Official Analytical Chemists]
Arlington, Virginia 22201 USA

DIN EN ISO (1998)
Bestimmung von 33 Elementen durch induktiv gekoppelte Plasma-Atom-Emissionsspektrometrie (ISO 11885:1997; E22, 1998-04; Deutsche Fassung EN ISO 11885:1997), Beuth Verlag GmbH

EEC (1986)
Directive de la Commission du 9 avril 1986 fixant la méthode de calcul de la valeur énergétique des aliments composés destinés à la volaille , Journal Officiel des Communautés Européennes, L 130, 53-54

GINDLER, E.M. AND J.D. KING (1972)
Determination of calcium concentration in human serum. Am. J Clin. Pathol. 58 , 376-382

GRIMMERSOFT (1995)
StatBoxPro, Version 5.0, Manuel dutilisation

GfE (Gesellschaft für Ernährungsphysiologie), 1999. Empfehlung zur Energie – und Nährstoffversorgung der Legehennen und Masthühner (Broiler), DLG-Verlag, Frankfurt am Main

ORIGINLAB CORPORATION (2002)
Origin Version 7, Programming Guide

VDLUFA (1976, 1997)
Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, Hrsg
Handbuch der landwirtschaftlichen Versuchs- und Untersuchungsmethodik (Methodenbuch).
Band III: Die chemische Untersuchung von Futtermitteln, 3. Auflage 1976 mit 1. Erg. Lief.1983, 2. Erg. Lief. 1993, 3. Erg. Lief. 1997, VDLUFA-Verlag, Darmstadt

Table 1: Composition of the basal diet

Ingredients (%)	
Maize	59.1
Soybean meal (50 %CP)	36.8
Soybean oil	1.50
DL-Methionin	0.20
DCP	0.30
$CaCO_3$	0.67
Sand	0.31
NaCl	0.10
Premix [1] without Vitamin D_3	1.00
Avatec	0.06
Calculated content:	
ME_N (MJ/kg) [2]	12.7
Crude protein (g/kg)	215
Calcium (g/kg)	6.0
Total P (g/kg)	4.1
Analyzed content:	
ME_N(MJ/kg) [3]	12.6
Crude protein (g/kg)	222
Calcium (g/kg)	5.6
Total P (g/kg)	3.8
Phytate P (g/kg)	3.0
Non Phytate-P (g/kg)	0.8

[1] without Avatec
[2] Calculated with EC-equation based on values from nutritional tables
[3] Calculated with EC-equation based on analyzed crude nutrients

Table 2: Analysed P and Ca concentrations in samples of the experimental diets

Treatment	Product	Total P ($g.kg^{-1}$ feed)		Ca ($g.kg^{-1}$ feed)	
		expected	measured	expected	measured
A	Negative control	4.1	3.8	6.0	5.6
H	Positive control	5.6	4.9	6.0	5.7

Table 3: Analysed product activities in samples of the experimental diets

Treatment	Product	Dose ($U.kg^{-1}$)	Phytase content ($U.kg^{-1}$ feed) before processing	Phytase content ($U.kg^{-1}$ feed) after processing
A	Negative control	-	**Below LOQ**	**LOQ***
B	IPA Phytase (L)	500	-	**500**
C	IPA Phytase (L)	1000	-	**983**
D	IPA Phytase (L)	2000	-	**2170**
E	IPA Phytase (s. coated)	500	**599**	**531**
F	IPA Phytase (s. coated)	1000	**1021**	**1445**
G	IPA Phytase (s. coated)	2000	**2493**	**1900**
H	Positive control	-	**Below LOQ**	**LOQ***

Table 4: Performance of broiler chickens (day 8 to day 22) fed different supplemental levels of phytase, mean ± stdev

Product	**Negative control**	**IPA Phytase (L)**			**IPA Phytase salt coated**			**Positive control**
Treatment	A	B	C	D	E	F	G	H
Dose (U/kg)	-	500	1000	2000	500	1000	2000	+1.5 g p/kg
cages x birds	11 x 8	12 x 8	12 x 8	12 x 8	12 x 8	12 x 8	12 x 8	12 x 8
Day 8-22								
Weight gain (g/bird)	**303** ^{D} ±26.6 *100.0*	**596** ^{BC} ±65.5 *197.0*	**651** ^{AB} ±34.8 *215.1*	**684** ^{A} ±66.5 *226.2*	**558** ^{C} ±44.3 *184.3*	**615** ^{B} ±58.9 *203.3*	**653** ^{AB} ±52.2 *216.0*	**623** ^{B} ±49.5 *205.8*
Feed intake (g/bird)	**600** ^{D} ±44.6 *100.0*	**870** ^{BC} ±93.2 *145.1*	**941** ^{AB} ±46.6 *156.9*	**973** ^{A} ±64.6 *162.2*	**831** ^{C} ±55.6 *138.6*	**896** ^{B} ±76.1 *149.3*	**932** ^{AB} ±56.3 *155.5*	**909** ^{AB} ±51.5 *151.6*
Feed conversion (g feed/g gain)	**1.988** ^{A} ±0.129 *100.0*	**1.461** ^{B} ±0.025 *73.5*	**1.446** ^{B} ±0.035 *72.8*	**1.426** ^{B} ±0.071 *71.7*	**1.492** ^{B} ±0.034 *75.1*	**1.458** ^{B} ±0.038 *73.3*	**1.430** ^{B} ±0.059 *71.9*	**1.463** ^{B} ±0.046 *73.6*
Mortality (%	44.8	10.4	9.4	16.7	10.4	9.4	17.7	11.5

Newman-Kuls test: Means within a row, not sharing a common s uperscript, are significantly different (p<0.05).

Table 5: Apparent utilization of phosphorus and calcium in male broiler chickens fed different supplemental levels of phytase, mean ± stdev.

Product	**Negative control**	**IPA Phytase (L)**			**IPA Phytase salt coated**			**Positive control**
Treatment	A	B	C	D	E	F	G	H
Dose (U/kg)	-	500	1000	2000	500	1000	2000	+1.5 g P /kg
Cages x birds	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4
Dry matter intake (g / bird / day)	31.7 ^{B} ±4.2	51.6 ^{A} ±1.9	56.9 ^{A} ±4.3	50.9 ^{A} ±5.9	50.2 ^{A} ±5.9	51.5 ^{A} ±3.6	51.2 ^{A} ±5.4	51.1 ^{A} ±3.6
Phosphorus								
Apparent P utilization (% of intake)	**44.5** ^{D} ±3.4	**64.5** ^{C} ±2.7	**70.7** ^{B} ±2.9	**77.9** ^{A} ±1.8	**62.1** ^{C} ±1.8	**69.5** ^{B} ±3.5	**71.1** ^{B} ±5.9	**47.8** ^{D} ±2.3
%	*100.0*	*144.9*	*158.9*	*175.1*	*139.6*	*156.2*	*159.8*	*107.4*
P in excreta (g/kg DM faeces)	**9.5** ^{B} ±0.7	**5.9** ^{C} ±0.4	**4.8** ^{D} ±0.4	**3.7** ^{E} ±0.3	**6.4** ^{C} ±0.3	**5.1** ^{D} ±0.5	**4.8** ^{D} ±0.9	**10.9** ^{A} ±0.5
%	*100.0*	*62.1*	*50.5*	*38.9*	*67.4*	*53.7*	*50.5*	*114.7*
Calcium								
Apparent Ca utilization (% of intake)	**32.8** ^{D} ±6.1	**56.0** ^{B} ±3.4	**61.3** ^{AB} ±3.7	**66.7** ^{A} ±2.4	**48.8** ^{C} ±2.4	**56.8** ^{B} ±4.8	**67.1** ^{A} ±5.1	**50.3** ^{C} ±2.6
%	*100.0*	*170.7*	*186.9*	*203.4*	*148.8*	*173.2*	*204.6*	*153.4*

Newman-Kuls test: Means within a row, not sharing a common superscript, are significantly different ($p<0.05$)

Table 6: Concentrations of inorganic phosphorus (P_i) in the plasma of male broiler chickens fed different supplemental levels of phytase, mean ± stdev.

Product	**Negative control**	**IPA Phytase (L)**			**IPA Phytase salt coated**			**Positive control**
Treatment	A	B	C	D	E	F	G	H
Dose (U/kg)	-	500	1000	2000	500	1000	2000	+1.5 g P /kg
cages x birds	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4	4 x 4
P_i (mmol/L)	**1.14** ^{C} ±0.18	**1.41** ^{C} ±0.18	**2.24** ^{A} ±0.21	**2.42** ^{A} ±0.09	**1.22** ^{C} ±0.15	**1.76** ^{B} ±0.38	**2.26** ^{A} ±0.12	**1.77** ^{B} ±0.19
%	*100.0*	*123.6*	*196.5*	*212.3*	*107.0*	*154.4*	*198.2*	*155.3*
Ca (mmol/L)	**2.95** ^{A} ±0.12	**2.76** ^{BC} ±0.06	**2.60** ^{D} ±0.10	**2.54** ^{D} ±0.08	**2.83** ^{B} ±0.07	**2.69** ^{BCD} ±0.14	**2.53** ^{D} ±0.11	**2.63** ^{CD} ±0.09
%	*100.0*	*93.6*	*88.1*	*86.1*	*95.9*	*91.2*	*85.8*	*89.2*

Newman Kuls test: Means within a row, not sharing a common superscript, are significantly different (p≤0.05)

Table 7: Tibia quality of male broiler chickens fed different supplemental levels of phytase, mean ± stdev.

Product	Negative control	IPA Phytase (L)			IPA Phytase salt coated			Positive control
Treatment	A	B	C	D	E	F	G	H
Dose	-	500	1000	2000	500	1000	2000	+1.5 g P /kg
cages x birds	6 x 4	6 x 4	6 x 4	6 x 4	6 x 4	6 x 4	6 x 4	6 x 4
Tibia strength (N)	**73.9** ^{C1} ±9.4	**155.5** ^{B} ±16.7	**197.0** ^{AB} ±30.7	**221.0** ^{A} ±27.5	**162.7** ^{AB} ±18.3	**187.0** ^{AB} ±40.4	**214.1** ^{A} ±26.2	**171.5** ^{B} ±25.8
%	*100*	*210.3*	*266.4*	*298.9*	*220.1*	*252.9*	*289.6*	*231.9*
Tibia ash (%	**37.2** ^{D} ±2.05	**46.6** ^{C} ±1.12	**50.0** ^{AB} ±0.78	**50.8** ^{A} ±0.66	**45.7** ^{C} ±2.19	**48.7** ^{AB} ±1.24	**50.5** ^{A} ±0.58	**48.3** ^{B} ±1.42
%	*100*	*125.3*	*134.3*	*136.6*	*122.8*	*130.9*	*135.7*	*129.9*
Toe ash (%	**20.5** ^{B} ±2.32	**32.2** ^{A} ±2.63	**32.7** ^{A} ±3.83	**35.0** ^{A} ±3.98	**33.4** ^{A} ±1.59	**33.7** ^{A} ±4.59	**37.3** ^{A} ±3.41	**33.1** ^{A} ±3.31
%	*100*	*157.1*	*159.5*	*170.7*	*162.7*	*164.4*	*181.9*	*161.4*

Newman-Kuls test: Means within a row, not sharing a co mmon superscript, are significantly different (p≤0.05).

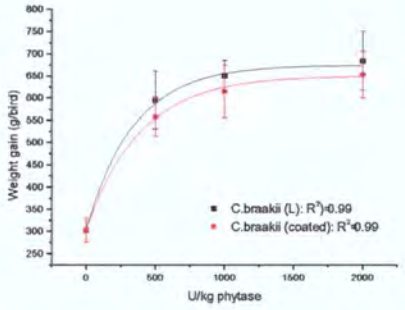


Figure 1: Effect of IPA phytase on weight gain

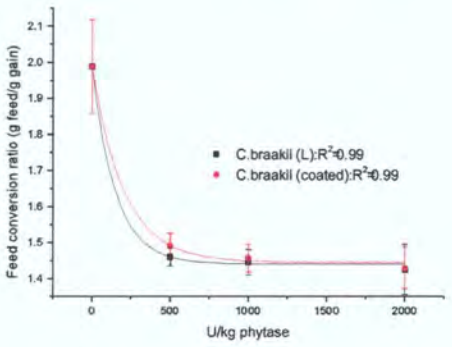


Figure 2: Effect of IPA phytase on feed conversion ratio

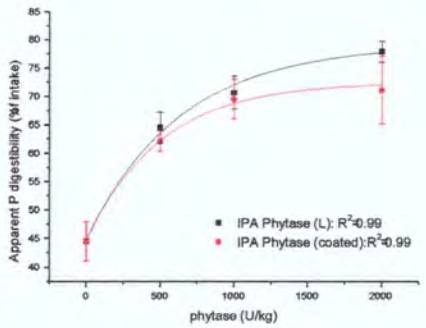


Figure 3: Effect of IPA phytase on apparent phosphorus utilisation

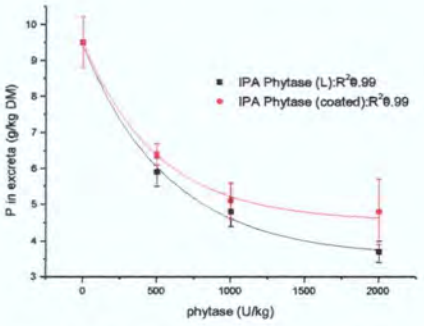


Figure 4: Effect of IPA phytase on P excretion

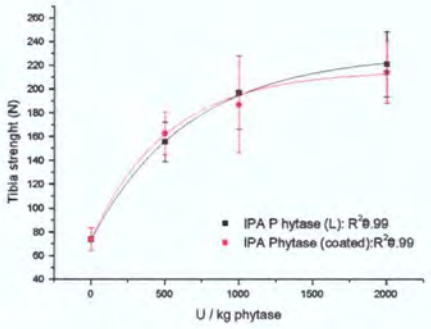


Figure 5: Effect of IPA phytase on tibia strength

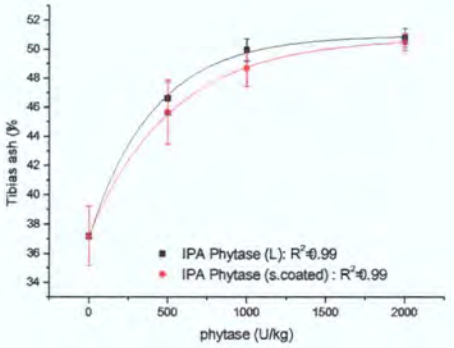


Figure 6: Effect of IPA phytase on tibia ash

Table 8: Non linear regression describing the supplementation of IPA Phytase liquid on *various parameters*

IPA Phytase liquid	a	b	k	R^2
Weight gain (g/bird)	302.6	372.7	0.0029	0.99
Feed conversion ratio (g feed/g gain)	2.0	-0.5	0.0066	0.99
Apparent P utilization (%	44.8	34.3	0.0016	0.99
P in excreta (g/kg DM faeces)	6.9	-5.9	0.0017	0.99
Tibia ash (%	37.1	13.9	0.0024	0.99
Tibia strength (N)	73.9	155.5	0.0015	0.99

Table 9: Non linear regression describing the supplementation of IPA Phytase salt coated on various parameters

IPA Phytase salt coated	a	b	k	R^2
Weight gain (g/bird)	302.6	348.6	0.0026	0.99
Feed conversion ratio (g feed/g gain)	2.0	-0.5	0.0049	0.99
Apparent P utilization (%	44.4	28.2	0.0020	0.99
P in excreta (g/kg DM faeces)	9.5	-5.0	0.0020	0.99
Tibia ash (%	37.2	13.6	0.0019	0.99
Tibia strength (N)	74.0	141.5	0.0019	0.99

Raw data of Trial BE-07/09

I. INTRODUCTION

The following documentation summarizes supplementary raw data concerning the trial BE-07/09 performed 7-April-2009 to 29-April 2009 at the Research Center for Animal Nutrition (NRD/CA, DSM Nutritional Products France, F-68128 Village-Neuf). This trial was reported under the following title: Comparison of two formulations of a microbial 6-phytase included at graded levels on growth performance and phosphorus utilization of broiler chickens (BE-07/09), (Philipps et al.2009).

REFERENCES

P. PHILIPPS, R. AURELI and Z. Nasir. (2009):
Comparison of two formulations of a microbial 6-phytase included at graded levels on growth performance and phosphorus utilization of broiler chickens (BE-07/09). (DSM Report No 00001184, Regulatory Document, 09-October-2009

II. Raw data of Trial BE-07/09

Petra Philipps, Raffaella Aureli and Zahid Nasir

Comparison of two formulations of a microbial 6-phytase included at graded levels on growth performance and phosphorus utilization of broiler chickens (BE-07/09). (DSM Report No 00001184, Regulatory Document, 09-October-2009

RDR 00001184

Analytical data on feed
Animal performance data
Data on apparent utilization of phosphorus
Data on calcium and inorganic phosphorus in plasma
Data on tibia strength and tibia/toe ash

09-October-2009

(Petra Philipps)

DSM Nutritional Products
B.P.170
F-68305 Saint-Louis cedex
France

2.1 Analytical data on feed

(see also tables 1,2 and 3 of report 00001184)

2.1.1 Nutrient content in feed

2.1.2 Ca/P

2.1.3 Phytate in feed

2.1.4 Phytase activity in feed

Service Volaille
BE-07/09 : Aliment A

Document Interne

Analyses d'aliments									
Echantillons	Matière sèche = MS en %	Cendres en %		Fibres en %		Graisses en %		Protéines en %	
			100% MS		100% MS		100% MS		100% MS
A	88.46	5.06	5.72	3.01	3.40	5.48	6.20	22.208	25.104

Echantillons	Matière sèche = MS en %	Amidon en %		Sucre en %	
			100% MS		100% MS
A	88.46	39.82	45.01	4.59	5.19

554

03/06/2009

SERVICE VOLAILLE
EXPERIENCE BE-07/09
ALIMENTS

Document Interne

CALCIUM - PHOSPHORE ICP						
Echantillons	gCa/100g MS	gCa/100g MF	gP/100g MS	gP/100g MF	MS%	C%
A	0.58	0.51	0.47	0.41	87.11	5.12
B	0.63	0.55	0.40	0.34	86.89	5.05
C	0.67	0.59	0.42	0.36	87.14	5.03
D	0.68	0.59	0.46	0.40	87.11	5.07
E	0.72	0.63	0.44	0.38	87.12	5.05
F	0.61	0.53	0.43	0.38	87.19	5.04
G	0.64	0.55	0.44	0.38	87.29	4.99
H	0.66	0.57	0.56	0.49	87.16	4.88

555

29/05/2009

DSM NUTRITINAL PRODUCTS
PHYTATE-P/NON PHYTATE-P
BE-07/09

BE-07/09_ 4.1 gP/kg	Composition de l'aliment	% P Phytique	g P Brut analysé	% P Phytique x g P Brut analysé	x % dans l'aliment
mais	0.5906	**0.66**	2.20	**1.452**	**0.8576**
soja	0.368	**0.6**	5.10	**3.06**	**1.12608**
P bicalcique		**0**		**0**	**0**
g/kg Phytate P					1.9836312
µg/g Phytate P					1983.6312
mg/g Phytate					**7.034**

	mg /g Phytate	
	calculé	analysé (AOAC)
ALIMENT	7.03	10.73

Valeurs théoriques

Phosphore total	Phytate-P (mg/g)	Non Phytate-P (mg/g)
3.80	3.03	0.77

Analytical Service

DSM
Nutritional

Analytical Research Center

To:	Dr. Petra Philipps	NRD/CA
Copies:		
From:	**Dr. Kurt Vogel**	
	Bldg. 203-20a	**Tel. +41 – 61 – 815 8665**
	CH-4002 Basel	**Fax +41 – 61 – 815 8440**
		kurt.vogel@dsm.com
Date:	08. April 2009	

Request: BE-07/09_1 & 2

Liebe Petra

Unten findest Du die ersten Resultate von Deinen Futterproben. Das Futter mit der L-Form sieht gut aus. Die Proben mit der festen Formulierung haben sehr grosse Streuung. Wir werden diese nach Ostern nochmals messen. Bitte betrachte diese Werte nur als vorläufige Information.

Viele Grüsse

Dr. P. Philipps, NRD/CA
IPA pellets
Request: **BE - 07-09 - 1 & 2**
Th 6562
Received: 06.04.2009

analyzed
07.04.2009
SJ; LJ 00290
LAB_ORD_
*25755_*

		declared	AVERAGE [Units : kg]	STDEV	CV (%)
Treatment **A** *mash*	1	0	below LOQ		
Treatment **A** *pellet*	2		below LOQ		
***C. braakii* phytase (L)** Lot PPQ 28432					
Treatment **B** *pellet*	3	500	471 528	500	40	8
Treatment **C** *pellet*	5	1000	936 1030	983	67	7
Treatment **D** *pellet*	5	2000	2203 2138	2170	46	2
***C. braakii* salt coated** Lot PPQ 29773					
Treatment **E** *mash*	6	500	598 600	599	2	0
Treatment **E** *pellet*	7		629 434	531	138	26
Treatment **F** *mash*	8	1000	1199 844	1021	251	25
Treatment **F** *pellet*	9		937 1953	1445	719	50
Treatment **G** *mash*	10	2000	2659 2327	2493	234	9
Treatment **G** *pellet*	11		2473 1328	1900	810	43
Treatment **H** *mash*	12	0	below LOQ		
Treatment **H** *pellet*	13		below LOQ		

2.2 Animal performance data

(see also table 4 of report 00001184)

2.2.1 Raw data on growth performance and feed consumption on a weekly base

4 pages

	N° cage	Sexe	Nombre d'animaux à J15	Nombre d'animaux à J22	Poids des morts de J8 à J15 en g	Poids des morts de J15 à J22 en g	Poids des animaux à J8 en g	Poids des animaux à J15 en g	Poids des animaux à J22 en g	Poids brut d'aliment à J8 en g	Poids brut d'aliment à J15 en g	Poids brut d'aliment à J22 en g
TT	-	S	N2	N3	M1	M2	TG1	TG2	TG3	FB1	FB2	FB3
A	1	Mâles	8	2		1651	1354	2160	758	18314	16805	15747
B	2	Mâles	8	7		265	1327	2862	5280	18166	15994	12059
C	3	Mâles	8	7		311	1322	2968	5921	18161	15820	11212
D	4	Mâles	5	5	334		1318	1726	4329	18310	16955	13436
E	5	Mâles	7	6	142	389	1353	2349	4263	18262	16538	13217
F	6	Mâles	8	7		346	1373	2716	5258	18094	16052	11882
G	7	Mâles	7	7	143		1338	2732	6250	18034	15826	10902
H	8	Mâles	7	7	131		1352	2629	5869	18119	16105	11448
A	9	Mâles	6	3	400	717	1310	1698	1377	18242	16742	15588
B	10	Mâles	8	8			1343	2582	5679	18103	16192	11801
C	11	Mâles	8	7		420	1400	2663	5349	18130	16171	11855
D	12	Mâles	6	6	331		1371	2076	4527	18172	16575	13061
E	13	Mâles	8	8			1362	2869	5778	18211	16084	11878
F	14	Mâles	7	7	133		1333	2319	5145	18255	16550	12589
G	15	Mâles	7	6	142	420	1335	2439	4988	18164	16279	12084
H	16	Mâles	7	7	148		1421	2621	5774	18293	16350	12024
A	17	Mâles	8	5		853	1327	2332	2192	18116	16405	14706
B	18	Mâles	8	6		817	1330	3000	4846	18100	15724	11776
C	19	Mâles	8	6		1203	1436	2780	4842	18133	16151	11681
D	20	Mâles	6	6	292		1344	2430	5300	18092	16228	12180
E	21	Mâles	7	7	104		1342	2498	5014	18102	16139	12404
F	22	Mâles	8	8			1367	2761	5869	18086	16058	11636
G	23	Mâles	7	7	177		1348	2555	5627	18259	16354	12135
H	24	Mâles	6	6	495		1351	2357	5177	18235	16152	12083
A	25	Mâles	7	3	140	1124	1394	2087	1442	18302	16785	15562
B	26	Mâles	8	8			1330	2862	5944	18289	16094	11815
C	27	Mâles	8	8			1330	3055	6908	18154	15688	10100
D	28	Mâles	6	6	286		1334	2214	4893	18325	16643	12747
E	29	Mâles	8	8			1431	3051	5975	18208	15893	11450
F	30	Mâles	8	8			1337	3151	6473	18245	15740	10530
G	31	Mâles	7	7	162		1344	2537	5674	18125	16250	11965
H	32	Mâles	7	6	171	175	1318	2191	4050	18222	16584	13412
A	33	Mâles	7	4	136	866	1401	2136	1857	18129	16521	15127
B	34	Mâles	8	8			1330	2720	5310	18218	16193	12320
C	35	Mâles	8	8			1362	2996	6501	18107	15823	10665
D	36	Mâles	8	8			1349	3217	6923	18077	15492	9963
E	37	Mâles	8	8			1355	2810	5627	18255	16139	11865
F	38	Mâles	8	8			1372	2879	5382	18207	16029	12015
G	39	Mâles	7	6	176	535	1303	2704	5348	18146	16022	11657
H	40	Mâles	7	7	159		1356	2653	5509	18384	16332	12129
A	41	Mâles	8	4		1333	1414	2560	1877	18210	16375	14569
B	42	Mâles	8	7		204	1332	2987	6068	18109	15752	11037
C	43	Mâles	5	5	553		1455	2014	4006	18259	16653	13608
D	44	Mâles	7	7	155		1354	2734	6047	18386	16351	11810
E	45	Mâles	8	8			1379	2909	5649	18296	16016	11685
F	46	Mâles	7	7	193		1386	2542	5272	18180	16229	12273
G	47	Mâles	7	6	143	208	1299	2422	4854	18168	16313	12399
H	48	Mâles	8	8			1334	2941	6270	18105	15820	10927
A	49	Mâles	8	6		694	1333	2585	2983	18115	16131	13936
B	50	Mâles	7	6	207	477	1397	2507	4826	18054	16089	12171
C	51	Mâles	8	8			1369	3069	6661	18334	15892	10491
D	52	Mâles	7	7	170		1358	3015	6740	18049	15689	10550
E	53	Mâles	5	5	466		1300	1585	3505	18115	16850	14056
F	54	Mâles	7	7	212		1383	2670	5545	18071	15928	11767
G	55	Mâles	6	6	299		1339	2041	4771	18211	16661	12966
H	56	Mâles	7	7	155		1326	2550	5201	18371	16394	12428
A	57	Mâles	7	5	261	476	1336	2080	2332	18096	16365	14595
B	58	Mâles	8	8			1309	2684	5216	18138	16126	12308
C	59	Mâles	7	7	177		1417	2520	5835	18104	16200	11838
D	60	Mâles	7	7	140		1342	2816	6679	18052	15882	10742
E	61	Mâles	8	6		856	1466	2965	4980	18581	16384	12150
F	62	Mâles	7	7	148		1407	2716	5820	18119	16009	11617
G	63	Mâles	6	5	515	507	1328	2333	4083	18118	15995	12679
H	64	Mâles	7	7	130		1353	2352	5273	18097	15276	11957
A	65	Mâles	8	5		786	1342	2292	2267	18346	16687	14878
B	66	Mâles	8	8			1336	2664	5789	18041	15844	11569
C	67	Mâles	8	7		219	1389	3002	6157	18357	16112	11361
D	68	Mâles	7	7	146		1337	2589	5994	18028	16086	11458
E	69	Mâles	8	8			1354	3050	6510	18270	15875	10852
F	70	Mâles	7	6	141	469	1394	2611	5072	18054	16013	11848
G	71	Mâles	7	7	144		1344	2675	6122	18079	16013	11295
H	72	Mâles	8	8			1338	2903	6226	18183	15873	10933
A	73	Mâles	6	5	316	294	1428	1937	2742	18087	16590	14554
B	74	Mâles	6	5	451	232	1427	2061	4169	18102	16385	12930
C	75	Mâles	8	8			1450	3114	6342	18184	15734	10663
D	76	Mâles	7	7	149		1647	2492	5444	18176	16304	11972
E	77	Mâles	8	8			1350	2733	5664	18197	16178	11796
F	78	Mâles	8	7		449	1389	3083	5910	18051	15643	10761
G	79	Mâles	6	6	285		1349	2067	4206	18089	16521	13192
H	80	Mâles	7	7	159		1397	2567	5637	18193	16180	11623
A	81	Mâles	8	5		846	1407	2547	2360	18123	16265	14669
B	82	Mâles	8	8			1399	3085	6463	18124	15796	10819
C	83	Mâles	8	8			1423	3158	6688	18205	15860	10479
D	84	Mâles	7	7	144		1350	2929	5893	18171	15784	11130
E	85	Mâles	7	7	254		1405	2385	4711	18395	16526	12942

F	86	Mâles	8	8			1385	2892	6343	18384	16150	11068
G	87	Mâles	8	8			1317	3092	6400	18033	15555	10402
H	88	Mâles	8	8			1322	3047	6662	18059	15744	10431
A	89	Mâles	7	5	248	617	1374	2162	2311	19121	17412	15741
B	90	Mâles	8	7		256	1321	3039	5648	18377	15941	11578
C	91	Mâles	7	7	152		1377	2716	5822	18339	16283	11649
D	92	Mâles	7	7	138		1353	2753	5798	17011	14920	10563
E	93	Mâles	7	7	180		1384	2476	5055	18540	16614	12712
F	94	Mâles	8	7		467	1321	3163	6131	18358	15853	10968
G	95	Mâles	8	8			1302	3016	6463	18328	15957	10944
H	96	Mâles	7	7	143		1345	2641	5672	18096	16052	11625

TT	N° cage	Sexe	Gain de poids de J8 à J15 en g par animal	Gain de poids de J15 à J22 en g par animal	Gain de poids de J8 à J22 en g par animal	Consommation d'aliment de J8 à J15 par animal	Consommation d'aliment de J15 à J22 par animal	Consommation d'aliment de J8 à J22 par animal	Indice de consommation de J8 à J15	Indice de consommation de J15 à J22	Indice de consommation de J8 à J22
	-	S	WG1	WG2	WGT	AL1	AL2	ALT	IC1	IC2	ICT
A	1	Mâles									
A	9	Mâles	119.3	176.0	295.3	227.0	512.9	661.8	1.904	2.914	2.242
A	17	Mâles	125.6	146.9	272.5	213.9	350.0	540.9	1.702	2.383	1.985
A	25	Mâles	123.9	182.5	306.4	225.6	466.0	639.9	1.821	2.553	2.088
A	33	Mâles	130.0	159.1	289.1	240.0	377.8	595.3	1.846	2.375	2.059
A	41	Mâles	143.3	149.3	292.5	229.4	414.7	593.0	1.601	2.778	2.027
A	49	Mâles	156.5	174.0	330.5	248.0	349.8	589.3	1.585	2.010	1.783
A	57	Mâles	130.1	169.3	299.4	224.2	411.5	604.8	1.722	2.431	2.020
A	65	Mâles	118.8	166.9	285.7	207.4	396.7	579.0	1.746	2.377	2.027
A	73	Mâles	144.3	225.6	369.9	251.9	417.9	679.2	1.815	1.853	1.836
A	81	Mâles	142.5	153.6	296.1	232.3	372.1	568.5	1.630	2.422	1.920
A	89	Mâles	137.1	153.3	290.5	226.2	334.5	544.8	1.650	2.181	1.876
B	2	Mâles	191.9	396.5	588.4	271.5	581.6	851.9	1.415	1.467	1.448
B	10	Mâles	154.9	387.1	542.0	238.9	548.9	787.8	1.542	1.418	1.453
B	18	Mâles	208.8	432.7	641.4	297.0	641.4	936.1	1.423	1.483	1.459
B	26	Mâles	191.5	385.3	576.8	274.4	559.9	834.3	1.433	1.453	1.446
B	34	Mâles	173.8	323.8	497.5	253.1	484.1	737.3	1.457	1.495	1.482
B	42	Mâles	206.9	493.5	700.4	294.6	708.3	1002.6	1.424	1.435	1.432
B	50	Mâles	183.5	446.2	629.7	273.8	625.2	900.7	1.492	1.401	1.430
B	58	Mâles	171.9	316.5	488.4	251.5	477.3	728.8	1.463	1.508	1.492
B	66	Mâles	191.0	365.6	556.6	274.6	534.4	809.0	1.438	1.462	1.453
B	74	Mâles	168.5	487.0	655.4	261.8	725.2	989.7	1.554	1.489	1.510
B	82	Mâles	210.8	422.3	633.0	291.0	622.1	913.1	1.381	1.473	1.443
B	90	Mâles	214.8	427.0	641.7	304.5	650.2	952.0	1.418	1.523	1.484
C	3	Mâles	205.8	474.9	680.6	292.6	670.4	963.2	1.422	1.412	1.415
C	11	Mâles	157.9	431.3	589.1	244.9	599.3	846.2	1.551	1.390	1.436
C	19	Mâles	168.0	459.5	627.5	247.8	629.1	876.4	1.475	1.369	1.400
C	27	Mâles	215.6	481.6	697.3	308.3	698.5	1006.8	1.430	1.450	1.444
C	35	Mâles	204.3	438.1	642.4	265.5	644.8	930.3	1.398	1.472	1.448
C	43	Mâles	220.9	398.4	619.3	319.1	609.0	928.0	1.444	1.529	1.498
C	51	Mâles	212.5	449.0	661.5	305.3	675.1	980.4	1.436	1.504	1.482
C	59	Mâles	182.9	473.6	656.4	272.0	651.7	923.7	1.488	1.376	1.407
C	67	Mâles	201.6	504.3	705.9	280.6	710.1	990.3	1.392	1.408	1.403
C	75	Mâles	208.0	403.5	611.5	306.3	608.9	915.1	1.472	1.509	1.497
C	83	Mâles	216.9	441.3	658.1	293.1	672.6	965.8	1.352	1.524	1.467
C	91	Mâles	215.9	443.7	659.6	297.7	662.0	959.9	1.379	1.492	1.455
D	4	Mâles	180.5	520.6	701.1	329.5	703.8	1021.5	1.826	1.352	1.457
D	12	Mâles	174.6	408.5	583.1	269.2	585.7	854.7	1.542	1.434	1.466
D	20	Mâles	237.0	478.3	715.3	320.6	674.7	995.5	1.353	1.410	1.392
D	28	Mâles	202.3	446.5	648.8	291.8	649.3	941.2	1.443	1.454	1.451
D	36	Mâles	233.5	463.3	696.8	323.1	691.1	1014.3	1.384	1.492	1.456
D	44	Mâles	221.3	473.3	694.6	293.4	648.7	942.2	1.326	1.371	1.356
D	52	Mâles	261.0	532.1	793.1	337.1	734.1	1071.2	1.292	1.380	1.351
D	60	Mâles	234.5	551.9	786.4	315.3	734.3	1049.6	1.344	1.331	1.335
D	68	Mâles	202.7	486.4	689.2	281.6	661.1	942.7	1.389	1.359	1.368
D	76	Mâles	150.1	421.7	571.8	282.7	616.9	899.1	1.883	1.467	1.572
D	84	Mâles	249.7	423.4	673.1	345.9	664.9	1011.2	1.385	1.570	1.502
D	92	Mâles	224.2	435.0	659.2	304.8	622.4	927.4	1.360	1.431	1.407
E	5	Mâles	166.4	374.9	541.4	252.2	540.7	793.7	1.515	1.442	1.466
E	13	Mâles	185.9	363.6	549.5	265.9	525.8	791.8	1.430	1.446	1.441
E	21	Mâles	189.1	359.4	548.5	294.6	533.6	827.7	1.558	1.484	1.509
E	29	Mâles	202.5	365.5	568.0	289.4	555.4	844.8	1.429	1.519	1.487
E	37	Mâles	181.9	352.1	534.0	264.5	534.3	798.8	1.454	1.517	1.496
E	45	Mâles	191.3	342.5	533.8	285.0	541.4	826.4	1.490	1.581	1.548
E	53	Mâles	154.5	384.0	538.5	260.2	558.8	818.3	1.684	1.455	1.520
E	61	Mâles	187.4	459.4	646.8	274.6	677.5	951.8	1.466	1.475	1.472
E	69	Mâles	212.0	432.5	644.5	299.4	627.9	927.3	1.412	1.452	1.439
E	77	Mâles	172.9	366.4	539.3	252.4	547.8	800.1	1.460	1.496	1.484
E	85	Mâles	165.1	332.3	497.4	250.0	512.0	761.8	1.515	1.541	1.532
E	93	Mâles	180.7	368.4	549.1	273.6	557.4	831.1	1.514	1.513	1.513
F	6	Mâles	167.9	411.6	579.5	255.3	594.4	850.9	1.520	1.444	1.468
F	14	Mâles	164.7	403.7	568.4	250.9	568.7	819.2	1.524	1.409	1.441
F	22	Mâles	174.3	388.5	562.8	253.5	552.8	806.3	1.455	1.423	1.433
F	30	Mâles	226.8	415.3	642.0	313.1	651.3	964.4	1.381	1.568	1.502
F	38	Mâles	188.4	312.9	501.3	272.3	501.8	774.0	1.445	1.604	1.544
F	46	Mâles	189.9	390.0	579.9	274.6	565.1	839.8	1.446	1.449	1.448
F	54	Mâles	208.6	410.7	619.3	298.2	594.4	892.5	1.430	1.447	1.441
F	62	Mâles	212.1	443.4	655.6	307.2	627.4	934.5	1.448	1.415	1.428
F	70	Mâles	198.8	472.3	671.1	298.7	671.4	971.3	1.503	1.422	1.447
F	78	Mâles	211.8	458.9	670.7	301.0	683.9	983.7	1.421	1.490	1.467
F	86	Mâles	188.4	431.4	619.8	279.3	635.3	914.5	1.482	1.473	1.476
F	94	Mâles	232.8	478.0	710.7	313.1	683.7	995.3	1.345	1.430	1.400
G	7	Mâles	223.0	502.6	725.6	320.4	703.4	1023.7	1.437	1.400	1.411
G	15	Mâles	181.6	482.9	664.5	274.7	685.6	961.6	1.513	1.420	1.447
G	23	Mâles	196.5	438.9	635.4	270.5	602.7	873.2	1.376	1.373	1.374
G	31	Mâles	194.4	448.1	642.6	269.0	612.1	881.2	1.384	1.366	1.371
G	39	Mâles	223.4	505.0	728.5	300.9	693.5	993.9	1.347	1.373	1.364
G	47	Mâles	183.6	463.0	646.6	269.1	686.4	955.0	1.465	1.483	1.477
G	55	Mâles	172.8	455.0	627.8	267.6	616.0	882.7	1.548	1.354	1.406
G	63	Mâles	222.8	427.8	650.6	311.2	628.5	936.9	1.397	1.469	1.440

G	71	Mâles	214.1	492.4	706.6	299.9	674.0	973.9	1.401	1.369	1.378
G	79	Mâles	175.9	356.5	532.4	274.9	554.8	829.7	1.563	1.556	1.559
G	87	Mâles	221.9	413.5	635.4	309.8	644.1	953.9	1.396	1.558	1.501
G	95	Mâles	214.3	430.9	645.1	296.4	626.6	923.0	1.383	1.454	1.431
H	8	Mâles	206.6	462.9	669.4	295.5	665.3	960.8	1.430	1.437	1.435
H	16	Mâles	196.8	450.4	647.2	283.7	618.0	901.5	1.441	1.372	1.393
H	24	Mâles	224.0	470.0	694.0	310.8	678.2	988.0	1.388	1.443	1.424
H	32	Mâles	148.3	362.0	510.3	232.6	564.5	797.4	1.569	1.559	1.563
H	40	Mâles	209.5	408.0	617.5	295.3	600.4	895.7	1.409	1.472	1.451
H	48	Mâles	200.9	416.1	617.0	285.8	611.6	897.3	1.422	1.470	1.454
H	56	Mâles	198.5	378.7	577.3	284.5	566.6	851.3	1.434	1.496	1.475
H	64	Mâles	166.9	417.3	584.2	269.2	615.6	884.2	1.613	1.475	1.514
H	72	Mâles	195.6	415.4	611.0	288.8	617.5	906.3	1.476	1.487	1.483
H	80	Mâles	192.1	438.6	630.7	291.0	651.0	941.9	1.515	1.484	1.494
H	88	Mâles	215.6	454.4	670.0	289.4	664.1	953.5	1.342	1.462	1.423
H	96	Mâles	209.2	433.0	642.2	297.1	632.4	929.6	1.420	1.461	1.448

2.3 Data on apparent utilization of phosphorus

(see also table 5 of report 00001184)

4 pages

TRAITEMENTS	N° éch	TARE	Aliment brut J1	Aliment brut J5	Matière sèche aliment en %	Taux P en % de MS aliment	Taux Ca en % de MS aliment	Quantité de fèces produite en 4 jours en g	MS de fèces frais en %	Taux P en % de MS fèces	Taux Ca en % de MS fèces	Nombre d'animaux à J1	Nombre d'animaux à J5	Nombre d'animaux X nombre de jours	Poids des morts
TT	-	T	FED1	FED5	DMFE	PHOFE	CAFE	FA4	DMFA	PHOFA	CAFA	N1	N5	FT	M
A1	1	413	17275	16322	87.12	0.44	0.65	1057	33.45	0.96	1.56	8	8	32	
A9	2	411	17125	16294	87.12	0.44	0.65	1020	31.31	0.92	1.68	7	5	26	390
A17	3	412	16961	15804	87.12	0.44	0.65	1326	29.82	0.96	1.86	8	6	30	581
A49	4	401	16771	15395	87.12	0.44	0.65	1375	31.76	0.84	1.50	8	8	32	
A57	5	412	16805	15815	87.12	0.44	0.65	1063	32.41	1.03	1.77	7	6	27	208
A65	6	401	17219	15990	87.12	0.44	0.65	1293	29.76	1.00	1.81	8	8	32	
B2	7	405	16807	14924	87.12	0.44	0.65	2248	23.41	0.59	1.16	8	8	32	
B10	8	412	16955	15109	87.12	0.44	0.65	2270	22.77	0.58	1.13	8	8	32	
B18	9	412	16547	14635	87.12	0.44	0.65	2202	25.16	0.65	1.14	8	7	31	272
B50	10	413	16827	15072	87.12	0.44	0.65	2153	22.90	0.61	1.09	8	7	29	207
B58	11	415	16875	15082	87.12	0.44	0.65	2074	24.22	0.61	1.06	8	8	32	
B66	12	428	16672	14722	87.12	0.44	0.65	2241	24.60	0.53	0.95	8	8	32	
C3	13	416	16698	14631	87.12	0.44	0.65	2410	24.44	0.56	1.09	8	7	31	311
C11	14	417	16952	15051	87.12	0.44	0.65	2238	24.45	0.48	0.97	8	8	32	
C19	15	420	16928	14991	87.12	0.44	0.65	2290	24.55	0.44	0.84	8	8	32	
C51	16	417	16805	14469	87.12	0.44	0.65	2979	20.64	0.46	0.92	8	8	32	
C59	17	417	16933	15103	87.12	0.44	0.65	2207	23.86	0.49	0.94	7	7	28	
C67	18	417	16983	14913	87.12	0.44	0.65	2367	24.30	0.46	0.90	8	7	31	219
D4	19	412	17481	16112	87.12	0.44	0.65	1696	23.71	0.41	0.89	7	7	28	
D12	20	412	17203	15708	87.12	0.44	0.65	1841	23.56	0.40	0.87	7	7	28	
D20	21	412	16953	15157	87.12	0.44	0.65	2334	22.33	0.38	0.86	8	8	32	
D52	22	414	16579	14470	87.12	0.44	0.65	2892	20.35	0.37	0.77	8	8	32	
D60	23	412	16708	14636	87.12	0.44	0.65	2646	21.69	0.33	0.76	8	8	32	
D68	24	412	16871	14904	87.12	0.44	0.65	2524	20.46	0.34	0.77	8	8	32	
E5	25	428	17191	15607	87.12	0.44	0.65	1769	26.06	0.67	1.34	8	8	32	
E13	26	411	16886	14923	87.12	0.44	0.65	2289	24.22	0.66	1.42	8	8	32	
E21	27	415	16844	15166	87.12	0.44	0.65	1999	24.21	0.64	1.25	7	7	28	
E53	28	406	17325	16102	87.12	0.44	0.65	1461	25.66	0.57	1.23	7	7	28	
E61	29	415	17232	15188	87.12	0.44	0.65	2358	24.62	0.66	1.22	8	8	32	
E69	30	411	16749	14587	87.12	0.44	0.65	2952	19.94	0.62	1.16	8	8	32	
F6	31	417	16826	14880	87.12	0.44	0.65	2566	21.08	0.54	1.06	8	8	32	
F14	32	417	17240	15588	87.12	0.44	0.65	1957	24.44	0.54	1.08	8	8	32	
F22	33	416	16826	14942	87.12	0.44	0.65	2284	23.89	0.55	1.23	8	8	32	
F54	34	419	16695	14843	87.12	0.44	0.65	2348	22.12	0.53	1.07	8	7	29	212
F62	35	417	16807	14861	87.12	0.44	0.65	2351	23.00	0.46	0.96	8	8	32	
F70	36	416	16783	14894	87.12	0.44	0.65	1961	27.87	0.42	0.95	8	8	32	
G7	37	403	16675	14619	87.12	0.44	0.65	2454	24.29	0.41	0.85	8	8	32	
G15	38	414	17002	15159	87.12	0.44	0.65	2291	22.95	0.52	0.97	8	8	32	
G23	39	411	17129	15054	87.12	0.44	0.65	2327	23.16	0.39	0.82	8	8	32	
G55	40	402	17258	15720	87.12	0.44	0.65	1888	22.70	0.41	0.67	8	8	32	
G63	41	411	16735	15142	87.12	0.44	0.65	1969	24.61	0.56	0.81	8	6	28	845
G71	42	413	16820	14859	87.12	0.44	0.65	2356	23.14	0.59	0.72	8	8	32	
H8	43	412	16897	14944	87.16	0.56	0.66	2250	25.33	1.15	1.31	8	8	32	
H16	44	409	17119	15268	87.16	0.56	0.66	2198	24.48	1.03	1.21	8	8	32	
H24	45	415	16890	15153	87.16	0.56	0.66	2134	24.02	1.05	1.20	8	7	29	351
H56	46	413	17142	15388	87.16	0.56	0.66	2342	20.78	1.08	1.16	8	8	32	
H64	47	415	16978	15272	87.16	0.56	0.66	2091	22.46	1.14	1.26	8	8	32	
H72	48	415	16731	14666	87.16	0.56	0.66	2780	21.13	1.08	1.19	8	8	32	

	N° éch	Quantité d'aliment ingéré en g de MS par jour et animal	Rétention du P en g	Coefficient d'utilisation apparente du P en %	Rétention du Ca en g	Coefficient d'utilisation apparente du Ca en %	Phosphore dans l'excrétion (g/kg de MS)	Calcium dans l'excrétion (g/kg de MS)
TT	-	DMI	RPHO	RPHOP	RCA	RCAP		
A1	1	25.945	0.050	43.557	0.063	37.616	9.57	15.63
A9	2	27.845	0.055	45.067	0.058	31.777	9.18	16.84
A17	3	33.599	0.060	40.873	0.050	22.768	9.62	18.56
A49	4	37.462	0.084	50.896	0.099	40.494	8.37	14.99
A57	5	31.944	0.060	42.578	0.070	33.549	10.33	17.66
A65	6	33.460	0.065	43.815	0.067	30.785	9.97	18.15
B2	7	51.265	0.146	64.618	0.177	53.060	5.92	11.60
B10	8	50.257	0.145	65.616	0.177	54.238	5.75	11.31
B18	9	53.733	0.142	59.887	0.184	52.667	6.53	11.38
B50	10	52.723	0.149	64.065	0.193	56.192	6.07	10.93
B58	11	48.814	0.139	64.527	0.184	58.126	6.07	10.58
B66	12	53.089	0.159	68.112	0.212	61.434	5.34	9.55
C3	13	58.089	0.167	65.358	0.207	54.783	5.63	10.86
C11	14	51.755	0.161	70.777	0.201	59.702	4.78	9.74
C19	15	52.735	0.169	72.870	0.223	64.915	4.39	8.38
C51	16	63.598	0.204	72.786	0.262	63.294	4.61	9.18
C59	17	56.939	0.175	69.951	0.227	61.464	4.94	9.35
C67	18	58.174	0.186	72.591	0.241	63.768	4.59	8.96
D4	19	42.595	0.143	76.424	0.180	64.953	4.06	8.92
D12	20	46.516	0.157	76.797	0.198	65.422	3.95	8.70
D20	21	48.896	0.165	76.615	0.203	63.855	3.75	8.57
D52	22	57.418	0.195	77.130	0.252	67.591	3.67	7.68
D60	23	56.410	0.199	79.992	0.251	68.418	3.28	7.65
D68	24	53.552	0.190	80.459	0.244	70.095	3.41	7.71
E5	25	43.124	0.116	61.278	0.134	47.815	6.73	13.39
E13	26	53.443	0.141	60.054	0.146	41.950	6.61	14.19
E21	27	52.210	0.142	61.744	0.168	49.437	6.42	12.53
E53	28	38.053	0.112	67.136	0.129	52.100	5.69	12.25
E61	29	55.648	0.147	60.015	0.179	49.498	6.55	12.22
E69	30	58.860	0.161	62.306	0.199	51.917	6.16	11.62
F6	31	52.980	0.157	67.337	0.194	56.332	5.38	10.62
F14	32	44.976	0.134	67.907	0.166	56.634	5.40	10.78
F22	33	51.292	0.149	66.176	0.161	48.404	5.47	12.34
F54	34	55.637	0.166	67.940	0.205	56.638	5.33	10.66
F62	35	52.980	0.169	72.678	0.211	61.384	4.58	9.57
F70	36	51.428	0.170	75.094	0.206	61.586	4.19	9.54
G7	37	55.975	0.182	73.807	0.231	63.494	4.14	8.53
G15	38	50.176	0.151	68.380	0.195	59.803	5.19	9.74
G23	39	56.492	0.195	78.334	0.253	68.925	3.88	8.23
G55	40	41.872	0.141	76.307	0.201	73.954	4.14	6.72
G63	41	49.565	0.142	64.883	0.211	65.575	5.59	8.10
G71	42	53.388	0.152	64.771	0.245	70.707	5.89	7.24
H8	43	53.195	0.131	43.971	0.161	45.825	11.47	13.07
H16	44	50.417	0.141	50.036	0.166	50.034	10.31	12.15
H24	45	52.206	0.142	48.711	0.174	50.467	10.53	11.99
H56	46	47.775	0.132	49.223	0.170	53.920	10.84	11.60
H64	47	46.467	0.127	48.652	0.158	51.598	11.36	12.62
H72	48	56.245	0.146	46.285	0.186	50.138	10.84	11.85

		Quantité d'aliment ingéré en g de MS par jour et animal	Rétention du P en g	Coefficient d'utilisation apparente du P en %	Rétention du Ca en g	Coefficient d'utilisation apparente du Ca en %	Phosphore dans l'excrétion (g/kg de MS)	Calcium dans l'excrétion (g/kg de MS)
A	**Mean**	**31.7**	**0.06**	**44.5**	**0.07**	**32.8**	**9.5**	**17.0**
	Stdv	4.2	0.01	3.4	0.02	6.1	0.7	1.4
B	**Mean**	**51.6**	**0.15**	**64.5**	**0.19**	**56.0**	**5.9**	**10.9**
	Stdv	1.9	0.01	2.7	0.01	3.4	0.4	0.8
C	**Mean**	**56.9**	**0.18**	**70.7**	**0.23**	**61.3**	**4.8**	**9.4**
	Stdv	4.3	0.02	2.9	0.02	3.7	0.4	0.8
D	**Mean**	**50.9**	**0.17**	**77.9**	**0.22**	**66.7**	**3.7**	**8.2**
	Stdv	5.9	0.02	1.8	0.03	2.4	0.3	0.6
E	**Mean**	**50.2**	**0.14**	**62.1**	**0.16**	**48.8**	**6.4**	**12.7**
	Stdv	5.9	0.02	1.8	0.03	2.4	0.3	0.6
F	**Mean**	**51.5**	**0.16**	**69.5**	**0.19**	**56.8**	**5.1**	**10.6**
	Stdv	3.6	0.01	3.5	0.02	4.8	0.5	1.0
G	**Mean**	**51.2**	**0.16**	**71.1**	**0.22**	**67.1**	**4.8**	**8.1**
	Stdv	5.4	0.02	5.9	0.02	5.1	0.9	1.1
H	**Mean**	**51.1**	**0.14**	**47.8**	**0.17**	**50.3**	**10.9**	**12.2**
	Stdv	3.6	0.01	2.3	0.01	2.6	0.5	0.5

SERVICE VOLAILLE
EXPERIENCE BE-07/09
FECES FRAICHES

Document Interne

CALCIUM -PHOSPHORE						
Feces	gCa/100g MS	gCa/100g MF	gP/100g MS	gP/100g MF	MS%	C%
1	1.56	0.52	0.96	0.32	33.45	5.38
2	1.68	0.53	0.92	0.29	31.31	5.07
3	1.86	0.55	0.96	0.29	29.82	4.79
4	1.50	0.48	0.84	0.27	31.76	4.91
5	1.77	0.57	1.03	0.33	32.41	5.18
6	1.81	0.54	1.00	0.30	29.76	4.66
7	1.16	0.27	0.59	0.14	23.41	3.18
8	1.13	0.26	0.58	0.13	22.77	3.06
9	1.14	0.29	0.65	0.16	25.16	3.39
10	1.09	0.25	0.61	0.14	22.90	3.13
11	1.06	0.26	0.61	0.15	24.22	3.33
12	0.95	0.23	0.53	0.13	24.60	3.29
13	1.09	0.27	0.56	0.14	24.44	3.18
14	0.97	0.24	0.48	0.12	24.45	3.10
15	0.84	0.21	0.44	0.11	24.55	3.06
16	0.92	0.19	0.46	0.10	20.64	2.63
17	0.94	0.22	0.49	0.12	23.86	3.12
18	0.90	0.22	0.46	0.11	24.30	3.15
19	0.89	0.21	0.41	0.10	23.71	2.96
20	0.87	0.20	0.40	0.09	23.56	2.98
21	0.86	0.19	0.38	0.08	22.33	2.72
22	0.77	0.16	0.37	0.07	20.35	2.52
23	0.76	0.17	0.33	0.07	21.69	2.69
24	0.77	0.16	0.34	0.07	20.46	2.55
25	1.34	0.35	0.67	0.18	26.06	3.65
26	1.42	0.34	0.66	0.16	24.22	3.52
27	1.25	0.30	0.64	0.16	24.21	3.46
28	1.23	0.31	0.57	0.15	25.66	3.59
29	1.22	0.30	0.66	0.16	24.62	3.41
30	1.16	0.23	0.62	0.12	19.94	2.71
31	1.06	0.22	0.54	0.11	21.08	2.76
32	1.08	0.26	0.54	0.13	24.44	3.29
33	1.23	0.29	0.55	0.13	23.89	3.25
34	1.07	0.24	0.53	0.12	22.12	2.96
35	0.96	0.22	0.46	0.11	23.00	3.04
36	0.95	0.27	0.42	0.12	27.87	3.66
37	0.85	0.21	0.41	0.10	24.29	2.95
38	0.97	0.22	0.52	0.12	22.95	2.93
39	0.82	0.19	0.39	0.09	23.16	2.87
40	0.67	0.15	0.41	0.09	22.70	2.76
41	0.81	0.20	0.56	0.14	24.61	2.99
42	0.72	0.17	0.59	0.14	23.14	2.72
43	1.31	0.33	1.15	0.29	25.33	3.37
44	1.21	0.30	1.03	0.25	24.48	3.33
45	1.20	0.29	1.05	0.25	24.02	3.20
46	1.16	0.24	1.08	0.23	20.78	2.71
47	1.26	0.28	1.14	0.26	22.46	2.93
48	1.19	0.25	1.08	0.23	21.13	2.71

29/05/2009

2.4 Data on calcium and inorganic phosphorus in plasma

(see also table 6 of report 00001184)

5 pages

TRAITEMENTS	Taux de P plasmatique en mg/dl	Taux de Ca plasmatique en mg/dl	Taux de P plasmatique en mmol/L	Taux de Ca plasmatique en mmol/L
A	3.41	11.08	1.10	2.76
A	3.78	11.74	1.22	2.93
A	3.60	11.42	1.16	2.85
A	4.43	12.30	1.43	3.07
A	2.86	12.06	0.92	3.01
A	3.04	12.30	0.98	3.07
B	3.82	11.46	1.23	2.86
B	4.24	10.92	1.37	2.72
B	4.81	11.16	1.55	2.78
B	4.63	11.16	1.50	2.78
B	5.00	10.87	1.61	2.71
B	3.65	10.76	1.18	2.68
C	6.18	10.68	2.00	2.66
C	7.59	9.91	2.45	2.47
C	6.73	10.39	2.17	2.59
C	7.63	10.13	2.46	2.53
C	6.26	10.38	2.02	2.59
C	7.27	11.01	2.35	2.75
D	7.31	10.63	2.36	2.65
D	7.68	10.10	2.48	2.52
D	7.19	9.77	2.32	2.44
D	7.79	10.37	2.52	2.59
D	7.76	9.86	2.51	2.46
D	7.24	10.33	2.34	2.58
E	3.28	11.49	1.06	2.87
E	3.57	11.33	1.15	2.83
E	3.34	10.81	1.08	2.70
E	3.98	11.47	1.29	2.86
E	4.21	11.57	1.36	2.89
E	4.35	11.27	1.40	2.81
F	5.22	11.15	1.69	2.78
F	4.09	11.59	1.32	2.89
F	4.31	11.10	1.39	2.77
F	6.66	10.14	2.15	2.53
F	6.91	10.21	2.23	2.55
F	5.48	10.56	1.77	2.63
G	6.94	10.12	2.24	2.52
G	6.49	10.67	2.10	2.66
G	6.71	10.43	2.17	2.60
G	7.28	9.81	2.35	2.45
G	7.46	9.44	2.41	2.36
G	7.19	10.43	2.32	2.60
H	5.18	10.72	1.67	2.67
H	5.12	10.55	1.65	2.63
H	5.46	10.97	1.76	2.74
H	5.75	10.19	1.86	2.54
H	4.89	10.79	1.58	2.69
H	6.50	10.06	2.10	2.51

Calcium- Phosphore dans les plasma

BE-07/09

N° éch.	Phosphore mg/dl Moyenne	Calcium (mg/dl) Moyenne
1	0.98	13.36
2	3.94	10.45
3	2.65	10.73
4	6.08	9.77
5	3.31	12.86
6	5.86	10.41
7	3.60	12.91
8	2.36	10.80
9	2.62	13.03
10	3.08	12.13
11	6.43	9.98
12	2.27	10.57
13	4.80	13.14
14	3.22	12.12
15	4.41	12.74
16	5.28	11.19
17	2.10	10.86
18	3.24	12.56
19	3.67	11.39
20	2.45	13.45
21	1.99	12.38
22	4.13	11.20
23	2.98	12.67
24	3.06	12.95
25	3.85	12.10
26	2.70	12.60
27	3.08	10.73
28	5.64	10.41
29	5.83	10.78
30	3.28	11.74
31	5.59	9.81
32	2.27	11.36
33	4.23	11.62
34	4.06	10.92
35	2.99	11.64
36	7.95	10.47
37	3.93	11.53
38	5.56	10.82
39	4.22	10.51
40	4.81	11.80
41	3.89	11.87
42	4.57	10.43
43	6.77	10.71
44	4.77	10.49
45	4.28	10.91
46	2.38	11.19
47	4.13	10.44
48	3.83	10.49

49	8.27	9.94
50	6.17	10.94
51	3.59	11.33
52	6.69	10.49
53	7.42	10.52
54	8.14	9.83
55	7.43	9.07
56	7.36	10.22
57	7.65	10.67
58	5.21	10.51
59	6.46	10.89
60	7.59	9.48
61	8.02	10.03
62	7.31	10.01
63	7.12	10.27
64	8.08	10.20
65	7.62	10.35
66	7.23	10.11
67	5.28	10.61
68	4.91	10.45
69	8.71	10.79
70	5.67	11.10
71	6.89	11.14
72	7.80	10.99
73	7.46	10.94
74	6.32	10.95
75	8.57	9.80
76	6.90	10.84
77	7.91	9.79
78	7.74	9.63
79	7.45	10.98
80	7.63	10.01
81	8.18	10.26
82	6.83	9.70
83	6.83	9.29
84	6.92	9.82
85	7.66	10.02
86	8.02	10.93
87	8.50	10.85
88	7.00	9.69
89	7.57	10.52
90	8.37	9.80
91	7.63	9.92
92	7.48	9.21
93	7.61	10.10
94	6.38	11.23
95	7.71	9.52
96	7.26	10.50
97	2.69	11.66
98	3.97	11.07
99	3.94	11.20
100	2.53	12.04

101	4.20	11.66
102	3.62	12.20
103	3.64	10.66
104	2.83	10.82
105	2.82	10.97
106	3.48	10.55
107	2.72	11.05
108	4.34	10.68
109	4.22	11.53
110	5.72	11.35
111	3.08	11.53
112	2.92	11.47
113	2.94	11.44
114	2.96	11.00
115	5.47	11.12
116	5.46	12.75
117	6.15	11.79
118	2.70	10.85
119	4.53	11.33
120	4.01	11.11
121	7.23	9.86
122	3.20	11.00
123	4.36	12.00
124	6.10	11.73
125	3.95	12.45
126	3.56	11.81
127	4.35	11.90
128	4.50	10.21
129	5.75	10.88
130	2.97	11.16
131	4.83	11.26
132	3.70	11.11
133	7.64	9.88
134	5.26	9.93
135	6.16	11.14
136	7.58	9.63
137	7.36	9.56
138	8.06	10.26
139	5.67	11.30
140	6.57	9.71
141	5.03	10.30
142	4.70	10.88
143	4.24	10.49
144	7.97	10.57
145	5.76	9.05
146	6.71	9.42
147	7.19	11.13
148	8.09	10.90
149	5.75	10.26
150	5.92	10.98
151	6.56	10.65
152	7.72	10.80

153	5.24	11.73
154	7.74	10.61
155	5.85	9.76
156	8.01	9.64
157	7.19	9.71
158	7.32	9.80
159	7.31	9.65
160	7.31	10.06
161	7.62	8.51
162	7.09	9.99
163	7.93	9.58
164	7.20	9.70
165	7.01	10.90
166	6.97	10.57
167	7.01	10.55
168	7.77	9.70
169	5.49	10.06
170	3.66	11.41
171	7.06	9.53
172	4.51	11.90
173	5.82	10.92
174	4.28	10.93
175	5.46	9.77
176	4.91	10.57
177	5.56	11.08
178	3.51	10.72
179	5.51	10.85
180	7.25	11.23
181	4.79	11.10
182	6.81	9.63
183	6.56	9.21
184	4.83	10.82
185	4.08	10.92
186	6.18	9.70
187	5.57	11.52
188	3.74	11.02
189	5.93	9.73
190	7.18	9.04
191	5.28	11.08
192	7.64	10.42

2.5 Data on tibia strength and tibia/toe ash

(see also table 7 of report 00001184)

TRAITEMENTS	Résistance osseuse en N	Taux de cendres en %	Taux de cendres en %_orteils
A	67.34	36.18	18.02
A	81.20	37.24	18.20
A	73.10	34.30	23.57
A	73.93	36.69	19.36
A	87.20	40.41	22.43
A	60.80	38.23	21.48
B	165.90	48.30	34.86
B	155.10	45.66	32.21
B	135.50	45.49	27.90
B	136.80	47.62	34.04
B	177.90	46.21	30.49
B	161.70	46.30	33.86
C	177.80	49.29	37.20
C	186.50	48.95	29.16
C	256.20	49.98	31.88
C	200.60	50.50	34.72
C	171.70	49.90	35.77
C	189.00	51.08	27.56
D	216.70	50.92	31.98
D	195.80	49.66	42.67
D	266.10	50.99	35.11
D	236.10	50.91	34.56
D	219.80	51.64	33.79
D	191.50	50.50	31.94
E	149.00	45.04	32.31
E	164.90	43.45	34.49
E	137.50	43.37	33.26
E	191.10	48.36	31.83
E	166.60	48.14	36.00
E	167.30	45.54	32.39
F	149.00	48.34	31.04
F	199.30	46.89	27.18
F	154.70	48.15	30.95
F	157.50	50.68	37.86
F	251.40	49.02	37.50
F	209.80	48.88	37.77
G	227.60	49.86	37.23
G	201.30	50.05	40.20
G	204.90	50.21	32.55
G	174.40	51.39	34.37
G	250.00	50.94	41.59
G	226.30	50.39	37.87
H	191.40	48.93	35.29
H	156.00	49.95	37.74
H	172.30	46.95	33.29
H	210.10	48.50	31.79
H	160.10	46.19	27.98
H	138.80	49.16	32.55

III. Trial Protocol Data Sheet

Trial Protocol Data Sheet

According to EFSA Journal (2008) 778, 5-13 Technical guidance: Tolerance and efficacy studies in target animals
Data sheet to be filled out by the applicant and signed by the study director and then added to each trial report concerning safety and efficacy of the additive for the target animal

For terrestrial animals

Identification of the additive: IPA phytase (CT) and (L) Batch number: PPQ 29773 and PPQ 28432 Trial ID: BE-07/09 Location: Research Center for Animal Nutrition (DSM Nutritional Products France, F-68128 Village-Neuf) Start date and exact duration of the study:April-7-2009 to April-29-2009, 2 weeks(1 week pre-trial period)
Number of treatment groups (+ control(s)): 8 (+2) Replicates per group: 12 Total number of animals: 768 Animals per replicate: 8
Dose(s) of the additive/active substance(s)/agent(s) (mg/Units of activity/CFU kg^{-1} complete feed/L^{-1} water) Intended:(CT) & (L):0/500/1000/2000 $U.kg^{-1}$ Analysed: -/531/1445/1900 and -/500/983/2170 $U.kg^{-1}$ Substances used for comparative purposes: Intended dose: Analysed:
Animal species/category: Broiler Breed: Ross PM3 Identification procedure: per cage number Sex: Males Age at start:8 days Body weight at start: 173 g Physiological stage: Growing General health: normal (P-deficient basal diet)
Additional Information for field trials: Location and size of herd or flock: Feeding and rearing conditions: Method of feeding:
Diets (type(s)): low phosphorus basal diet Presentation of the diet: Mash ☐ Pellet ☒ Extruded ☐ Other Composition (main feedingstuffs): 59.1% Maize/ 36.8% SBM Nutrient content (relevant nutrients and energy content) Intended values: per kg: 12.7 MJ/ME, 215 g Crude protein, 4.1 g total P, 6.0 g Calcium Analysed values: per kg: 12.6 MJ/ME, 222 g Crude protein, 3.8 g total P, 5.6 g Calcium, 0.8 g Non Phytate P
Date and nature of the examinations performed: growth performance, apparent phosphorus utilization, bone quality, plasma Method(s) of statistical evaluation used: one-factorial analysis of variance (factor: treatment), Newman-Keuls test, non-linear regression analysis Therapeutic/preventive treatments (reason, timing, kind, duration): nothing to report Timing and prevalence of any undesirable consequences of treatment: nothing to report

Date	Signature Study Director
09-October-2009	

efsa

European Food Safety Authority

FEEDAP UNIT

ANNEX I[1]

TRIAL PROTOCOL DATA SHEET: FOR TERRESTRIAL ANIMALS

Identification of the additive: **IPA phytase (CT) and (L)**	Batch number: **PPQ 29773 and PPQ 28432**
Trial ID: **BE-07/09**	Location: **DSM Nutrtional Products France; Research Centre for Animal Nutrion & Health, F-68128 Village-Neuf**
Start date and exact duration of the study: **April-7-2009 to April-29-2009, 2 weeks(1 week pre-trial period)**	
Number of treatment groups (+ control(s)): **6 (+2)**	Replicates per group: **12**
Total number of animals: **768**	Animals per replicate: **8**
Dose(s) of the additive/active substance(s)/agent(s) (mg/Units of activity/CFU kg^{-1} complete feed/L^{-1} water)	
Intended: **0/500/1000/2000 U.kg-1**	Analysed: : **-/531/1445/1900 and -/500/983/2170 U.kg-1**
Substances used for comparative purposes:	
Intended dose:	Analysed:
Animal species/category: **Broiler**	
Breed: **ROSS PM3**	Identification procedure: **per cage number**
Sex: **Males** Age at start: **8 days**	Body weight at start: **173 g**
Physiological stage: **Growing**	General health: **Normal (P-deficient basal diet)**
Additional information for field trials:	
Location and size of herd or flock:	
Feeding and rearing conditions:	
Method of feeding:	
Diets (type(s)): **low phosphorus basal diet**	
Presentation of the diet: Mash ☐ Pellet ☒ Extruded ☐ Other	
Composition (main feedingstuffs): **): 59.1% Maize/ 36.8% SBM**	
Nutrient content (relevant nutrients and energy content)	
Intended values: **per kg: 12.7 MJ/ME, 215 g Crude protein, 4.1 g total P, 6.0 g Calcium**	
Analysed values: **per kg: 12.6 MJ/ME, 222 g Crude protein, 3.8 g total P, 5.6 g Calcium, 0.8 g Non Phytate P**	
Date and nature of the examinations performed: **Growth performance, app. P utilisation, bone quality, plasma**	
Method(s) of statistical evaluation used: **one-factoral analysis of variance (factor: treatment), Newman-Keuls-test, non-linear regression analysis**	
Therapeutic/preventive treatments (reason, timing, kind, duration): **Nothing to report**	
Timing and prevalence of any undesirable consequences of treatment: **Nothing to report**	

[1] Please submit this form using a common word processing format (e.g. MS Word).

FEEDAP UNIT

Date **18-Feb-2010**	Signature Study Director
	Petra Pleijgt

† In case the concentration of the additive in complete feed/water may reflect insufficient accuracy, the dose of the additive can be given per animal day^{-1} or mg kg^{-1} body weight or as concentration in complementary feed.

TAB

ANNEX 25

Annex 25

Francesch, M. et *al.* (2009). Report No. 00000960: Dose response and tolerance study with IPA Mash phytase (RONOZYME® HiPhos) in laying hens fed a maize-based diet. 2009

REPORT No. 00000960
Regulatory Document

DSM

Document Date: 22 June, 2009

Author(s): M. Francesch[1] and JBroz [2]

[1] Department of Animal Nutrition, IRTA, Centre Mas de Bover, Constanti (Spain)
[2] Animal Nutrition and Health R&D, DSM Nutritional Products Ltd, Basel

Title: **Dose response and tolerance study with IPA phytase in laying hens fed a maize-based diet**

Project No. 6106

Summary

An experiment was conducted to evaluate the dose related effects of IPA phytase (M) on performance, tibia characteristics, apparent ileal phosphorus digestibility and P excretion in laying hens fed a maize-soybean meal based diet low in non-phytate P. Furthermore, the tolerance of hens to IPA phytase when administered at 10 times the maximum recommended dose during 8 weeks was determined. A total of 288 brown hens (Hy-Line strain) were used and allocated into 96 cages, with 3 hens per cage. A negative control diet containing 0.1% non-phytate P was supplemented with IPA phytase at 0, 500, 1000, 2000, 4000 and 40,000 U/kg, respectively. Each dietary treatment was assigned to 16 replicates. Experimental feeds were provided from 52 to 59 weeks of age and performance parameters such as body weight, egg production, egg weight, feed intake and conversion and mortality were recorded. P excretion was measured after 7 weeks of feeding experimental diets and ileal P digestibility and tibia P percentage and strength were determined at the end of study. Finally, blood samples from 1 bird per cage from treatments fed 0, 4000 and 40,000 U/kg were taken for heamatology and blood biochemistry measurements. Performance parameters were not affected by phytase supplementation during 8 weeks of trial period. The apparent ileal P digestibility responded to IPA phytase supplementation in a linear manner up to 4000 U/kg diet (P<0.0001) and was improved from 36.7% in the negative control to 57.7% in treatment receiving 4000 U/kg. Further increase of phytase dosage from 4000 to 40,000 U/kg increased P digestibility to 74.5% (P<0.01). The P concentration in excreta was reduced linearly (P<0.01) with the increasing phytase dose. No adverse effects of IPA phytase overdose (40,000 U/kg) on performance, mortality, as well as on all examined heamatological and blood biochemical parameters were observed. However, the concentration of inorganic P in blood serum was significantly elevated due to phytase addition (P<0.01).

This report consists of Pages I – II and 1 - 43

Distribution

Ms. R. Aureli, NRD/CA
Dr. M. Eggersdorfer, NRD
Dr. F. Fru, NRD/PA
Mr. J-F. Hocquet, NBD/RG
Dr. A.-M. Künter, NRD/CA
Dr. JPheiffer, NRD/PA
Dr. P. Philipps, NRD/CA
Mr. J-P. Ruckebusch, ANH

Approved

Name	Signature	Date
Main Author	signed by	
Dr. JBroz, NRD/CA	JBroz	23.06.2009
Principal Scientist / Competence Mgr	signed by	
Dr. JBroz, NRD/CA	JBroz	23.06.2009
Research Center Head	signed by	
Dr. A.-M. Künter, NRD/CA	A.-M. Künter	25.06.2009
Project Manager	signed by	
Dr. F. Fru, NRD/PA	F. Fru	23.06.2009

Nomenclature and Structural Formula

IPA phytase (M), enzyme product containing bacterial 6-phytase (EC 3.1.3.26), produced by a submerged fermentation of a genetically modified *Aspergillus oryzae* strain. Lot PPQ28656 was used in this study, manufactured by Novozymes A/S, Bagsvaerd, Denmark.

IRTA
RESEARCH & TECHNOLOGY
FOOD & AGRICULTURE

FINAL REPORT OF THE CONTRACT SIGNED WITH:

Company: DSM Nutritional Products

Title: Dose response and tolerance study with IPA phytase in laying hens fed a maize-based diet

Experiment number: G-129

Contract Code:	2 2 5 4 1
Organic Code:	0 6 0 2

Author: Dr. Maria Francesch

Center: IRTA - RECERCA I TECNOLOGIA AGROALIMENTÀRIES
Monogastric Nutrition
Mas de Bover
Ctra. Reus-El Morell km. 3.8, E-43120 Constantí (Tarragona), Spain
Tel. 977 32 84 24 Fax 977 34 40 55

Number of pages: 43

Date: 19/06/2009

TABLE OF CONTENTS

SUMMARY

An experiment was conducted to evaluate the dose related effects of dietary **IPA phytase** (6-phytase expressed in *Aspergillus oryzae*) on performance, tibia ash and phosphorus (P) content and strength, apparent ileal P digestibility and P excretion, in laying hens fed a maize-soybean based diet, low in non-phytate phosphorus (NPP). Furthermore, the tolerance of laying hens to IPA phytase, when administered at 10 times the maximum recommended dose during 56 days, was determined. A total of 288 Hy-Line brown laying hens 52 weeks old were used, allocated into 96 cages at 3 laying hens per cage, with a total of 16 replicates per dietary treatment. A negative control diet, low in NPP (0.1%), was supplemented with phytase at 0, 500, 1000, 2000, 4000 and 40000 U/kg feed. Experimental feeds were provided from 52 to 59 weeks of age and performance parameters, such as weight gain, egg production, egg weight, feed consumption, and percentage of dirty, faulty and broken eggs were recorded. Phosphorus excretion was measured after seven weeks of feeding experimental diets and ileal P digestibility and tibia ash and P concentrations and strength at week 59. Blood samples from one bird per cage from treatments fed 0, 4000 and 40000 phytase U/kg were taken for haematological and biochemical measurements. Data were subjected to a linear and quadratic regression analysis to evaluate the enzyme dose response, including only the levels up to 4000 phytase U/kg of feed. A linear contrast was used to compare the maximum recommended vs. tolerance dose. No significant differences among treatments were observed for rate of lay, egg mass production, egg weight, feed intake, feed conversion, percentage of broken, faulty or dirty eggs and eggshell strength ($P > 0.1$). All hens lost weight over the 8 weeks of the experiment, but there was no significant effect of treatment. Tibia ash and P concentrations and bone strength were not significantly affected by phytase supplementation. The apparent ileal P digestibility responded to phytase supplementation in a linear manner up to 4000 phytase U/kg ($P<0.0001$), from 36.7% in the negative control to 57.7% with 4000 phytase U/kg of feed. Further increase of phytase dosage from 4000 to 40000 U/kg increased P digestibility from 57.7% to 74.5% ($P<0.01$). The P excretion was reduced linearly ($P <0.01$) with the increase of phytase dose. Further increase of phytase dosage from 4000 U/kg to 40000 U/kg reduced excreta P content from 0.74% to 0.66% ($P < 0.05$). No significant changes related to phytase addition at 4000 U/kg or 40000 U/kg feed (ten-fold maximum recommended dose) were detected in haematological and biochemical blood characteristics, with the exception of serum phosphorus concentration ($P<0.01$). Phytase increased significantly phosphorus concentration ($P <0.05$) and there was not difference between the supplementation of 4000 or 40000 phytase U/Kg. Results of this experiment suggest that IPA phytase supplementation was efficacious in increasing apparent ileal P digestibility and in reducing phosphorus excretion of laying hens, fed a maize-soybean based diet, low in non-phytate phosphorus. The response to phytase supplementation was linear. No adverse effects of the use of 40000 U/kg level of phytase on performance, mortality or haematological-biochemical characteristics were observed, with respect to the maximum recommended level (4000 U/kg). The response beyond the phytase level of 4000 U/kg feed was in many cases considerable.

RESPONSIBILITIES

Study director

Dr. Maria Francesch
IRTA - **Monogastric Nutrition**
Ctra. Reus-El Morell, km. 3.8, E-43120 Constantí (Tarragona)
Tel: +34 977 32 84 24 Fax: +34 977 34 40 55
E.mail: maria.francesch@irta.cat

Study monitor

Dr Jiri Broz
DSM Nutritional Products
P.O. Box 2676
CH-4002 Basel, Switzerland
Tel. +41 61 815 87 35
E-mail: jiri.broz@dsm.com

Daily monitor

Ramon Salvadó
IRTA - **Monogastric Nutrition**
Ctra. Reus-El Morell, km. 3.8, E-43120 Constantí (Tarragona)

Stockworkers

Alfonso Mejias and Joan Martí
IRTA - **Monogastric Nutrition**
Ctra. Reus-El Morell, km. 3.8, E-43120 Constantí (Tarragona)

Feed preparation

Lluís Padrell and Andreu Vilalta
IRTA - **Monogastric Nutrition**
Ctra. Reus-El Morell, km. 3.8, E-43120 Constantí (Tarragona)

Laboratory analysis

Dr. Anna Pérez
IRTA - **Monogastric Nutrition**
Ctra. Reus-El Morell, km. 3.8, E-43120 Constantí (Tarragona)

OBJECTIVES

1. To evaluate the dose related effects of dietary administration of IPA phytase on performance, tibia ash and P contents and strength, and apparent ileal P digestibility and P excretion, in laying hens fed a maize-soybean based diet, low in non-phytate phosphorus.
2. To evaluate the tolerance of laying hens to IPA phytase, when administered at 10 times the maximum recommended dose during eight weeks.

METHODOLOGY

Site of the experiment

The trial was carried out in the laying hen unit, at the Mas de Bover Center, Ctra. Reus-El Morell, km. 3.8, E-43120 Constantí (Tarragona, Spain).

Duration of the experiment

The experiment started on the 28-10-08 and it lasted 56 days of laying period.

Test product

- Name of the product tested: **IPA Phytase (M)**
- Active ingredient: 6-phytase expressed in *Aspergillus oryzae*, 57085 U/g product (analyzed)
- Lot number: PPQ 28656
- Manufactured by: Novozymes A/S, Denmark
- Supplied by: DSM Nutritional Products Ltd, Basel, Switzerland
- Level of inclusion in the diet: 0, 500, 1000, 2000, 4000 and 40000 phytase U/kg feed, corresponding to 8.8, 17.6, 35.2, 70.4 and 704.0 mg/kg of IPA phytase, respectively.

Animals and housing

A total of two hundred and eighty eight brown hens of the Hy-Line strain were used. Hens were 52 weeks old at the beginning of the experiment and they were allocated into 96 cages, at 3 laying hens per cage. The health status of the hens was good and the average rate of lay of the flock, recorded during the week before to start the experiment, was 82% (± 9.5%).

The experimental room was a windowless room provided with programmable light and ventilation by extraction. The lighting programme was 16 h light per day during the experiment.

Feeding program

Laying hens were fed a single negative control experimental diet, based on maize-soybean meal, low in NPP (0.10%) and without inorganic P supplementation. The ingredient and the calculated nutrient composition of the experimental diet are shown in Table 1.

The premix and experimental feeds were manufactured at the Feed Mill of IRTA. All feed ingredients, except fat, salt, dicalcium phosphate, calcium carbonate, vitamin and mineral premix were ground through a 25 CV hammer mill until the particles passed through a 6 mm sieve. The mixer was a 1000 L capacity horizontal mixer, and the mixing time was 5 min. Phytase preparation was added on top of the mix, premixed with an aliquot of 100 g, then 1 kg and 10 kg of the mix in a small mixer.

Feed and water were provided *ad-libitum* throughout the experiment and diets were supplied in mash form.

The feed used in this trial did not contain any antibiotic growth promoter, nor any other feed additive or NSP enzymes.

Two kg of sample from each batch of feed and treatment were taken and divided into three sets. One set of samples (500 g each) was dispatched to IRTA Laboratory, another set of samples (1000 g) was sent to Biopract GmbH (Berlin) and the last one was kept in the fridge.

Treatments and experimental design

There were six experimental treatments replicated 16 times each and allocated at random by blocks. Each replicate consisted of 3 laying hens.

The arrangement of treatments was:

Treatment	Name	Phytase U/kg	IPA Phytase (M) mg/kg[(1)]
T-1	Negative control	-	-
T-2	IPA Phytase	500	8.8
T-3	IPA Phytase	1000	17.6
T-4	IPA Phytase	2000	35.2
T-5	IPA Phytase	4000	70.4
T-6	IPA Phytase	40000	704

[(1)]Calculated on the basis of current phytase activity in the product (57 085 U/g).

Controls

Chemical analyses

Quality control of the manufactured feed was performed by determining: dry matter (AOAC, 2000, method 925.09), crude protein (Dumas procedure by means of a Nitrogen/protein analyzer FP-528 LECO, AOAC 2000, method 968.06), ether extract (Buchi Extraction System B-811, AOAC 2000, method 920.39), chloride (AOAC 2000, method 969.10) and total P (AOAC, 2000). Finally, phytase concentration in feeds was measured using the method of Engelen et al. (1994). One unit of phytase (U) is the activity that releases 1 μml phosphate (PO_4^{-3}) from phytate per minute at pH 5.5 and 37°C. One set of samples was also sent to Biopract GmbH, Berlin, for phytase analysis.

Performance

Body weight was recorded at the beginning and at the end of the trial. Total egg production, egg weight and percentage of dirty, faulty (shell less and misshape) and broken eggs were recorded in relation to the total egg production every two days. Feed consumption was recorded every four weeks. Mortality, abnormal clinical signs and causes of mortality were monitored daily.

Eggshell quality

Eggshell strength was measured every four weeks over a total of 32 eggs from each treatment, by means of an egg force reader (Orka Food Technology, Israel).

Bone ash and strength

At the end of the experiment, the bone mineralization was assessed by measuring tibia ash and P percentage and tibia breaking strength in 16 birds per treatment (one bird per cage). To determine the percentage of tibia ash, the right leg was removed as drumstick and autoclaved, following the procedure described by Hall et al. (2003). Bones were dried for 24 h at 100 °C, weighed and dry-ashed for 24 h in a muffle furnace at 600 °C. Ash weight was expressed as a proportion of dry bone weight. Following this, total P (analyzed colorimetrically by the vanadomolybdate method, AOAC, 2000) was measured in ash. In the left leg, tibia breaking strength was measured in a MTS Alliance RT/5 material testing system.

Ileal balance study

Experimental diets containing 5 g/kg of titanium dioxide as a tracer were supplied during the last week of the experiment. Following this, all laying hens were sacrificed by cervical dislocation (according to the procedure num. 3884 approved by

the Ethical Commission of IRTA). Ileal chyme samples from the last 25 cm preceding the ileo-cecal junction were removed and pooled per cage.

In feeds and ileal samples, titanium dioxide and total P were measured. The apparent ileal P digestibility was calculated by the relation:

$$\% \text{ Digestibility} = (1 - [(Ti_{feed} / Ti_{digesta}) \times (P_{digesta} / P_{feed})]) \times 100$$

Where: Ti_{feed} = marker concentration in feed; $Ti_{digesta}$ = marker concentration in digesta; $P_{digesta}$ = phosphorus concentration in digesta; P_{feed} = phosphorus concentration in feed.

Titanium dioxide concentration was analysed according to Short et al. (1996). Total P concentration was determined colorimetrically using the vanadomolybdate procedure (AOAC, 2000).

Phosphorus excretion

Excreta from each cage was collected and dried to measure total phosphorus content (AOAC, 2000), after seven weeks of feeding experimental diets.

Blood examinations

Before sacrificing layers for ileal contents sampling and bone mineralization measurements, sixteen laying hens per treatment (one hen each block) from T-1 (negative control), T-5 (4000 phytase U/kg - maximum recommended dose) and T-6 (40000 phytase U/kg - tolerance dose) treatments, were bled by cardiac puncture, according to the experimental procedure num. 1563, approved by the Ethical Commission of IRTA. Haematology measurements were done on blood samples, including erythrocytes count, haematocrit, haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). In addition glucose, alkaline phosphatase, aspartate amino transferase (GOT) alanine aminotransferase (GPT), gamma glutamin transpeptidase (GGT), uric acid, albumin, total proteins, calcium and P serum concentrations were determined. These measurements were performed in the Laboratori Domingo, Laboratori de Análisis Clínicos S.L., Pau Casals 11, baixos, 43003 Tarragona (Spain).

Statistical analysis

Data were analysed as a randomised complete block design with a two-way analysis of the variance (16 blocks and 6 treatments) using the General Linear Model (GLM) procedure of SAS. Treatment means were compared by using Duncan's multiple range test. Moreover, data were subjected to a linear and quadratic regression analysis to evaluate the enzyme dose response, including only the levels up to 4000 phytase U/kg of feed. A linear contrast was used to compare the maximum recommended

dose and the tolerance dose of phytase (4000 vs. 40000 phytase U/kg). Angular transformation was used to analyze data expressed as percentages. Statements of significance were based on a probability of $P \leq 0.05$ and a significant trend at $P < 0.1$.

RESULTS AND DISCUSSION

The analytical composition of experimental feeds used throughout the experiment is presented in Table 2. Within batches, results showed a good uniformity in the analytical composition among the experimental diets. Crude protein measurements were higher than the expected (19.8 % on average vs. 17 %), but they were consistently higher across the diets. This might be due to higher protein content in the ingredients used. Analysed crude fat and total P content in feeds were nearby the expected values (4.1 % of crude fat and 0.32 % of total phosphorus).

In-feed determination of IPA phytase equivalent, expressed as U/kg feed is shown in Table 3. In overall, phytase values measured in both laboratories were quite similar and slightly higher than the expected ones up to 4000 U/kg. At 40000 U/Kg, IRTA value was 14 % lower and Biopract value 16 % higher than the expected level, but the accuracy of the methods is lower at high doses of phytase. These recoveries were within acceptable limits, taking account the relative standard deviation of the analysis and the errors introduced by enzyme application and sampling.

The effects of phytase on performance from 52 to 59 weeks are shown in Table 4. No significant differences among treatments were observed in rate of lay, egg mass production, egg weight, feed intake, feed conversion and percentage of broken, faulty or dirty eggs ($P > 0.1$). This lack of significant response to phytase supplementation to a low NPP diet on performance might suggest that eight weeks of P depletion might not be enough to show signs of P deficiency when layers are older than 52 weeks. Although differences did not reach significance, it was noticed a numerically higher rate of lay, egg mass production and feed intake, and numerically better feed conversion in hens fed higher doses of phytase, both 4000 and 40000 U/kg.

No birds died during the experiment.

Body weights at the beginning and at the end of the experiment and weight gain are shown in Table 5. There were no significant differences among treatments in body weight at the beginning or at the end of the experiment ($P > 0.1$). All hens lost weight over the 8 weeks of the experiment, but there were no significant effect of treatment ($P > 0.1$).

Egg shell strength was measured at the end of week 4 and 8 (Table 6). No significant differences between treatments were observed ($P > 0.1$).

Tibia ash and P concentrations (Table 7) were not significantly affected by phytase supplementation ($P > 0.1$). Bone strength measurements (breaking force and bone

strength, Table 8) were not significantly affected by phytase supplementation (P > 0.1), however bone strength tended to be higher with phytase supplementation. There were no significant differences for bone strength values between the use of 4000 or 40000 phytase units (P>0.1).

Results of ileal phosphorus digestibility are presented in Table 9. The apparent availability of P responded to phytase supplementation in a linear manner up to 4000 phytase U/kg (P<0.0001), from 36.7% in the negative control to 57.7% with 4000 phytase U/kg of feed (Figure 1). Further increase of phytase from 4000 U/kg to 40000 U/kg (tolerance dose) increased ileal P digestibility from 57.7% to 74.5% (P<0.0001).

The P concentration in dry excreta (Table 10) was significantly affected by phytase supplementation. The P excretion was reduced linearly (P <0.01) with the increase of phytase dose. Further increase of phytase from 4000 U/kg to 40000 U/kg (tolerance dose) reduced P excretion from 0.74% to 0.66% (P < 0.05).

No significant changes related to phytase addition at 4000 U/kg (maximum recommended dose) or 40000 U/kg feed (ten-fold maximum recommended dose) were detected in haematological and biochemical blood parameters (Table 11), when comparing with the negative control, with the exception of serum phosphorus concentration (P<0.01). Phytase increased significantly serum phosphorus concentration (P <0.05) and there was not difference between the supplementation of 4000 or 40000 phytase U/Kg.

CONCLUSIONS

Results of this experiment indicated that **IPA phytase** supplementation was efficacious in increasing apparent ileal P digestibility and in reducing P excretion in laying hens, fed a maize-soybean based diet low in non-phytate phosphorus. The response to phytase supplementation was linear.

No adverse effects of the use of 40000 U/kg level of phytase on performance, mortality or haematological-biochemical characteristics were observed, with respect to the maximum recommended level (4000 U/kg). The response beyond the 4000 U/kg of phytase level was in many cases considerable.

REFERENCES

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS (2000) Official methods of analysis. 17th ed., Association of Official Analytical Chemist. Washington D. C.

HALL, L. E., R. B. SHIRLEY, R. I. BAKALLI, S. E. AGGREY, G. M. PESTI, and H. M. EDWARDS, Jr. 2003. Power of two methods for the estimation of bone ash of broilers. Poult. Sci. 82:414-418.

ENGELEN, A.J., VAN DER HEEFT, F.C., RANDSDORP, P.H.G., & SMIT, E.L.C. (1994) Simple and rapid determination of phytase activity. Journal of AOAC, 77: 760-764.

SHORT, F. J., GORTON, P., WISEMAN, J. & BOORMAN, K. N. (1996) Determination of titanium dioxide added as an inert marker in chicken digestibility studies. Animal Feed Science and Technology, 59:215-221.

Signatures:

Dr. Maria Francesch Study researcher	Dr. Enric Esteve Director **Monogastric Nutrition**	Dr. Joaquim Brufau Director Mas de Bover
Date: 19/6/09	Date: 19/6/09	Date: 19/06/09

APENDIX I. TABLES AND FIGURES

Table 1. Composition of experimental diet

Ingredients	(%)
Maize	53.3
Soybean meal (48% CP)	22.9
Cassava	8.0
Sunflower meal (37% CP)	4.0
Soybean oil	1.7
DL-Methionine	0.130
L-lysine HCl	0.013
Calcium carbonate	9.123
Dicalcium phosphate	-
Salt	0.400
Carophyll red	0.003
Carophyll yellow	0.002
Mineral & vitamin mix	0.400
Calculated nutrient composition:	
M.E. (kcal/kg)	2700
Crude protein (%)	17.0
Crude fat (%)	4.1
Crude fibre (%)	3.3
Lysine (%)	0.850
Methionine (%)	0.400
Met + Cys (%)	0.687
Threonine (%)	0.626
Tryptophan (%)	0.182
Calcium (%)	3.60
Total phosphorus	0.33
Non-phytate phosphorus (%)	0.10

[1]One kg of feed contains: Vitamin A: 8000 UI; Vitamin D_3: 1600 UI; Vitamin E: 20 mg; Vitamin K_3: 2 mg; Vitamin B_1: 1.5 mg; Vitamin B_2: 4 mg; Vitamin B_6: 3 mg; Vitamin B_{12}: 11 μg; Folic acid: 0.35 mg; Biotin: 150 μg; Calcium pantothenate: 10 mg; Nicotinic acid: 20 mg; Mn: 30 mg; Zn: 50 mg; I: 0.3 mg; Fe: 50 mg; Cu: 6 mg; Se: 0.1 mg; Ethoxyquin: 125 mg.

Table 2. Analytical composition of experimental diets (on air basis)

	Dry matter (%)	Crude protein (%)	Crude fat (%)	Chloride (%)	Total P (%)
T-1	89.4	19.3	4.0	0.47	0.33
T-2	89.5	19.2	4.3	0.48	0.31
T-3	89.3	20.1	4.4	0.47	0.31
T-4	89.5	20.1	4.2	0.46	0.31
T-5	89.4	19.9	3.9	0.43	0.32
T-6	89.7	19.9	3.8	0.41	0.32

Table 3. In-feed determination of IPA Phytase equivalent, expressed as U/kg

	Expected values (U/kg)	Enzyme recovery (U/kg)	
		IRTA laboratory	Biopract laboratory
T-1	0	-	55
T-2	500	563	635
T-3	1000	1008	1351
T-4	2000	2125	2098
T-5	4000	4107	4586
T-6	40000	34377	46400

Table 4. Effect of phytase on performance from 52 to 59 weeks

Treatment	Rate of lay (%)	Egg mass (g/day)	Egg weight (g)	Feed intake (g/day)	[1] Feed conversion	Broken eggs (%)	Faulty eggs (%)	Dirty eggs (%)
T-1 (0 U/kg)	85.7	55.4	64.6	107.2	1.946	0.68	0.83	1.52
T-2 (500 U/kg)	86.3	54.9	63.6	105.0	1.923	0.34	0.15	0.91
T-3 (1000 U/kg)	85.0	55.1	64.8	107.0	1.953	0.15	0.08	1.70
T-4 (2000 U/kg)	86.7	55.3	63.9	107.1	1.949	0.53	0.80	1.48
T-5 (4000 U/kg)	89.2	57.3	64.3	109.7	1.914	0.15	0.27	2.58
T-6 (40000 U/kg)	89.2	57.2	64.2	109.3	1.917	0.30	0.30	2.54
SE [2]	1.63	1.13	0.70	1.50	0.0348	0.143	0.305	0.475
P>F	0.322	0.457	0.833	0.283	0.938	0.152	0.315	0.182
4000 vs. 40000 U/kg	0.880	0.964	0.915	0.853	0.949	0.565	0.813	0.627
Linear [3]	0.114	0.152	0.913	0.090	0.599	0.182	0.860	0.090
Quadratic [3]	0.607	0.462	0.724	0.495	0.612	0.798	0.738	0.832

Values are means of sixteen replicates of 3 laying hens. [1] g feed/ g egg. [2] Pooled standard error. [3] Using treatments T-1 through T-5.

Table 5. Effect of phytase on body weight and body weight gain

Treatment	Phytase (U/kg)	Body weight Week 52 (g)	Body weight Week 59 (g)	Weight gain (g)
T-1	0	1838	1772	-65.8
T-2	500	1782	1743	-38.2
T-3	1000	1826	1784	-41.3
T-4	2000	1822	1788	-34.7
T-5	4000	1811	1766	-44.9
T-6	40000	1838	1796	-41.8
SE [1]		24.7	22.6	10.4
P>F		0.599	0.624	0.360
4000 vs. 40000 U/kg		0.437	0.343	0.832
Linear [2]		0.859	0.841	0.423
Quadratic [2]		0.961	0.426	0.082

Values are means of sixteen replicates of 3 laying hens. [1] Pooled standard error. [2] Using treatments T-1 through T-5.

Table 6. Effect of phytase on eggshell strength

Treatment	Phytase (U/kg)	Eggshell strength(Kg/cm^2)	
		Week 55	Week 59
T-1	0	3.652	3.340
T-2	500	3.802	3.680
T-3	1000	3.640	3.652
T-4	2000	3.708	3.523
T-5	4000	3.654	3.241
T-6	40000	3.623	3.697
SE [1]		0.1237	0.1443
P>F		0.917	0.119
4000 vs. 40000 U/kg		0.862	<0.05
Linear [2]		0.965	0.153
Quadratic [2]		0.410	0.203

Values are means of 48 eggs per treatment. [1] Pooled standard error. [2] Using treatments T-1 through T-5.

Table 7. Effect of phytase on tibia ash and phosphorus content

Treatment	Phytase (U/kg)	Ash (%)	Phosphorus (%DM)
T-1	0	46.7	7.52
T-2	500	46.9	7.64
T-3	1000	46.7	7.56
T-4	2000	46.1	7.48
T-5	4000	45.3	7.38
T-6	40000	46.6	7.60
SE [1]		0.85	0.142
P>F		0.803	0.827
4000 vs. 40000 U/kg		0.291	0.302
Linear [2]		0.130	0.199
Quadratic [2]		0.862	0.836

Values are means from sixteen birds per treatment. [1] Pooled standard error. [2] Using treatments T-1 through T-5.

Table 8. Effect of phytase on tibia breaking strength

Treatment	Phytase(U/kg)	Breaking force (Kg)	Bone strength (Kg/mm^2)
T-1	0	29.3	27.7
T-2	500	28.3	28.6
T-3	1000	29.9	30.7
T-4	2000	28.8	29.4
T-5	4000	27.0	28.8
T-6	40000	28.0	28.4
SE [1]		1.64	1.28
P>F		0.853	0.656
4000 vs. 40000 U/kg		0.659	0.831
Linear [2]		0.300	0.776
Quadratic [2]		0.583	0.234

Values are means of sixteen birds per treatment. Values within a column not sharing a common superscript are statistically different ($P<0.05$). [1]Pooled standard error. [2]Using treatments T-1 through T-5.

Table 9. Effect of phytase on apparent ileal phosphorus digestibility

Treatment	Phytase (U/kg)	(%)	(g/kg feed)
T-1	0	36.7 [d]	1.15 [d]
T-2	500	44.4 [c]	1.40 [c]
T-3	1000	44.6 [c]	1.40 [c]
T-4	2000	49.6 [c]	1.56 [c]
T-5	4000	57.7 [b]	1.82 [b]
T-6	40000	74.5 [a]	2.34 [a]
SE [(1)]		2.12	0.0667
P>F		<0.0001	<0.0001
4000 vs. 40 000 U/kg		<0.0001	<0.0001
Linear [(2)]		<0.0001	<0.0001
Quadratic [(2)]		0.346	0.342

Values are least square means of sixteen replicates per treatment and each replicate was a pool from three laying hens. [(1)] Pooled standard error. [(2)] Using treatments T-1 through T-5.

Figure 1. Effect of phytase on ileal phosphorus digestibility

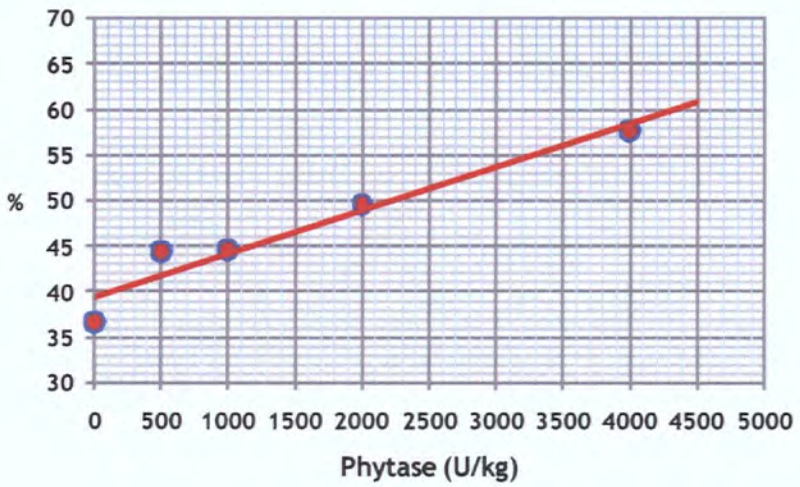


Table 10. Effect of phytase on excreta phosphorus content (on dry matter basis)

Treatment	Phytase (U/kg)	% Phosphorus [1]
T-1	0	0.86 [a]
T-2	500	0.81 [ab]
T-3	1000	0.77 [b]
T-4	2000	0.76 [b]
T-5	4000	0.74 [b]
T-6	40000	0.66 [c]
SE [1]		0.030
P>F		<0.001
4000 vs. 40000 U/kg		<0.05
Linear [2]		<0.01
Quadratic [2]		0.103

Values are means of sixteen samples per treatment. Values within a column not sharing a common superscript are statistically different (P<0.05). [1] Pooled standard error. [2] Using treatments T-1 through T-5.

Table 11. Tolerance test: haematological and biochemical characteristics

	T-1 -	T-5 4000 U/kg	T-6 40000 U/kg	SE[1]	Pr>F
Erythrocytes (10E12/L)	2.52	2.46	2.46	0.046	0.596
Haemoglobin (g/dL)	12.1	11.8	11.7	0.217	0.408
Haematocrit (%)	32.8	31.6	31.7	0.599	0.320
Mean corpuscular volume (fL)	130	129	129	0.7	0.220
Mean corpuscular haemoglobin (ρg)	48.2	48.0	47.6	0.39	0.538
Mean corpuscular haemoglobin concentration (g/dLl)	37.0	37.4	37.0	0.24	0.530
Glucose (mg/dL)	230	232	233	5.6	0.937
Urates (mg/dL)	6.34	6.32	6.39	0.275	0.982
Total protein (g/dL)	5.26	5.32	5.41	0.156	0.796
Albumin (g/dL)	1.71	1.71	1.74	0.045	0.815
Calcium (mg/dL)	28.6	30.1	30.4	1.04	0.442
Phosphorus (mg/dL)	4.63 [b]	6.13 [a]	6.37 [a]	0.332	**<0.01**
Aspartate aminotranferase (GOT) (U/L)	178	183	200	11.8	0.350
Alanine aminotranferase (GPT) (U/L)	1.60	3.13	1.81	0.59	0.142
Gamma glutamyltransferase (GGT) (U/L)	25.0	20.1	22.1	1.51	0.081
Alcaline phosphatase (U/L)	1144	991	864	190.1	0.571

Values are least-square means of blood samples from sixteen laying hens per treatment. Values within a row not sharing a common superscript are statistically different (P<0.05). [1] Pooled standard error.

APENDIX II. RAW DATA

Table 12. Performance raw data from 52 to 59 weeks

Pen	Trt	Block	Daily feed intake	Rate of lay	Broken eggs	Faulty eggs	Dirty eggs	Egg weight	FCR	Egg mass
1	1	1	111.84	94.55	0.00	0.00	0.00	66.90	1.768	63.25
2	2	1	106.31	94.55	0.00	0.00	0.61	59.61	1.886	56.36
3	3	1	104.70	86.67	0.00	0.61	1.21	68.00	1.777	58.93
4	4	1	106.92	87.88	0.00	0.00	2.42	65.62	1.854	57.66
5	5	1	115.33	92.73	0.00	0.00	0.61	65.60	1.896	60.83
6	6	1	102.54	96.36	0.61	0.00	1.82	61.96	1.717	59.71
7	1	2	105.87	92.12	1.21	0.00	0.00	61.12	1.880	56.31
8	3	2	105.81	90.30	0.00	0.00	1.21	60.17	1.948	54.33
9	4	2	106.40	91.52	0.61	0.61	0.61	60.64	1.917	55.50
10	6	2	112.54	86.67	0.00	0.00	1.21	66.26	1.960	57.42
11	5	2	104.71	90.30	0.00	0.00	0.00	60.96	1.902	55.05
12	2	2	102.45	75.76	0.61	0.00	0.61	65.58	2.062	49.68
13	3	3	102.93	86.06	0.00	0.00	2.42	65.27	1.832	56.18
14	4	3	98.25	90.91	0.61	0.00	0.00	61.49	1.758	55.90
15	5	3	109.19	82.42	0.00	0.00	0.61	70.52	1.878	58.13
16	6	3	100.14	92.12	0.00	0.00	0.00	67.56	1.609	62.24
17	1	3	108.81	90.91	1.21	0.00	3.03	65.77	1.820	59.79
18	2	3	99.87	91.52	1.21	0.00	3.03	64.65	1.688	59.16
19	2	4	105.57	93.33	0.00	0.00	0.00	62.53	1.809	58.36
20	1	4	103.63	87.27	0.00	0.61	0.00	66.80	1.778	58.30
21	3	4	108.46	85.45	0.00	0.00	2.42	66.57	1.907	56.88
22	5	4	120.29	92.12	0.61	0.00	5.45	66.10	1.976	60.89
23	4	4	109.34	85.45	0.00	0.00	0.00	62.77	2.038	53.64
24	6	4	108.31	72.73	0.61	0.00	2.42	63.78	2.335	46.39
25	2	5	107.24	88.48	0.61	1.21	1.82	64.23	1.887	56.83
26	1	5	107.78	93.33	0.00	0.00	0.61	62.25	1.855	58.10
27	6	5	108.29	92.12	0.00	0.00	2.42	60.01	1.959	55.28
28	5	5	100.53	89.70	0.00	0.61	5.45	60.62	1.849	54.37
29	3	5	113.71	83.64	0.61	0.00	0.00	66.00	2.060	55.20
30	4	5	104.52	90.30	1.21	0.00	1.82	63.84	1.813	57.65
31	1	6	96.45	76.36	0.00	0.00	0.00	65.06	1.941	49.68
32	3	6	107.35	73.33	0.00	0.61	3.03	65.92	2.221	48.34
33	5	6	111.43	89.70	0.61	1.21	0.61	63.00	1.972	56.51
34	2	6	102.06	83.64	0.00	0.00	1.21	62.09	1.965	51.93
35	4	6	111.05	89.70	0.61	0.00	3.03	62.79	1.972	56.32
36	6	6	111.40	90.91	0.61	0.61	1.21	63.06	1.943	57.33
37	1	7	104.43	88.48	0.00	0.61	1.82	64.17	1.839	56.78
38	2	7	99.83	82.42	0.00	0.00	0.00	65.48	1.850	53.97
39	3	7	105.64	81.82	0.00	0.00	1.21	67.92	1.901	55.57
40	5	7	110.80	90.30	0.61	0.00	0.00	59.36	2.067	53.60
41	6	7	108.88	89.70	0.00	0.00	5.45	68.42	1.774	61.37
42	4	7	117.03	80.00	1.82	7.27	0.00	61.04	2.396	48.84
43	1	8	97.18	78.18	0.00	0.61	0.00	62.23	1.997	48.66
44	3	8	88.07	64.24	0.00	0.00	1.21	58.12	2.359	37.34
45	2	8	105.59	76.36	0.61	0.61	2.42	68.12	2.030	52.02

Contract code: *2 2 5 4 1*

46	5	8	109.88	83.03	0.00	0.61	0.00	66.44	1.992	55.16
47	6	8	113.76	92.12	0.00	0.00	0.61	64.58	1.912	59.49
48	4	8	109.79	90.91	0.00	0.00	1.21	63.04	1.916	57.31
49	3	9	106.01	87.27	0.61	0.00	0.61	65.83	1.845	57.45
50	5	9	99.01	84.85	0.00	0.61	3.03	63.72	1.831	54.06
51	2	9	115.35	87.27	0.00	0.00	0.00	69.31	1.907	60.48
52	1	9	109.53	83.64	2.42	1.21	1.82	64.30	2.037	53.78
53	6	9	112.10	88.48	0.00	0.00	0.00	65.27	1.941	57.75
54	4	9	103.32	81.21	0.00	0.00	1.21	64.28	1.979	52.20
55	1	10	114.16	80.00	1.21	0.00	2.42	71.55	1.995	57.24
56	2	10	102.95	86.06	0.61	0.00	1.82	60.85	1.966	52.37
57	3	10	111.77	91.52	0.61	0.00	4.24	66.30	1.842	60.68
58	5	10	116.32	95.15	0.00	0.00	12.12	63.55	1.924	60.47
59	4	10	99.62	71.52	0.61	2.42	0.61	66.50	2.095	47.56
60	6	10	107.59	83.03	2.42	2.42	8.48	63.70	2.034	52.89
61	2	11	110.44	93.94	0.00	0.00	0.00	63.72	1.845	59.86
62	1	11	119.59	88.48	0.00	0.00	0.00	63.18	2.139	55.90
63	4	11	116.28	90.91	1.21	0.61	5.45	64.57	1.981	58.70
64	5	11	113.83	90.91	0.00	0.00	4.85	66.97	1.870	60.88
65	6	11	104.10	93.33	0.00	0.00	6.06	60.86	1.833	56.80
66	3	11	114.53	88.48	0.00	0.00	0.00	66.47	1.947	58.81
67	3	12	120.07	87.88	0.00	0.00	5.45	64.67	2.113	56.83
68	1	12	114.59	92.73	0.00	1.82	7.88	67.19	1.839	62.30
69	6	12	111.53	91.52	0.00	1.21	2.42	64.71	1.883	59.22
70	2	12	104.77	82.42	0.00	0.00	0.61	59.79	2.126	49.28
71	4	12	106.56	90.91	0.00	0.00	1.21	64.69	1.812	58.81
72	5	12	105.01	90.30	0.00	0.00	3.64	60.66	1.917	54.78
73	3	13	106.32	84.85	0.00	0.00	0.00	65.16	1.923	55.29
74	1	13	100.53	86.67	0.61	0.00	0.00	64.18	1.807	55.62
75	2	13	96.50	71.52	0.61	0.00	1.21	63.09	2.139	45.12
76	4	13	113.77	92.12	0.00	0.00	1.21	65.22	1.894	60.08
77	5	13	112.25	90.91	0.61	0.00	0.00	64.48	1.915	58.62
78	6	13	110.59	96.36	0.00	0.61	2.42	59.19	1.939	57.04
79	3	14	110.62	96.97	0.00	0.00	1.21	59.81	1.907	57.99
80	5	14	105.41	84.24	0.00	0.00	1.21	66.95	1.869	56.40
81	6	14	110.32	89.70	0.61	0.00	3.64	64.87	1.896	58.19
82	1	14	108.83	79.39	3.03	8.48	2.42	61.66	2.223	48.95
83	2	14	110.56	92.73	0.00	0.00	0.00	63.62	1.874	58.99
84	4	14	93.01	66.06	1.21	0.61	2.42	65.20	2.159	43.07
85	1	15	102.62	71.52	1.21	0.00	0.61	63.03	2.277	45.07
86	3	15	96.38	78.79	0.00	0.00	2.42	65.19	1.876	51.37
87	2	15	99.28	86.06	0.00	0.00	0.61	60.90	1.894	52.41
88	5	15	108.95	86.67	0.00	0.61	1.82	64.98	1.935	56.32
89	4	15	107.25	93.94	0.61	1.21	1.21	69.71	1.638	65.48
90	6	15	119.83	87.88	0.00	0.00	0.61	65.62	2.078	57.67
91	6	16	106.53	84.85	0.00	0.00	1.82	67.29	1.866	57.10
92	1	16	109.86	87.88	0.00	0.00	3.64	64.34	1.943	56.55
93	3	16	108.92	92.73	0.61	0.00	0.61	65.80	1.785	61.01
94	2	16	111.77	95.15	1.21	0.61	0.61	64.08	1.833	60.97
95	5	16	111.86	93.94	0.00	0.61	1.82	64.91	1.835	60.98
96	4	16	111.13	93.33	0.00	0.00	1.21	60.71	1.961	56.66

Table 13. Body weight and weight gain raw data

Pen	Trt	Block	BW 52 w	BW 59 w	Weight gain
1	1	1	1823.3	1786.0	-37.3
2	2	1	1724.0	1645.0	-79.0
3	3	1	2028.3	1868.3	-160.0
4	4	1	1841.3	1748.3	-93.0
5	5	1	1798.7	1776.0	-22.7
6	6	1	1900.7	1830.0	-70.7
7	1	2	1759.0	1736.3	-22.7
8	3	2	1805.0	1746.7	-58.3
9	4	2	1745.0	1748.3	3.3
10	6	2	1901.3	1861.7	-39.7
11	5	2	1778.3	1740.0	-38.3
12	2	2	1808.3	1801.7	-6.7
13	3	3	1702.3	1705.7	3.3
14	4	3	1714.7	1748.3	33.7
15	5	3	1900.0	1828.0	-72.0
16	6	3	1900.3	1776.7	-123.7
17	1	3	1919.0	1871.7	-47.3
18	2	3	1665.3	1638.3	-27.0
19	2	4	1738.7	1675.0	-63.7
20	1	4	1848.3	1788.3	-60.0
21	3	4	1841.7	1846.7	5.0
22	5	4	1790.7	1765.0	-25.7
23	4	4	1707.0	1673.3	-33.7
24	6	4	1822.3	1823.3	1.0
25	2	5	1776.7	1768.3	-8.3
26	1	5	1796.0	1730.0	-66.0
27	6	5	1742.3	1698.3	-44.0
28	5	5	1735.0	1696.0	-39.0
29	3	5	2016.0	1928.3	-87.7
30	4	5	1924.7	1836.7	-88.0
31	1	6	1782.3	1648.3	-134.0
32	3	6	1836.7	1776.7	-60.0
33	5	6	1730.3	1715.0	-15.3
34	2	6	1745.0	1745.0	0.0
35	4	6	1819.0	1873.3	54.3
36	6	6	1763.3	1773.3	10.0
37	1	7	1634.3	1578.3	-56.0
38	2	7	1832.0	1751.7	-80.3
39	3	7	1777.0	1715.0	-62.0
40	5	7	1745.0	1710.0	-35.0
41	6	7	1968.3	1903.3	-65.0
42	4	7	1870.7	1876.7	6.0
43	1	8	1809.3	1673.3	-136.0
44	3	8	1592.0	1538.3	-53.7
45	2	8	1884.3	1771.7	-112.7
46	5	8	1933.3	1875.0	-58.3

Contract code: 2 2 5 4 1

47	6	8	1809.3	1770.0	-39.3
48	4	8	1786.7	1750.0	-36.7
49	3	9	1703.3	1705.0	1.7
50	5	9	1867.0	1711.7	-155.3
51	2	9	1770.0	1728.3	-41.7
52	1	9	1954.7	1781.7	-173.0
53	6	9	1756.0	1715.0	-41.0
54	4	9	1743.0	1686.7	-56.3
55	1	10	1863.3	1805.0	-58.3
56	2	10	1746.7	1703.3	-43.3
57	3	10	1897.0	1910.0	13.0
58	5	10	1846.7	1795.0	-51.7
59	4	10	1807.3	1753.3	-54.0
60	6	10	1898.7	1830.0	-68.7
61	2	11	1645.7	1731.7	86.0
62	1	11	1976.3	1955.0	-21.3
63	4	11	1774.7	1758.3	-16.3
64	5	11	1869.0	1856.7	-12.3
65	6	11	1684.3	1611.7	-72.7
66	3	11	1821.0	1791.7	-29.3
67	3	12	1980.0	1930.0	-50.0
68	1	12	1892.0	1866.7	-25.3
69	6	12	1991.3	1918.3	-73.0
70	2	12	1712.7	1708.3	-4.3
71	4	12	1764.3	1716.7	-47.7
72	5	12	1738.3	1693.3	-45.0
73	3	13	1684.3	1686.7	2.3
74	1	13	1756.7	1681.7	-75.0
75	2	13	1794.7	1743.3	-51.3
76	4	13	1976.3	1891.7	-84.7
77	5	13	1973.7	1890.0	-83.7
78	6	13	1819.7	1815.0	-4.7
79	3	14	1834.0	1798.3	-35.7
80	5	14	1678.3	1683.3	5.0
81	6	14	1779.3	1774.3	-5.0
82	1	14	1825.7	1758.3	-67.3
83	2	14	1786.3	1751.7	-34.7
84	4	14	1815.0	1758.3	-56.7
85	1	15	1893.3	1849.0	-44.3
86	3	15	1784.7	1715.0	-69.7
87	2	15	1778.3	1693.3	-85.0
88	5	15	1691.7	1696.7	5.0
89	4	15	1915.7	1873.3	-42.3
90	6	15	1921.7	1940.0	18.3
91	6	16	1748.3	1698.3	-50.0
92	1	16	1876.0	1846.7	-29.3
93	3	16	1907.3	1888.3	-19.0
94	2	16	2097.7	2038.3	-59.3
95	5	16	1894.0	1819.7	-74.3
96	4	16	1954.3	1911.7	-42.7

Table 14. Eggshell strength measurement raw data

Lot	Trt	Block	Egg number	Egg force week 56	Egg force week 59
1	1	1	1	2.828	3.180
1	1	1	2	3.322	3.095
1	1	1	3	3.570	2.278
2	2	1	1	3.038	3.066
2	2	1	2	3.366	2.778
2	2	1	3	3.591	2.834
3	3	1	1	4.171	3.663
3	3	1	2	3.578	3.716
3	3	1	3	3.367	3.500
4	4	1	1	3.162	2.844
4	4	1	2	2.938	4.479
4	4	1	3	3.336	3.274
5	5	1	1	3.365	3.753
5	5	1	2	3.541	2.953
5	5	1	3	3.741	2.894
6	6	1	1	3.535	3.233
6	6	1	2	3.247	3.110
6	6	1	3	3.178	2.898
7	1	2	1	4.469	3.158
7	1	2	2	3.833	3.608
7	1	2	3	3.354	3.590
8	3	2	1	3.278	2.963
8	3	2	2	3.609	3.554
8	3	2	3	3.084	3.328
9	4	2	1	4.011	3.881
9	4	2	2	3.872	3.072
9	4	2	3	3.287	3.006
10	6	2	1	3.975	3.874
10	6	2	2	2.974	3.940
10	6	2	3	3.170	4.720
11	5	2	1	3.000	3.008
11	5	2	2	3.912	3.381
11	5	2	3	1.704	2.460
12	2	2	1	3.270	4.058
12	2	2	2	3.966	3.537
12	2	2	3	3.528	4.622
13	3	3	1	2.962	3.539
13	3	3	2	4.948	2.299
13	3	3	3	2.094	3.896
14	4	3	1	4.025	3.734
14	4	3	2	4.284	3.237
14	4	3	3	5.067	3.474
15	5	3	1	4.993	4.714
15	5	3	2	3.902	3.561
15	5	3	3	4.063	2.408

Contract code: *2 2 5 4 1*

16	6	3	1	4.553	1.670
16	6	3	2	3.383	3.795
16	6	3	3	3.354	4.146
17	1	3	1	3.828	3.863
17	1	3	2	4.076	3.262
17	1	3	3	3.307	2.774
18	2	3	1	2.684	3.449
18	2	3	2	4.554	3.014
18	2	3	3	4.787	4.179
19	2	4	1	3.671	2.516
19	2	4	2	3.840	3.918
19	2	4	3	3.033	4.236
20	1	4	1	3.425	3.675
20	1	4	2	4.083	3.225
20	1	4	3	5.046	3.998
21	3	4	1	3.019	3.198
21	3	4	2	3.407	4.163
21	3	4	3	4.132	3.327
22	5	4	1	3.584	3.367
22	5	4	2	3.700	2.777
22	5	4	3	2.809	3.350
23	4	4	1	3.208	4.782
23	4	4	2	4.152	3.405
23	4	4	3	4.122	2.331
24	6	4	1	3.431	4.721
24	6	4	2	4.343	4.789
24	6	4	3	3.935	3.427
25	2	5	1	3.353	3.568
25	2	5	2	4.010	2.235
25	2	5	3	4.259	3.598
26	1	5	1	3.178	3.031
26	1	5	2	4.627	2.942
26	1	5	3	3.311	2.999
27	6	5	1	4.467	3.853
27	6	5	2	3.195	4.079
27	6	5	3	3.995	3.507
28	5	5	1	4.199	2.632
28	5	5	2	5.096	3.732
28	5	5	3	3.990	5.049
29	3	5	1	5.002	4.916
29	3	5	2	3.507	3.696
29	3	5	3	3.639	4.853
30	4	5	1	3.579	2.928
30	4	5	2	3.934	3.864
30	4	5	3	3.053	2.578
31	1	6	1	3.335	3.888
31	1	6	2	3.790	3.680
31	1	6	3	3.533	2.769
32	3	6	1	3.621	4.066
32	3	6	2	3.169	4.305
32	3	6	3	3.946	3.533

				Contract code: 2 2 5 4 1	
33	5	6	1	4.299	3.266
33	5	6	2	3.436	3.407
33	5	6	3	3.002	1.250
34	2	6	1	4.886	3.226
34	2	6	2	3.866	4.736
34	2	6	3	4.050	4.643
35	4	6	1	3.384	3.366
35	4	6	2	3.485	4.855
35	4	6	3	3.141	3.149
36	6	6	1	4.704	4.120
36	6	6	2	4.152	3.516
36	6	6	3	3.628	3.549
37	1	7	1	3.292	3.973
37	1	7	2	3.500	3.465
37	1	7	3	3.655	4.477
38	2	7	1	3.783	4.232
38	2	7	2	3.512	4.703
38	2	7	3	4.114	4.063
39	3	7	1	3.233	3.972
39	3	7	2	3.641	2.735
39	3	7	3	4.670	3.430
40	5	7	1	3.266	4.099
40	5	7	2	3.228	3.299
40	5	7	3	4.441	3.072
41	6	7	1	3.764	3.552
41	6	7	2	2.601	3.891
41	6	7	3	2.920	3.596
42	4	7	1	3.312	3.664
42	4	7	2	3.797	2.517
42	4	7	3	3.195	5.174
43	1	8	1	4.645	3.381
43	1	8	2	3.173	2.818
43	1	8	3	2.480	3.035
44	3	8	1	3.922	4.239
44	3	8	2	3.335	2.894
44	3	8	3	4.030	3.856
45	2	8	1	4.500	3.954
45	2	8	2	4.176	3.799
45	2	8	3	3.411	3.347
46	5	8	1	3.007	3.944
46	5	8	2	4.009	2.718
46	5	8	3	3.405	2.680
47	6	8	1	3.295	3.255
47	6	8	2	3.606	3.648
47	6	8	3	3.554	3.836
48	4	8	1	3.776	2.972
48	4	8	2	3.457	3.271
48	4	8	3	4.324	3.310
49	4	8	1	2.923	2.570
49	4	8	2	2.479	2.980
49	4	8	3	2.760	3.240

Contract code: *2 2 5 4 1*

50	4	8	1	3.382	5.098
50	4	8	2	3.193	2.734
50	4	8	3	2.769	4.365
51	4	8	1	3.998	4.000
51	4	8	2	3.725	5.217
51	4	8	3	2.901	3.180
52	4	8	1	3.950	3.937
52	4	8	2	4.132	3.819
52	4	8	3	3.540	2.664
53	4	8	1	3.700	3.823
53	4	8	2	3.777	2.686
53	4	8	3	4.336	4.364
54	4	8	1	3.467	3.774
54	4	8	2	4.191	2.923
54	4	8	3	3.768	3.384
55	4	8	1	2.933	1.520
55	4	8	2	3.503	2.202
55	4	8	3	4.493	3.953
56	4	8	1	2.847	3.244
56	4	8	2	3.473	2.416
56	4	8	3	5.105	1.638
57	4	8	1	3.962	3.238
57	4	8	2	3.468	3.567
57	4	8	3	3.391	3.946
58	4	8	1	3.975	2.966
58	4	8	2	3.791	3.066
58	4	8	3	4.404	3.670
59	4	8	1	4.183	4.119
59	4	8	2	4.522	4.464
59	4	8	3	4.397	3.351
60	4	8	1	4.112	5.346
60	4	8	2	4.040	3.169
60	4	8	3	3.473	4.670
61	4	8	1	4.573	3.973
61	4	8	2	3.171	2.999
61	4	8	3	2.505	3.860
62	4	8	1	3.610	3.870
62	4	8	2	3.326	3.811
62	4	8	3	4.323	3.551
63	4	8	1	3.037	3.273
63	4	8	2	3.400	3.953
63	4	8	3	3.701	3.292
64	4	8	1	4.035	3.264
64	4	8	2	4.476	4.416
64	4	8	3	5.053	2.295
65	4	8	1	3.852	2.963
65	4	8	2	3.990	3.477
65	4	8	3	3.538	3.165
66	4	8	1	3.286	4.070
66	4	8	2	3.123	4.013
66	4	8	3	4.032	2.791

Contract code: *2 2 5 4 1*

67	4	8	1	3.133	3.598
67	4	8	2	2.890	4.153
67	4	8	3	2.650	3.617
68	4	8	1	3.936	3.689
68	4	8	2	4.181	1.990
68	4	8	3	3.073	3.145
69	4	8	1	3.443	2.973
69	4	8	2	3.909	2.223
69	4	8	3	3.495	3.151
70	4	8	1	3.482	3.057
70	4	8	2	3.267	4.116
70	4	8	3	4.640	4.510
71	4	8	1	4.607	3.387
71	4	8	2	4.095	4.128
71	4	8	3	4.165	3.094
72	4	8	1	3.872	3.458
72	4	8	2	3.860	2.799
72	4	8	3	3.642	3.055
73	4	8	1	5.269	3.492
73	4	8	2	3.196	3.755
73	4	8	3	4.110	3.837
74	4	8	1	3.798	4.238
74	4	8	2	4.419	3.342
74	4	8	3	3.452	3.882
75	4	8	1	3.874	4.557
75	4	8	2	4.438	1.839
75	4	8	3	4.042	3.567
76	4	8	1	4.186	3.003
76	4	8	2	3.902	3.444
76	4	8	3	4.374	3.023
77	4	8	1	4.508	3.290
77	4	8	2	3.651	3.427
77	4	8	3	3.586	3.459
78	4	8	1	3.112	4.021
78	4	8	2	3.957	3.703
78	4	8	3	4.374	3.320
79	4	8	1	3.452	2.520
79	4	8	2	3.392	2.975
79	4	8	3	3.175	3.456
80	4	8	1	3.608	4.247
80	4	8	2	3.380	3.635
80	4	8	3	2.432	3.874
81	4	8	1	5.042	2.750
81	4	8	2	3.873	4.564
81	4	8	3	4.593	3.679
82	4	8	1	3.842	3.857
82	4	8	2	3.874	3.710
82	4	8	3	4.773	3.257
83	4	8	1	3.751	4.415
83	4	8	2	4.235	3.964
83	4	8	3	3.424	3.562

Contract code: *2 2 5 4 1*

84	4	8	1	3.437	4.386
84	4	8	2	3.713	4.168
84	4	8	3	3.969	2.602
85	4	8	1	4.780	2.952
85	4	8	2	3.472	3.185
85	4	8	3	3.141	3.195
86	4	8	1	3.466	3.516
86	4	8	2	4.790	2.541
86	4	8	3	5.005	4.881
87	4	8	1	4.428	5.205
87	4	8	2	4.164	1.781
87	4	8	3	3.605	3.812
88	4	8	1	4.307	4.186
88	4	8	2	2.891	3.282
88	4	8	3	4.216	3.693
89	4	8	1	3.500	2.935
89	4	8	2	3.349	3.396
89	4	8	3	3.390	3.417
90	4	8	1	2.738	3.026
90	4	8	2	3.967	3.598
90	4	8	3	3.062	2.476
91	4	8	1	4.227	3.171
91	4	8	2	3.417	4.853
91	4	8	3	4.333	3.060
92	4	8	1	5.136	4.193
92	4	8	2	4.656	4.516
92	4	8	3	3.961	4.121
93	4	8	1	2.564	2.062
93	4	8	2	3.082	3.212
93	4	8	3	3.758	3.584
94	4	8	1	3.045	3.152
94	4	8	2	3.996	3.930
94	4	8	3	2.856	4.434
95	4	8	1	3.260	2.044
95	4	8	2	4.963	1.954
95	4	8	3	3.177	4.314
96	4	8	1	3.295	2.799
96	4	8	2	4.849	2.839
96	4	8	3	3.076	3.657

Table 15. Tibia ash and phosphorus content raw data

Pen	Trt	Block	Ash (%)	P (%DM)
1	1	1	47.64	7.874
2	2	1	48.13	7.892
3	3	1	45.97	7.442
4	4	1	44.63	7.210
5	5	1	47.14	7.582
6	6	1	39.23	6.427
7	1	2	45.18	7.194
8	3	2	48.21	7.810
9	4	2	45.15	7.149
10	6	2	53.46	8.916
11	5	2	45.33	7.421
12	2	2	47.22	7.652
13	3	3	45.48	7.459
14	4	3	48.97	7.979
15	5	3	44.28	7.336
16	6	3	39.68	6.638
17	1	3	46.76	7.817
18	2	3	45.82	7.463
19	2	4	49.82	8.271
20	1	4	44.70	7.272
21	3	4	37.25	6.027
22	5	4	46.23	7.568
23	4	4	47.76	7.849
24	6	4	45.87	7.610
25	2	5	48.25	7.692
26	1	5	47.74	8.027
27	6	5	47.97	7.928
28	5	5	50.21	8.353
29	3	5	48.30	7.920
30	4	5	47.77	7.797
31	1	6	49.67	8.017
32	3	6	47.37	7.689
33	5	6	44.61	7.197
34	2	6	44.32	7.116
35	4	6	47.07	7.683
36	6	6	53.26	8.549
37	1	7	45.46	7.386
38	2	7	47.64	7.767
39	3	7	51.35	8.190
40	5	7	47.05	7.570
41	6	7	44.25	7.187
42	4	7	52.96	8.485
43	1	8	47.69	7.712
44	3	8	48.17	7.837
45	2	8	44.69	7.342
46	5	8	46.04	7.449

Contract code: *2 2 5 4 1*

47	6	8	46.51	7.689
48	4	8	51.37	8.508
49	3	9	45.20	7.258
50	5	9	47.62	7.841
51	2	9	46.78	7.642
52	1	9	41.18	6.597
53	6	9	45.82	7.516
54	4	9	47.31	7.602
55	1	10	47.41	7.718
56	2	10	49.46	8.025
57	3	10	49.19	8.018
58	5	10	45.40	7.378
59	4	10	50.87	8.169
60	6	10	47.87	7.769
61	2	11	47.03	7.612
62	1	11	48.26	7.517
63	4	11	47.78	7.735
64	5	11	45.43	7.230
65	6	11	45.66	7.163
66	3	11	47.71	7.753
67	3	12	45.27	7.404
68	1	12	47.29	7.571
69	6	12	50.09	7.958
70	2	12	44.05	7.218
71	4	12	33.61	5.576
72	5	12	47.38	7.747
73	3	13	49.26	7.831
74	1	13	49.41	7.789
75	2	13	47.98	7.700
76	4	13	49.11	8.016
77	5	13	40.18	6.527
78	6	13	42.43	6.868
79	3	14	44.60	7.129
80	5	14	41.43	6.599
81	6	14	43.19	6.683
82	1	14	49.16	7.856
83	2	14	45.73	7.862
84	4	14	37.54	6.113
85	1	15	42.38	6.743
86	3	15	49.98	7.994
87	2	15	48.34	7.699
88	5	15	44.57	7.209
89	4	15	41.85	6.826
90	6	15	48.35	7.852
91	6	16	52.65	8.801
92	1	16	46.51	7.370
93	3	16	44.40	7.166
94	2	16	44.77	7.306
95	5	16	42.68	7.018
96	4	16	43.09	7.035

Table 16. Tibia breaking strength measurement raw data

Pen	Trt	Block	Breaking force (Kg)	Bone strength (Kg/mm2)
1	1	1	28.686	23.131
2	2	1	20.204	22.038
3	3	1	33.326	36.405
4	4	1	24.964	20.225
5	5	1	36.016	29.285
6	6	1	21.935	23.903
7	1	2	28.457	39.087
8	3	2	18.985	31.471
9	4	2	29.252	33.641
10	6	2	42.845	33.837
11	5	2	27.375	32.577
12	2	2	34.150	29.392
13	3	3	33.986	24.856
14	4	3	35.046	30.062
15	5	3	32.233	24.666
16	6	3	16.902	26.132
17	1	3	23.626	18.099
18	2	3	20.047	23.250
19	2	4	24.523	29.185
20	1	4	29.591	29.961
21	3	4	37.029	32.709
22	5	4	27.710	20.586
23	4	4	31.122	32.149
24	6	4	31.472	29.221
25	2	5	41.997	33.389
26	1	5	19.565	22.726
27	6	5	26.070	24.585
28	5	5	27.669	26.933
29	3	5	29.209	28.330
30	4	5	31.540	25.078
31	1	6	41.444	30.457
32	3	6	33.652	34.773
33	5	6	21.861	27.159
34	2	6	31.835	31.562
35	4	6	46.573	37.770
36	6	6	32.849	30.052
37	1	7	36.310	31.722
38	2	7	26.138	28.599
39	3	7	42.025	41.692
40	5	7	16.033	23.337
41	6	7	32.895	29.846
42	4	7	41.895	49.088
43	1	8	29.719	27.140
44	3	8	26.026	26.397
45	2	8	31.828	22.760
46	5	8	23.210	31.962

47	6	8	22.336	28.289
48	4	8	25.476	24.011
49	3	9	33.117	31.530
50	5	9	37.412	33.856
51	2	9	30.226	27.706
52	1	9	14.248	20.887
53	6	9	27.275	27.642
54	4	9	26.414	30.369
55	1	10	22.950	22.448
56	2	10	29.203	33.304
57	3	10	22.329	31.853
58	5	10	22.674	31.531
59	4	10	27.639	30.757
60	6	10	23.305	28.138
61	2	11	24.331	25.612
62	1	11	27.826	24.763
63	4	11	21.649	24.117
64	5	11	22.729	32.043
65	6	11	22.932	28.201
66	3	11	20.207	30.556
67	3	12	38.380	34.574
68	1	12	29.964	25.392
69	6	12	39.459	42.270
70	2	12	27.368	32.751
71	4	12	20.314	22.694
72	5	12	27.887	26.156
73	3	13	27.519	28.242
74	1	13	29.350	29.652
75	2	13	28.108	32.330
76	4	13	27.383	28.345
77	5	13	25.631	41.935
78	6	13	23.140	29.226
79	3	14	30.399	26.149
80	5	14	23.878	21.026
81	6	14	25.642	24.849
82	1	14	36.810	36.726
83	2	14	25.251	31.517
84	4	14	19.486	21.733
85	1	15	29.808	29.714
86	3	15	26.506	29.364
87	2	15	26.104	25.931
88	5	15	33.353	34.058
89	4	15	31.057	35.441
90	6	15	22.618	20.532
91	6	16	36.400	28.103
92	1	16	40.044	31.179
93	3	16	25.601	22.939
94	2	16	31.322	28.065
95	5	16	26.014	23.925
96	4	16	21.621	25.274

Table 17. Ileal phosphorus digestibility raw data

Pen	Trt	Block	P digestibility (%)	P digestibility (g/kg)
1	1	1	42.91	1.35
2	2	1	R	R
3	3	1	48.41	1.52
4	4	1	36.11	1.14
5	5	1	47.11	1.48
6	6	1	74.88	2.36
7	1	2	30.85	0.97
8	3	2	46.46	1.46
9	4	2	59.78	1.88
10	6	2	78.17	2.46
11	5	2	51.99	1.64
12	2	2	50.42	1.59
13	3	3	48.26	1.52
14	4	3	44.18	1.39
15	5	3	40.18	1.27
16	6	3	75.15	2.37
17	1	3	37.10	1.17
18	2	3	46.62	1.47
19	2	4	45.11	1.42
20	1	4	30.29	0.95
21	3	4	54.04	1.70
22	5	4	58.35	1.84
23	4	4	42.42	1.34
24	6	4	67.91	2.14
25	2	5	42.75	1.35
26	1	5	39.46	1.24
27	6	5	68.27	2.15
28	5	5	67.12	2.11
29	3	5	51.25	1.61
30	4	5	64.00	2.02
31	1	6	38.58	1.22
32	3	6	49.73	1.57
33	5	6	63.82	2.01
34	2	6	37.49	1.18
35	4	6	57.04	1.80
36	6	6	74.09	2.33
37	1	7	36.49	1.15
38	2	7	49.79	1.57
39	3	7	49.90	1.57
40	5	7	56.78	1.79
41	6	7	82.67	2.60
42	4	7	66.23	2.09
43	1	8	39.89	1.26
44	3	8	30.73	0.97
45	2	8	57.06	1.80
46	5	8	40.84	1.29

47	6	8	71.72	2.26
48	4	8	52.04	1.64
49	3	9	55.73	1.76
50	5	9	57.83	1.82
51	2	9	41.20	1.30
52	1	9	37.24	1.17
53	6	9	76.91	2.42
54	4	9	42.88	1.35
55	1	10	39.59	1.25
56	2	10	56.25	1.77
57	3	10	28.41	0.89
58	5	10	70.19	2.21
59	4	10	56.94	1.79
60	6	10	65.19	2.05
61	2	11	50.10	1.58
62	1	11	35.53	1.12
63	4	11	39.17	1.23
64	5	11	62.40	1.97
65	6	11	74.08	2.33
66	3	11	31.38	0.99
67	3	12	40.85	1.29
68	1	12	32.30	1.02
69	6	12	76.86	2.42
70	2	12	R	R
71	4	12	43.66	1.38
72	5	12	62.78	1.98
73	3	13	29.58	0.93
74	1	13	R	R
75	2	13	32.64	1.03
76	4	13	43.27	1.36
77	5	13	51.12	1.61
78	6	13	76.26	2.40
79	3	14	45.88	1.45
80	5	14	62.91	1.98
81	6	14	79.36	2.50
82	1	14	37.74	1.19
83	2	14	32.61	1.03
84	4	14	53.69	1.69
85	1	15	R	R
86	3	15	50.40	1.59
87	2	15	45.43	1.43
88	5	15	64.39	2.03
89	4	15	57.88	1.82
90	6	15	76.13	2.40
91	6	16	73.79	2.32
92	1	16	38.32	1.21
93	3	16	52.23	1.65
94	2	16	38.82	1.22
95	5	16	66.05	2.08
96	4	16	34.93	1.10

Table 18. Phosphorus content in excreta raw data

Pen	Trt	Block	Phosphorus (%)
1	1	1	0.79
2	2	1	0.85
3	3	1	0.74
4	4	1	0.78
5	5	1	0.71
6	6	1	0.58
7	1	2	0.99
8	3	2	0.84
9	4	2	0.86
10	6	2	0.97
11	5	2	0.83
12	2	2	0.86
13	3	3	0.83
14	4	3	0.59
15	5	3	0.58
16	6	3	0.77
17	1	3	0.77
18	2	3	0.81
19	2	4	0.91
20	1	4	0.79
21	3	4	0.74
22	5	4	0.73
23	4	4	0.83
24	6	4	0.52
25	2	5	0.83
26	1	5	0.96
27	6	5	0.51
28	5	5	0.72
29	3	5	0.65
30	4	5	0.73
31	1	6	0.84
32	3	6	0.63
33	5	6	0.63
34	2	6	0.93
35	4	6	0.73
36	6	6	0.87
37	1	7	0.82
38	2	7	0.63
39	3	7	0.78
40	5	7	0.53
41	6	7	0.71
42	4	7	0.66
43	1	8	0.90
44	3	8	0.76
45	2	8	0.79
46	5	8	0.89

Contract code: *2 2 5 4 1*

47	6	8	0.68
48	4	8	0.65
49	3	9	0.79
50	5	9	1.00
51	2	9	0.77
52	1	9	0.77
53	6	9	0.61
54	4	9	0.74
55	1	10	0.84
56	2	10	0.83
57	3	10	0.68
58	5	10	0.75
59	4	10	0.70
60	6	10	0.47
61	2	11	0.70
62	1	11	1.04
63	4	11	0.56
64	5	11	0.70
65	6	11	0.67
66	3	11	0.82
67	3	12	0.86
68	1	12	0.69
69	6	12	0.48
70	2	12	0.82
71	4	12	1.17
72	5	12	0.56
73	3	13	0.95
74	1	13	0.90
75	2	13	0.89
76	4	13	0.86
77	5	13	0.81
78	6	13	0.83
79	3	14	0.60
80	5	14	0.88
81	6	14	0.50
82	1	14	0.88
83	2	14	0.70
84	4	14	0.78
85	1	15	0.96
86	3	15	0.79
87	2	15	0.90
88	5	15	0.64
89	4	15	1.02
90	6	15	0.81
91	6	16	0.56
92	1	16	0.88
93	3	16	0.78
94	2	16	0.81
95	5	16	0.93
96	4	16	0.57

Table 19. Haematological and biochemical characteristics raw data

Pen	Trt	Block	Erythrocytes	Haemoglobin	Haematocrit	VCM	HCM	CHCM	Glucose	Urates	Total protein	Albumine	Calcium	Phosphorus	GOT	GPT	GGT	Alcaline phosphatase
			10E12/L	g/dL	%	fL	pg	g/dL	mg/dL	mg/dL	g/dL	g/dL	mg/dL	mg/dL	U/L	U/L	U/L	U/L
1	1	1	2.84	13.5	36.1	127	47.7	37.5	255	5.4	4.48	1.52	21.5	3.81	194	1	27	4480
5	5	1	2.56	12.2	32.3	127	47.7	37.7	247	4.9	5.00	1.58	28.2	5.48	155	1	20	418
6	6	1	2.69	12.8	34.2	127	47.4	37.3	240	6.0	5.70	1.91	34.0	6.24	173	1	23	664
7	1	2	2.44	11.8	32.1	132	48.1	36.6	211	7.1	4.87	1.55	24.9	3.73	178	2	29	770
10	6	2	2.54	12.3	33.6	133	48.4	36.5	227	6.0	5.37	1.71	30.5	6.02	166	2	22	941
11	5	2	2.51	12.0	31.9	127	47.6	37.5	194	7.8	5.75	1.79	28.6	5.72	165	13	6	2015
15	5	3	2.39	11.7	31.9	133	48.8	36.6	237	5.2	4.73	1.62	27.3	6.90	172	1	17	462
16	6	3	1.90	9.0	24.2	127	47.6	37.4	255	6.8	5.90	1.93	35.7	8.74	149	1	15	933
17	1	3	2.47	11.8	32.2	131	47.9	36.7	188	3.9	3.84	1.37	23.3	3.36	124	1	22	715
20	1	4	2.52	12.0	32.4	129	47.7	37.1	175	6.5	5.88	1.80	27.5	4.94	208	2	19	746
22	5	4	R	R	R	R	R	R	217	8.5	6.50	1.85	38.4	8.76	168	2	15	295
24	6	4	2.46	12.2	32.4	131	49.6	37.8	226	6.5	5.32	1.67	27.3	5.46	155	2	32	552
26	1	5	2.62	12.2	34.6	132	46.8	35.4	241	6.9	5.54	1.71	27.3	4.10	246	1	30	888
27	6	5	2.76	13.7	35.5	129	49.5	38.5	241	8.1	5.98	1.96	33.4	7.62	187	1	19	1285
28	5	5	2.05	10.3	26.1	127	50.0	39.3	239	5.6	4.62	1.70	30.0	4.52	170	2	23	482
31	1	6	2.43	12.0	32.1	132	49.3	37.4	206	9.0	4.79	1.63	25.2	3.48	151	2	18	1700
33	5	6	2.61	12.7	33.3	128	48.6	38.1	251	8.6	5.66	2.11	33.5	8.71	178	12	19	872
36	6	6	2.48	12.3	30.6	123	49.4	40.1	232	7.9	6.01	1.78	35.1	6.98	167	1	29	540
37	1	7	2.45	12.6	32.8	133	51.5	38.6	240	7.1	5.93	1.87	35.4	6.65	150	2	27	236
40	5	7	2.79	13.1	35.2	126	46.9	37.2	253	4.8	4.94	1.54	27.8	4.49	194	1	16	1467
41	6	7	2.33	10.6	29.6	127	45.6	35.9	232	6.2	4.77	1.74	28.2	6.27	239	4	31	805
43	1	8	2.43	11.4	30.9	127	46.7	36.8	250	6.2	5.14	1.59	25.4	3.92	189	1	19	841
46	5	8	2.60	12.1	33.5	129	46.4	36.1	199	6.0	5.81	1.86	32.7	6.96	173	2	22	1268
47	6	8	2.65	12.8	34.5	130	48.4	37.2	228	7.2	5.63	1.96	31.9	6.89	224	2	20	926
50	5	9	2.41	11.8	31.6	131	48.7	37.2	241	6.8	4.36	1.44	23.6	4.97	154	1	15	1346

52	1	9	2.72	13.0	35.1	129	47.9	37.1	227	6.4	5.47	1.77	31.6	5.75	195	1	15	448
53	6	9	2.58	12.5	33.3	129	48.3	37.5	171	4.0	3.98	1.14	22.7	4.06	116	1	13	458
58	5	10	2.43	12.1	31.7	130	49.7	38.2	218	5.2	5.76	1.76	29.6	4.50	221	2	32	1280
60	6	10	2.47	11.1	30.5	123	45.1	36.5	255	7.0	5.32	1.86	30.4	6.21	218	3	24	546
62	1	11	2.33	11.9	31.1	133	50.9	38.1	257	7.6	5.19	1.86	31.5	4.51	157	1	19	1131
64	5	11	2.39	11.4	31.3	131	47.7	36.3	246	6.7	4.95	1.64	28.4	6.16	195	2	18	672
65	6	11	2.44	11.6	31.1	127	47.4	37.2	226	5.9	5.47	1.72	31.5	5.96	182	3	21	1000
68	1	12	2.47	11.8	32.1	130	47.5	36.7	225	5.8	6.27	1.85	33.8	7.17	197	3	31	516
69	6	12	2.11	10.3	28.4	134	48.7	36.3	257	4.6	5.54	1.75	34.7	7.72	289	1	17	656
72	5	12	2.45	11.7	31.8	130	47.6	36.7	255	4.2	5.32	1.85	31.6	5.81	181	2	32	656
74	1	13	2.44	11.9	31.8	130	48.7	37.3	240	5.1	5.05	1.72	29.6	4.39	124	2	32	1202
77	5	13	2.54	12.1	32.2	127	47.4	37.4	206	4.9	5.53	1.71	27.2	5.65	205	2	18	1583
78	6	13	2.51	11.8	32.5	129	47.1	36.4	234	6.7	6.28	1.60	26.5	6.27	390	3	17	465
80	5	14	2.45	12.2	31.8	130	49.6	38.3	240	8.3	5.10	1.52	33.8	7.12	184	2	19	808
81	6	14	2.70	12.2	34.1	126	45.3	35.9	230	8.4	4.90	1.80	31.4	6.51	184	1	25	1020
82	1	14	2.60	12.6	33.9	131	48.5	37.1	234	7.6	5.84	1.72	32.9	6.09	176	2	26	632
85	1	15	2.48	12.1	33.2	134	49.0	36.6	245	5.1	5.35	1.77	31.9	4.06	164	1	31	1776
88	5	15	2.35	11.0	29.7	127	46.7	36.9	240	7.8	5.15	1.71	30.2	6.69	236	1	25	927
90	6	15	2.33	11.0	30.4	130	47.1	36.1	254	6.1	5.04	1.67	27.1	5.86	190	1	27	2093
91	6	16	2.45	11.5	32.0	131	47.0	36.0	221	4.8	5.29	1.70	26.2	5.06	176	2	18	936
92	1	16	2.54	11.5	31.8	125	45.4	36.2	256	5.6	5.11	1.81	28.0	4.40	185	2	23	1099
95	5	16	2.36	10.9	29.9	127	46.4	36.6	222	5.8	5.94	1.69	30.2	5.62	169	4	25	1311

European Food Safety Authority

FEEDAP UNIT

ANNEX C [1]

TRIAL PROTOCOL DATA SHEET: FOR TERRESTRIAL ANIMALS

Identification of the additive: **IPA Phytase (M)**	Batch number: **PPQ 28656**
Trial ID: **G-129**	Location: **IRTA, Spain**
Start date and exact duration of the study: **28-10-08, duration 56 days**	
Number of treatment groups (+ control(s)): **6**	Replicates per group: **16**
Total number of animals: **288**	Animals per replicate: **3**
Dose(s) of the additive/active substance(s)/agent(s) (mg/Units of activity/CFU kg^{-1} complete feed/L^{-1} water)	
Intended: **0, 500, 1000, 2000, 4000 and †40000 phytase U/kg feed**	Analysed: **28, 599, 1180, 2112, 4347 and 40389 U/kg**
Substances used for comparative purposes:	
Intended dose:	Analysed:
Animal species/category: **Laying hens**	
Breed: **brown Hy-Line strain**	Identification procedure: **Pen**
Sex: **females** Age at start: **52 weeks**	Body weight at start: **1820 g**
Physiological stage: **laying**	General health: **good**
Additional information for field trials:	
Location and size of herd or flock:	
Feeding and rearing conditions:	
Method of feeding:	
Diets (type(s)): **Layer diet**	
Presentation of the diet: Mash ☒ Pellet ☐ Extruded ☐ Other	
Composition (main feedingstuffs): **Maize + soybean meal**	
Nutrient content (relevant nutrients and energy content)	
Intended values: **2700 Kcal/kg, 17% CP, 0.85% Lys, 0.63% Met+Cys, 0.18 Trp, 4.1% fat, 0.33% P**	
Analysed values: **19.8% CP, 4.1% fat and 0.33 P**	
Date and nature of the examinations performed: **Performance (8 weeks), tibia ash, P and strength, ileal P digestibility, excreta P content, blood analysis after feeding experimental diets for 56 days**	
Method(s) of statistical evaluation used: **ANOVA and linear and quadratic regression analysis**	
Therapeutic/preventive treatments (reason, timing, kind, duration): **Not applied**	
Timing and prevalence of any undesirable consequences of treatment: **No undesirable effects**	
Date: 19-6-09	Signature Study Director: Marta Francesch

† In case the concentration of the additive in complete feed/water may reflect insufficient accuracy, the dose of the additive can be given per animal day^{-1} or mg kg^{-1} body weight or as concentration in complementary feed.

[1] Please submit this form using a common word processing format (e.g. MS Word).

TAB

ANNEX 26

Annex 26

Kwakernaa, K. et *al.* (2009). Report No. 00000959: IPA mash phytase (RONOZYME® HiPhos) improves ileal P- and Ca-absorption in laying hens. 2009

REPORT No. 00000959
Regulatory Document

DSM

Document Date:	22 June, 2009
Author(s):	C. Kwakernaak[1], J.D. van der Klis[1] and J. Broz[2]
	[1] Schothorst Feed Research, Lelystad (Netherlands) [2] Animal Nutrition and Health R&D, DSM Nutritional Products Ltd, Basel
Title:	**IPA Mash phytase improves ileal P- and Ca-absorption in laying hens**
Project No.	6106

Summary

An experiment was carried out to determine the effect of IPA Mash phytase on the apparent ileal phosphorus (P) and calcium (Ca) absorption in laying hens. Graded phytase levels of 0, 500, 1000 and 2000 U/kg were added to a maize/soya-based, P-deficient basal diet containing 33.6 g Ca, 3.2 g P and 2.1 g phytate-P per kg. A positive control was involved as well, which received the basal diet supplemented with 1 g P from DCP per kg. The experimental diets were fed to laying hens from the 26th to the 28th life week (15 days). All experimental diets were fed *ad libitum* in mash form. Each dietary treatment was assigned to 6 replicates, each consisting of 4 cages with four hens per cage. Ileal absorption of P and Ca and tibia ash content were determined as response parameters at the last day of trial. The results of this study confirmed that IPA Mash phytase markedly improved apparent ileal P absorption. The lowest dietary inclusion level (500 U/kg) already improved ileal P absorption significantly compared to the P-deficient negative control (+80%). An increase of the dietary level to 1000 U/kg resulted in further significant improvement of P absorption (+109% versus NC). Phytase supplementation also numerically improved ileal Ca absorption, resulting in significantly increased tibia ash contents at phytase levels of 1000 and 2000 U/kg diet, respectively. Based on an exponential fitted dose-response curve, phytase inclusion at 500 U/kg diet was equal to 0.66 g absorbable P from DCP. The absorption coefficient for P from DCP was found to be 64% in this particular study. When using the exponential curve, 1 g P from DCP was equal to 469 U/kg.

This report consists of Pages I – II and 1 - 22

Distribution

Ms. R. Aureli, NRD/CA
Dr. M. Eggersdorfer, NRD
Dr. F. Fru, NRD/PA
Mr. J.-F. Hecquet, NBD/RG
Dr. A.-M. Klünter, NRD/CA
Dr. J. Pheiffer, NRD/PA
Dr. P. Philipps, NRD/CA
Mr. J.-P. Ruckebusch, ANH

Approved

Name	Signature	Date
Main Author	signed by	
Dr. J. Broz, NRD/CA	J. Broz	23.06.2009
Principal Scientist / Competence Mgr	signed by	
Dr. J. Broz, NRD/CA	J. Broz	23.06.2009
Research Center Head	signed by	
Dr. A.-M. Klünter, NRD/CA	A.-M. Klünter	25.06.2009
Project Manager	signed by	
Dr. F. Fru, NRD/PA	F. Fru	23.06.2009

Nomenclature and Structural Formula

IPA Mash phytase (M), enzyme product containing bacterial 6-phytase (EC 3.1.3.26), produced by a submerged fermentation of a genetically modified *Aspergillus oryzae* strain. Lot PPQ 28656 was used in this study, manufactured by Novozymes A/S, Bagsvaerd, Denmark.

Schothorst Feed Research

IPA Mash Phytase Improves Ileal P- and Ca-absorption in Laying Hens

Trial report
nr. 965

May 2009

Authors:
ing. C. Kwakernaak
dr. ir. J.D. van der Klis

630

Schothorst Feed Research

Confidential
Report: nr. 965

IPA Mash Phytase Improves Ileal P- and Ca-absorption in Laying Hens

(Experiment PLE-48, Project Code PA 08-27)

Client: DSM Nutritional Products Ltd.

Key words: Laying hens, layers, phytase, IPA Mash, Ca and P absorption

Authors:
ing. C. Kwakernaak
dr. ir. J.D. van der Klis

Contents

1 Introduction

The use of phytase in pig and poultry nutrition has become common practise to improve the availability of phytate-P and reduce phosphorus excretion into the environment. The efficacy of phytase in pigs and poultry has been clearly demonstrated (as reviewed by Coelho and Kornegay, 1996). New phytase products are continuously developed. IPA Mash is a phytase developed jointly by Novozymes and DSM Nutritional Products. As part of the registration dossier, the bio-efficacy of this product has to be determined with the target species.

In the current experiment, the bio-efficacy was determined of IPA Mash phytase with laying hens fed a corn/soy diet. Graded levels of the test product were applied by mixing it into mash layer diets.

2 Objectives

This study was carried out to determine the dose-response relationship of IPA Mash phytase in a layer diet on:

- Absorption coefficient of P and Ca measured in the 28th life week
- Tibia ash contents in the 28th life week
- Production performance of layers from the 26th till the 28th life week.

3 Sponsor

The study was carried out on request of:

DSM Nutritional Products Ltd
Building 241/ office 302
PO Box 2576
CH-4002 Basel
Switzerland
Tel: 41 61 815 8735
Fax: 41 61 815 8870
Study monitor: Dr J. Broz
Email: jiri.broz@dsm.com
Study director : C. Kwakernaak BSc.
Email: ckwakernaak@shothorst.nl

Date of execution of the animal experiment:

Start of the animal experiment :	3 November 2008
End of the animal experiment:	8 January 2009.

4 Material and methods

4.1 Experimental design

This trial was carried out as a complete randomised block design with five dietary treatments and six replicates per treatment for calcium and phosphorus absorption. Each replicate comprised four cages with four hens per cage (16 laying hens per replicate). Graded levels of the test product were added to a phosphorus deficient basal diet. The unsupplemented P-deficient basal diet was also fed as such (negative control diet) and was supplemented with 1.0 g P from dicalcium phosphate dihydrate (DCP) as positive control diet.

Table 1 Experimental dietary treatments

Treatment Group	Test product	Dose level
1	Negative control	-
2	As 1, plus test product	500 U/kg
3	As 1, plus test product	1000 U/kg
4	As 1, plus test product	2000 U/kg
5	Positive control	+1 g DCP-P/kg

[1] *The test product was added to diets 2, 3, and 4 on basis of 'top-dressing'. The added amount of test product was calculated based on the analysed phytase activity per g IPA Mash phytase preparation (see test material)*

4.2 Test material

The sponsor delivered the test product, according the following specifications:

Test product	:	IPA Mash phytase (M)
Producer	:	Novozymes A/S, Denmark
Supplier	:	Sponsor
Physical form	:	Powder
Active ingredient	:	6-Phytase
Analysed phytase activity*	:	60 700 U/g added test product (Lot PPQ 28656)
Production strain	:	Aspergillus oryzae
Storage conditions	:	Dry and protected from sun, 0-25°C.
Safety	:	See MSDS
Administration route	:	Orally, though feed
Administration duration:	:	23-25 weeks of age (15 days)

* *One U (Phytase unit) is defined as the amount of enzyme that releases 1 µmol of inorganic phosphate from sodium phytate per minute at pH 5.5 and 37 °C.*

4.3 Animal Origin

Animal	:	Poultry
Type	:	Laying hens
Breed/strain	:	ISA white
Sex	:	Female
Number of birds	:	480 (5 diets * 6 reps * 16 hens)
Origin	:	Registered supplier
Age at arrival	:	18 weeks of age

4.4 Animals, management and procedures

A total of 480 laying hens were delivered at Schothorst Feed Research at 16 weeks of age. After arrival the birds were allotted to 120 battery cages (0.25 m^2/ 4 birds per cage) divided over two tier levels. Target ambient temperature in the animal house was 21-22°C. The lighting schedule was gradually increased to 16 h light alternated with 8 h darkness at 21 weeks of age. Day and night periods were switched, because of the intention to sample ileal chyme during eggshell formation. After arrival the hens received a commercial pre-layer diet. When egg production started, the diet was switched to a commercial layer diet (mash). Starting in week 26 the experimental diets were fed after a two day transition period in which a 50/50 mixture of the commercial layer diet and the experimental diets was fed. Subsequently, the experimental diets (mash) were fed until the end of the experiment. Water and feed were supplied for *ad libitum* intake during the entire experiment.

The experiment was divided into three periods:
- Pre-experimental of 7 weeks:
- Transition-period of two days
- Experimental period of 15 days

Layers were fully vaccinated during the rearing period, prior to arrival at the test facility. During the experiment no vaccinations were applied.

4.5 Experimental diets

Diet composition

The experimental diets were based on a P-deficient (negative control) maize/soya- diet, formulated to contain 37 g Ca, 3.6 g P and 1.2 g absorbable P per kg diet (CVB, 2006). The calculated phytate P content was approx. 2.5 g/kg. Next a positive control diet was formulated by adding 1.0 g P with dicalcium phosphate, dihydrate (DCP) in exchange for limestone and diamol. The feed composition for both control diets is given in Appendix I. Chromium

oxide (Cr_2O_3) was added as an inert dietary marker. Besides a low absorbable P content, other dietary nutrients, minerals and vitamin levels were adequate to meet the hens' requirements.

Diet manufacturing
The diets were produced in a feed mill specialised in preparation of experimental diets of Arkervaart-Twente at Leusden, under the responsibility of Schothorst Feed Research. First a pre-basal diet was mixed, in a batch large enough to make all diets. Next, this batch was split into two parts to which the correct amounts of maize starch, limestone, diamol, marker and DCP were added to obtain the negative and positive control diets. The negative control diet was finally split into four sub-batches to which the graded levels of the test product were added and mixed thoroughly.

Feed samples at manufacture
Mash samples of each diet were taken at equal intervals during production. These samples were split into three portions for analysis by Schothorst Feed Research, by the sponsor and for storage.

Diet analyses
The negative control diet was analysed for dry matter, crude ash, N, Ca, P and phytate-P. The positive control diet was analysed for dry matter, ash, Ca and P. Finally, all experimental diets were analysed for dry matter and Cr2O3. These analyses were performed by the laboratory of Schothorst Feed Research and carried out in duplicate. Enzyme activities in the test product and in the diets were analysed by Biopract GmbH, Magnusstrasse 11, D-12489 Berlin, Germany

Diet presentation
The experimental diets were fed as mash.

4.6 Measurements

PRE-EXPERIMENTAL PERIOD (flock characteristics)
- Body weight of laying hens at 20 weeks of age
- Pre-experimental production performance during the 25th life week: Feed intake and egg production parameters (laying rate, average egg weight, daily egg mass, feed conversion ratio (for double yolked eggs, broken or shell-less eggs the average weight of a normal egg was used)).

EXPERIMENTAL PERIOD

Key parameters

- Apparent ileal digestibility of organic matter and apparent ileal absorbability of Ca and P in laying hens measured per experimental unit in the 28th life week.
- Tibia ash content of the right tibia of six laying hens per experimental unit in the 28th life week.

  In the 28th life week all birds were removed, weighed and euthanized with T61. Subsequently, the content of the last 20 cm of the ileum from 1 to 21 cm proximal of the ileo-caecal junction was collected and pooled per experimental unit. Next, the right tibiae of five birds per experimental unit were collected.

Indicative parameters

- Feed intake per experimental unit from the 26th till the 28th life week (14 days).
- Egg production parameters per experimental unit from the 26th till the 28th life week (14 days).
- Feed conversion ratio per experimental unit from the 26th till the 28th life week (14 days).
- Body weight of the laying hens at the end of the experiment in the 28th life week.
- Mortality.

4.7 Chemical analyses in chyme and bone material

Ileal chyme samples were freeze-dried, grinded and analysed for dry matter, ash, Ca, P and Cr. All analyses were carried out in simplo. Muscle residues and cartilage were removed from tibia bones after cooking in the autoclave. Next, the ash content was determined in tibia after extraction with petroleum ether.

4.8 Calculations and statistical analysis

Raw data were analyzed for outliers. Significant outliers were not included in the statistical analysis. Next, the experimental data were analyzed by analysis of variance using Genstat statistical software according the following model:

$$Y_{ijk} = \mu + Bloc_i + Treatment_j + e_{ijk}$$

Where:

Y	Response parameter
μ	General mean
Bloc	Effect of Bloc (i=16)
Treatment	Effect of diet (j=15)
Error	Error term

The P-value of the statistical model and the LSD (least significant difference at P= 0.05) are given per response parameter. Effects with P≤ 0.05 are considered to be statistically significant, whereas 0.05< P≤ 0.10 is considered to be a near-significant trend. Dose response relationships were calculated by the following exponential regression model:

$$Y = A + B * R^{X} + error$$

Where:

Y	Response parameter
A	Upper asymptote value
B	Response compared to upper asymptote without phytase supplementation
R	Slope ratio coefficient
X	Analysed dietary phytase activity
Error	Error term

4.9 Schedule of events

Age hens (wks)	Diet	days Of experimental period	Activities
18	Pre-layer		
20	Layer		Body weight hens
22	Layer		
23	Layer		
24	Layer		Start 7-day pre-experimental measuring period
25	50/50% layer/exp. diet		Start transition period
	50/50% layer/exp. diet		
	Experimental diets	1	Start experimental period
	Experimental diets	2	
	Experimental diets	3	
	Experimental diets	4	
	Experimental diets	5	
26	Experimental diets	6	
	Experimental diets	7	
	Experimental diets	8	
	Experimental diets	9	
	Experimental diets	10	
	Experimental diets	11	
	Experimental diets	12	
27	Experimental diets	13	
	Experimental diets	14	End of experimental period
	Experimental diets	15	Body weight and chyme collection

4.10 Welfare and health

The experiment was carried out according to the guidelines of the Dutch law for animal experiments and after approval of the experimental protocol by the Animal Experimental and Ethics Committee.

5 Results and discussion

5.1 General

Healthy 18-wk-old laying hens arrived at the institute. Average body weight of the hens at 20 weeks of age was 1493 g. The flock performance in week 25 was as follows:

Average laying rate: 94%
Average egg weight: 55.2 g
Average egg mass per hen: 51.6 g/d
Average feed intake: 113 g/d
Average feed conversion ratio: 2.18

The experimental was carried out without any deviations from the protocol except for the age of the laying hens. Compared to the protocol the laying hens were two weeks older at the start of the pre-experimental measuring period for the flock performance. No mortality occurred during the experimental period.

5.2 Experimental diets

The analysed contents for dry matter, crude protein, marker (Cr_2O_3), ash, phosphorus (P), phytate P, calcium (Ca) and the phytase activity are given in Table 2.

Table 2 Analysed nutrients and phytase activity in the experimental diets

TRT. No.	Dose U/kg	DM g/kg	CP g/kg	Cr_2O_3 g/kg	Ash g/kg	P g/kg	Phytate-P g/kg	Ca g/kg	Activity U/kg
1	0	897	153	0.61	113	3.24	2.10	33.60	<50
2	500	896	n.a.	0.60	n.a.	n.a.	n.a.	n.a.	556
3	1000	890	n.a.	0.58	n.a.	n.a.	n.a.	n.a.	1086
4	2000	898	n.a.	0.65	n.a.	n.a.	n.a.	n.a.	2583
5	+1 g P	898	n.a.	0.65	110	4.16	n.a.	32.80	<50

n.a. = not analysed, as all diets were obtained from the same basal diets, without further supplements of these nutrients.

The difference in analysed P content between the negative and positive control diet was 0.92 g/kg which is close to the expected difference. The Ca and P contents in both control diets was approximately 10% lower than expected. This was confirmed by re-analyses and must be due to lower contents in the pre-basal diet. Because the dietary marker was added to the basal diet prior to making the sub-batches the concentration was similar in all diets. Digestibility values were calculated with the mean analysed Cr_2O_3 value in the diets (0.68 g/kg DM). The analysed phytase activity in both control diets and in diets 2 and 3 met the target activity. The analysed phytase activity in diet 4 was higher than intended. According to the feed manufacturing reports correct amounts of the phytase preparation were added to the basal diet. Maybe the high calcium levels interfered with the phytase analyses. Because of this, the target dose levels are presented in the tables with results.

5.3 Results for production performance

In Tables 3 and 4 the results for the production performance of the laying hens are given.

Table 3 Results for body weight (BW), body weight gain (BWG) and laying rate of the laying hens

		wk 20	wk 20-28	wk 25	wk 26-28	
TRT. No.	Dose U/kg	BW g	BWG g	Laying [1] rate %	Laying rate %	Laying rate change (%)
1	0	1493	69	97	94	-2.5
2	500	1495	120	97	96	-0.6
3	1000	1490	98	98	97	-0.7
4	2000	1494	122	98	96	-1.5
5	+1 g P	1494	104	98	96	-2.0
P value		*NS*	*NS*	*NS*	*NS*	*NS*
LSD		*26*	*44*	*3.1*	*2.7*	*4.6*

[1] *Average laying rate during the last three days of the pre-experimental period*
NS = non significant (P >0.10)

Table 4 Results for egg weight, feed intake (FI) and feed conversion ratio (FCR) of the laying hens during the experimental period from 26 to 28 weeks of age

		wk 26-28				
TRT. No.	Dose U/kg	egg weight g	egg mass g/d	FI g/d		FCR
1	0	56.3	53.2	97.3	c	1.83
2	500	56.1	53.8	101.7	a	1.89
3	1000	56.0	54.2	100.1	a	1.85
4	2000	56.4	54.2	101.2	a	1.87
5	+1 g P	56.2	53.9	100.0	a	1.86
P value		*NS*	*NS*	*0.02*		*NS*
LSD		*0.7*	*1.9*	*2.55*		*0.06*

NS = non significant (P >0.10)
a,b,c Mean values without a common letter indicate significant differences (P≤0.05) within a column

Results show that the average laying rate of all groups was approx. 97% at the start of the experimental period. During the experimental period average laying rate decreased. This appeared to be larger for the negative control diet than for the other groups and was accompanied with a lower feed intake of the negative control group. Mean egg weight did not differ among the treatments and was higher than in the pre-experimental period (which is normal with increasing hen age). Although performance results are only indicative parameters due to the length of the experimental period and the limited number of birds per treatment, the decrease in laying rate on the negative control diet and a lower body weight gain indicate a P-deficiency on the negative control. Increasing the P supply via phytase or DCP supplementation apparently minimized these effects.

5.4 Results for mineral absorption and tibia ash content

The results for the ileal organic matter digestibility and absorbability of P and Ca are given in Table 5. Digestibility of the organic matter (approx. 81%) did not differ among the treatment groups and was considered as normal.

Table 5 Results for apparent ileal digestibility of organic matter (OM) and absorption coefficients of P and Ca and tibia ash content in fat free dry matter of laying hens in the 28th life week

TRT. No.	Dose U/kg	dc. OM %	abs. P %		rel.	abs. Ca %	rel.	Tibia ash g/kg DM		rel.
1	0	81.7	25.8	d	**100**	57.4	**100**	518	b	**100**
2	500	80.9	46.3	b	180	57.9	101	527	ab	102
3	1000	80.7	53.7	a	209	59.1	103	532	a	103
4	2000	80.9	57.6	a	224	60.7	106	530	a	102
5	+1 g P	81.8	34.2	c	133	60.6	106	523	ab	101
P value		*NS*	*<0.001*			*NS*		*0.02*		
LSD		*1.12*	*3.90*			*4.47*		*9*		

NS = non significant (P >0.10)
a,b,c Mean values without a common letter indicate significant differences (P≤0.05) within a column

A significant positive response to the phytase supplementation was found for P absorption. Compared to the negative control group the group with the lowest phytase supplementation level of 500 U/kg already improved P absorption significantly from 26% to 46%. A dose level of 1000 U/kg resulted in a further significant improvement of P absorption up to 54%. Doubling the dose level to the highest inclusion level of 2000 U/kg gave further improvement of P absorption to 58%, but this increase could not be shown to be significant.

Total P absorption, P excretion and phytate P degradation at ileal level was calculated and given in Table 6. Assuming that the increased P absorption in the phytase supplemented diets is fully accounted for by phytate P degradation, it can be calculated that the degradation coefficient was increased to 32, 43 and 49% for the dose levels 500, 1000 and 2000 U/kg respectively.
P absorption of the positive control group was significantly higher compared to the negative control group. For the absorption of P from DCP a coefficient of 64% could be calculated. In broilers the retainable P content (as a percentage of total P) for DCP (dihydrate) is 78%.The lower value in layers is most probably due to differences in Ca and P metabolism in layers compared to broilers (Van der Klis et al 1997 also published a lower P absorbability from MCP in layers compared to broilers (59-70 versus 83%)).

Phytase supplementation improved calcium absorption non significantly, resulting in a significantly higher tibia ash content when 1000 or 2000 U/kg phytase was added to the diet. The lowest phytase inclusion level or extra DCP-P gave a numerical improvement of tibia ash contents compared to the negative control. The small response on tibia ash content is in agreement with a previous study (Report 843) and will most probably become more pronounced in a long-term study.

Table 6 Results for total apparent ileal P absorption and calculated phytate P degradation and P excretion of laying hens in the 28th life week

TRT. No.	Dose U/kg	P absorption g/kg diet		P absorption g/d		Phytate-P Degradation[1] %	P excretion g/kg diet	P excretion % of tot. P
1	0	0.84	c	0.06		0	2.41	74
2	500	1.50	b	0.13		32	1.74	54
3	1000	1.74	a	0.15		43	1.50	46
4	2000	1.87	a	0.15		49	1.37	42
5	+1 g P	1.42	b	0.12		0	2.74	66
P value		*<0.001*						
LSD		*0.13*						

[1] *Based on the assumption of 0 in the negative control diet*

a,b,c *Mean values without a common letter indicate significant differences (P≤0.05) within a column*

5.5 Dose-response relationship and P-equivalence

Assuming a linear dose-response relationship up to a phytase inclusion level of 500 U/kg, 100 U phytase/kg was equivalent to 0.13 g absorbable P. At higher dose levels no linear relationship could be assumed. For this reason an exponential dose-response curve was fitted (P<0.001) for the phytase activity and the increase in absorbable P per kg diet. The relationship is illustrated in Figure 1.

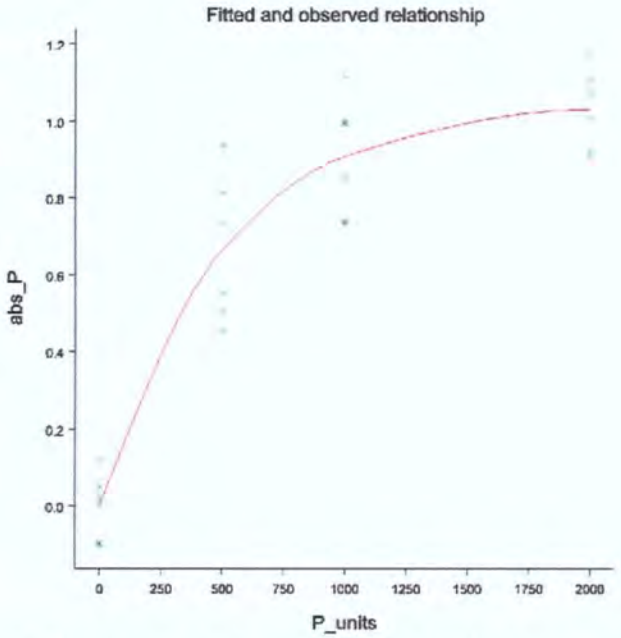


Figure 1 The exponential relationship between dosed phytase activity in the diet (Punits in U/kg diet) and the amount of absorbable P at ileal level (absP in g/kg diet intake) of laying hens in the 28 th life week

The equation of the relationship is:

absorbable P (g/kg diet) = 1.050 – 1.050 * 0.9980 $^{U/kg\ diet}$

Percentage variance accounted for (PVA) by the model is 90%

Based on the fitted dose response curve and linearity between the dietary P content and P retention for 1 g DCP-P it was calculated that 469 U/kg was equal to 1 g DCP-P/kg diet in laying hens.

6 Conclusions

From this experiment with laying hens it was concluded that the dietary supplementation of IPA Mash phytase improved apparent ileal P absorption and tibia ash contents in the 28th life week with the following details:

1. The lowest inclusion level of the phytase of 500 U/kg improved ileal P absorption significantly compared to the P-deficient negative control group (~~8~~0%). Next dose level step of 1000 U/kg resulted in a further significantly improvement of the ileal P absorption (~~2~~9% compared to the previous 500 U/kg s upplementation level). The highest dose level step of 2000 U/kg improved ileal P absorption with ~~1~~5% compared to the 1000 U/kg supplementation level. The latter increase was no longer statistically significant.
2. The ileal Ca absorption was numerically improved by dietary phytase supplementation, resulting in significantly higher tibia ash contents at an inclusion level of 1000 or 2000 U/kg compared to the negative control group.
3. Tibia ash content was significantly improved (2-3%) when 1000 or 2000 U/kg of the phytase was supplemented to the diet compared to the negative control group.
4. Based on an exponential dose-response curve 500 U of the phytase per kg diet was equal to 0.66 g absorbable P at ileal level.
5. The absorption coefficient for P from the dicalcium phosphate (dihydrate) was 64%. Based on the exponential dose response curve, 1 g P from DCP was equal to 469 U/kg.

7 References

Coelho, M.B. and E.T. Kornegay (1996). Phytase in animal nutrition and waste management. A BASF reference manual. ISBN 1-889640-03-04

Genstat 7 Reference Manual, Oxford University Press, UK

Kwakernaak, C. and J.D. van der Klis (2007). Efficacy of Ronozyme®NP phytase preparation in layer diets using P and Ca absorption as response parameters. Schothorst Report no. 843

Van der Klis, J.D., H.A.J. Versteegh, P.C.M. Simons and A.K. Kies (1997). The efficacy of phytase in corn-soybean meal-based diets for laying hens. Poultry Science 76:1535-1542

Veevoedertabel 2005, CVB, Lelystad, The Netherlands.

8. Summary

An experiment was carried out by Schothorst Feed Research to determine the effect of IPA Mash phytase on the apparent ileal calcium (Ca) and phosphorus (P) absorption in laying hens around peak production. The phytase product was delivered by DSM Nutritional Products Ltd. Graded levels of the test product (phytase levels of 0, 500, 1000 and 2000 U/kg) were added to a maize/soya P-deficient basal diet (negative control (NC) group), containing 33.6 g Ca, 3.2 g P and 2.1 g phytate-P per kg diet. A positive control (PC) was made by supplementing the NC diet with 1 g P from dicalcium phosphate dihydrate (DCP) per kg diet. The experimental diets were fed to the laying hens from 26th to the 28th life week (15 days). All experimental mash diets were fed for *ad libitum* intake. Each dietary treatment was assigned to six replicates, each replicate consisted of four cages with four hens per cage. Ileal absorption of P and Ca and tibia ash content were determined at the last day of the trial.

From this experiment it was concluded that the supplementation of IPA Mash phytase preparation to a laying hen diet improved apparent ileal P absorption. The lowest inclusion level of the phytase of 500 U/kg already improved ileal P absorption significantly compared to the P-deficient NC group (80%). Increasin g the dose level up to 1000 U/kg resulted in further significantly improvement of the P absorption (109% compared to NC). The highest dose level step of 2000 U/kg improved ileal P absorption with 124% compared to NC but this was not significantly different with the effect obtained with the 1000 U/kg group. Phytase supplementation also numerically improved ileal Ca absorption, resulting in an increased tibia ash content at an phytase inclusion level of at least 1000 U /kg diet. Based on an exponential fitted dose-response curve 500 U of the phytase preparation per kg diet was equal to 0.66 g absorbable P and 1 g P from DCP was calculated to be equal with 469 U/kg of IPA Mash phytase.

Appendix 1 Ingredients and calculated nutrient composition of the experimental diets

	Neg. Control Low aP g/kg	Pos. Control +1g DCP-P g/kg
Maize	500.0	500.0
Maize gluten meal	31.4	31.4
Maize gluten feed	18.7	18.7
Soybean meal	142.7	142.7
Sunflower seed meal	90.9	90.9
Animal fat	24.5	24.5
NaHCO3	2.6	2.6
Premix min+vit	10.0	10.0
Premix lys	2.95	2.95
Marker[1]	0.75	0.75
Corn starch	70.0	70.0
Limestone	79.3	79.3
Corn starch	18.17	18.17
Limestone	3.31	-
DCP[2]	0.00	5.50
Diamol	4.68	2.50
	1000.00	1000.00
Calculated nutrients (g/kg)		
Ca	37	37
P	**3.6**	**4.6**
Phytate P	2.5	2.5
Absorbable P	1.2	2.0
Na	1.6	1.6
K	6.5	6.5
Cl	1.6	1.6
AMEn (layers in kcal/kg)	2800	2800
Crude protein	156	156
Crude fat	50	50
dig. Lys	6.3	6.3
dig. Met€ys	5.7	5.7
dig. Thr	4.8	4.8
dig.Trp	1.4	1.4

[1] 0.75 g/kg Cr_2O_3 was added to all diets as inert marker.

[2] Aliphos®ical was used as dicalcium phosphate (dihydrate)

Appendix 2 Raw Data

RAW DATA PLE-48

Tier level; 1= bottom ; 2 = top
dc. = digestibility coefficient
abs. = absorption coefficient

Exp. Unit	Block level	Tier level	Diet Group	wk 28 dc. organic matter	wk 28 abs. P	wk 28 abs. Ca	wk 28 Tibia ash	wk 28 P absorption	wk 26-28					wk 20 BW	wk 28 BW	wk20-28 BWG	wk 25 Laying rate 3 days	wk25-28 Laying rate change
									Laying rate	Egg weight	Egg mass	FI	FCR					
No.				%	%	%	g/kg	g/kg diet	%	g	g/d	g/d	g/g	g	g	g	%	%
1	1	1	3	79,1	48,6	45,8	542,6	1,575	97,3	56,2	54,7	101,1	1,846	1469	1547	78,1	95,8	1,5
2	1	1	2	79,1	39,8	50,1	*	1,289	93,3	55,2	51,5	103,6	2,014	1519	1603	84,4	95,8	-2,5
3	1	1	4	78,7	54,2	50,1	523,8	1,756	97,8	56,1	54,8	102,2	1,866	1497	1613	115,6	95,8	1,9
4	1	1	1	78,9	22,7	43,8	519,7	0,735	96,4	56,1	54,1	97,9	1,807	1466	1538	71,9	91,7	4,8
5	1	1	5	79,5	29,5	50,8	522,8	1,229	96,9	56,4	54,6	102,3	1,873	1500	1597	96,9	95,8	1,0
6	2	1	1	80,0	22,8	51,4	517,0	0,737	97,3	57,7	56,1	100,6	1,792	1519	1597	78,1	100,0	-2,7
7	2	1	4	78,8	59,9	60,0	520,6	1,941	97,3	56,9	55,3	100,3	1,812	1513	1597	84,4	100,0	-2,7
8	2	1	5	79,7	33,6	60,0	519,3	1,397	97,3	56,4	54,9	100,7	1,835	1488	1572	84,4	97,9	-0,6
9	2	1	3	79,5	48,5	58,5	536,2	1,571	94,6	55,9	52,9	99,9	1,889	1531	1597	65,6	95,8	-1,2
10	2	1	2	80,1	42,8	52,0	517,2	1,387	98,2	56,4	55,4	101,9	1,840	1525	1616	90,6	100,0	-1,8
11	3	1	3	79,6	56,6	45,2	544,8	1,834	96,0	55,5	53,2	102,0	1,916	1478	1563	84,4	102,1	-6,1
12	3	1	1	80,9	26,5	52,4	511,8	0,857	89,7	56,0	50,3	92,8	1,843	1469	1519	50,0	97,9	-8,2
13	3	1	4	81,4	56,9	56,6	542,5	1,845	96,4	56,7	54,7	105,9	1,937	1459	1653	193,8	93,8	2,7
14	3	1	2	80,3	41,4	52,0	536,3	1,340	96,9	56,5	54,7	101,1	1,848	1519	1622	103,1	91,7	5,2
15	3	1	5	83,4	36,5	54,7	534,7	1,517	91,1	55,9	50,9	99,4	1,956	1469	1575	106,3	100,0	-8,9
16	4	2	1	83,9	29,5	54,7	507,6	0,957	93,3	56,4	52,6	96,0	1,825	1481	1553	71,9	95,8	-2,5

RAW DATA PLE-48

Tier level; 1= bottom ; 2 = top
dc. = digestibility coefficient
abs. = absorption coefficient

Exp. Unit	Block level	Tier level	Diet Group	wk 28 dc. organic matter	wk 28 abs. P	wk 28 abs. Ca	wk 28 Tibia ash	wk 28 P absorption	wk 26-28 Laying rate	wk 26-28 Egg weight	wk 26-28 Egg mass	wk 26-28 FI	wk 26-28 FCR	wk 20 BW	wk 28 BW	wk20-28 BWG	wk 25 Laying rate 3 days	wk25-28 Laying rate change
No.				%	%	%	g/kg	g/kg diet	%	g	g/d	g/d	g/g	g	g	g	%	%
17	4	2	4	82,8	62,1	54,0	521,6	2,013	98,2	56,7	55,6	99,2	1,783	1494	1591	96,9	100,0	-1,8
18	4	2	3	79,8	52,1	62,1	513,1	1,687	98,2	57,8	56,8	102,2	1,799	1484	1688	203,1	95,8	2,4
19	4	2	5	82,1	34,0	60,0	503,8	1,413	96,0	57,5	55,2	98,7	1,790	1497	1591	93,8	97,9	-1,9
20	4	2	2	82,0	54,7	58,3	522,0	1,772	94,2	56,6	53,3	99,1	1,860	1472	1581	109,4	100,0	-5,8
21	5	2	2	81,9	50,9	59,5	528,0	1,648	96,9	55,3	53,6	102,4	1,910	1459	1631	171,9	93,8	3,1
22	5	2	3	83,4	56,5	70,5	526,6	1,831	98,2	55,4	54,4	97,7	1,795	1466	1541	75,0	97,9	0,3
23	5	2	5	83,6	36,8	67,2	527,9	1,530	95,5	55,3	52,8	99,3	1,880	1509	1631	121,9	97,9	-2,4
24	5	2	4	82,0	53,7	66,2	534,7	1,739	92,0	55,5	51,0	98,7	1,934	1478	1606	128,1	100,0	-8,0
25	5	2	1	83,2	25,9	68,8	530,0	0,840	95,5	55,7	53,3	97,6	1,833	1494	1606	112,5	97,9	-2,4
26	6	2	2	82,0	48,5	75,4	529,4	1,571	96,4	56,4	54,3	102,2	1,880	1478	1638	159,4	97,9	-1,5
27	6	2	3	82,7	60,1	72,5	530,3	1,949	96,9	55,0	53,3	97,5	1,829	1513	1594	81,3	97,9	-1,0
28	6	2	5	82,4	34,6	71,0	528,0	1,440	98,7	55,9	55,1	99,4	1,802	1500	1622	121,9	97,9	0,7
29	6	2	1	83,3	27,2	73,4	*	0,883	94,2	55,7	52,5	98,7	1,881	1531	1559	28,1	97,9	-3,7
30	6	2	4	81,5	58,9	77,3	535,0	1,909	94,6	56,4	53,4	100,7	1,886	1522	1638	115,6	95,8	-1,2

** = missing values due to measurement error*

Annex C EFSA Trial Protocol Data Sheet

efsa
European Food Safety Authority

FEEDAP UNIT

ANNEX C [1]

TRIAL PROTOCOL DATA SHEET: FOR TERRESTRIAL ANIMALS

Identification of the additive: **IPA Mash Phytase**	Batch number: **Lot PPQ 28656**
Trial ID: **PLE-48**	Location: **Schothorst Feed Research**
Start date and exact duration of the study: **3 November 2008 - 9 January 2009 (66 days)**	
Number of treatment groups (+ control(s)): **5**	Replicates per group: **6**
Total number of animals: **480**	Animals per replicate: **16**
Dose(s) of the additive/active substance(s)/agent(s) (mg/Units of activity/CFU kg^{-1} complete feed/L^{-1} water)	
Intended: **0, 500, 1000, 2000, 0 U/kg**	Analysed: **<50, 556,1086, 2583, <50 U/kg**
+	
Substances used for comparative purposes: **dicalcium phosphate (anhydrous) (DCP)**	
Intended dose: **+1 g DCP-P per kg diet**	Analysed: **+0.92 g P per kg diet**
Animal species/category: **Laying hens**	
Breed: **LSL White**	Identification procedure: **Cage number**
Sex: **Female** Age at start: **18 weeks old**	Body weight at start: **1493 g (20 weeks of age)**
Physiological stage: **Egg laying**	General health: **Good**
Additional information for field trials:	
Location and size of herd or flock:	
Feeding and rearing conditions:	
Method of feeding:	
Diets (type(s)): **Low P laying hen diets**	
Presentation of the diet: Mash ☒ Pellet ☐ Extruded ☐ Other	
Composition (main feedingstuffs): **Maize (50%), Soybean meal (14%), Sunflower seed meal (9%), Corn starch (9%), limestone (8%)**	
Nutrient content (relevant nutrients and energy content)	
Intended values: **Negative control : CP=156 g/gkg; Ca=37 g/kg; P=3.6 g/kg; AMEn = 2800 kcal/kg**	
Positive control : CP=156 g/gkg; Ca=37 g/kg; P=4.6 g/kg; AMEn = 2800 kcal/kg	
Analysed values: **Negative control : CP=153 g/gkg; Ca=33.6 g/kg; P=3.2 g/kg**	
Positive control : Ca=32.8 g/kg; P=4.2 g/kg	
Date and nature of the examinations performed: **Body weight at wk 20 and 28, egg production parameters in week 25 and from 26-28 weeks of age, nutrient and mineral absorption and tibia-ash content in week 28**	
Method(s) of statistical evaluation used: **Effect of treatment means (diets) with ANOVA and dose-response relation with an exponential regression model both carried out with GENSTAT statistical software**	
Therapeutic/preventive treatments (reason, timing, kind, duration): **-**	

[1] Please submit this form using a common word processing format (e.g. MS Word).

Timing and prevalence of any undesirable consequences of treatment: -	
Date **17 May 2009**	Signature Study Director

† In case the concentration of the additive in complete feed/water may reflect insufficient accuracy, the dose of the additive can be given per animal day^{-1} or mg.kg^{-1} body weight or as concentration in complementary feed.

TAB

ANNEX 27

Annex 27

Aureli, R. et *al.* (2009). Report No. 00000099: Effect of graded levels of bacterial 6- phytase on apparent ileal digestibility of phosphorus in laying hens fed a maize-based diet low in phosphorus content (H-01/09). 2009

REPORT No.00000099
Regulatory Document

DSM

Document Date:	09-June-2009
Author(s):	Raffaella Aureli [1], Petra Philipps [1] and Jiri Broz [2]
	[1]NRD/CA, DSM Nutritional Products France
	[2]NRD/CA, DSM Nutritional Products Ltd, Switzerland
Title:	Effect of graded levels of bacterial 6- phytase on apparent ileal digestibility of phosphorus in laying hens fed a maize-based diet low in phosphorus content (H-01/09)
Project No.	6106
Compound No.	

Summary

An experiment was carried out to determine the effect of bacterial 6- phytase on the mineral digestibility at ileal level in laying hens. Graded levels of the test product (500, 1000 and 2000 U per kg feed) were added to a maize/soybean meal based, P deficient basal diet (negative control group) containing 2.9 g P total per kg feed. The basal diet was also supplemented with 1 g P from DCP per kg to obtain a positive control group. Each dietary treatment was assigned to 24 replicates; each replicate consisted of one cage with two hens of 23 weeks of age per cage. The laying hens were fed with the low phosphorus basal diet without enzyme supplementation until 25 weeks of age to induce a phosphorus deficiency. The apparent ileal digestibility of phosphorus was clearly improved by phytase supplementation in a dose-dependent manner compared to the negative control treatment. The effects were significant ($p<0.01$). Relative improvements from 19.8 % to 28.2 % were demonstrated with dietary inclusion levels of phytase of 500 to 2000 U per kg feed compared to the negative control. The response of the apparent ileal digestibility of phosphorus to the addition of the phytase could be described by a non-linear regression $y = 45.9 + 12.6\ (1-e^{-0.0019x})$, R^2 =0.98. Under the conditions of the present trial, tibia strength responded to phytase supplementation in a linear manner (R^2=0.99) with numerical improvement in a range of 12 % to 38 %.

This report consists of pages 1-10

Distribution

Dr. J.Broz, NRD/CA
Dr. M. Eggersdorfer, NRD
Dr. F. Fru, NRD/PA
Mr . J.-F. Hecquet, NBD/RA
Dr.A.-M. Kluenter, NRD/CA
Dr. J. Pheiffer, NRD/PA
Dr.P.Philipps, NRD/CA

Approved

Name	Signature	Date
Main Author R. Aureli NRD/CA		10/06/2009
Principal Scientist/Competence Mgr Dr. J. Broz NRD/CA		12.6.2009
Research center Head Dr.A.-M. Kluenter NRD/CA		10.6.2009
Project Manager Dr. F. Fru, NDR/PA		15.06.2009

Nomenclature and Structural Formula

A liquid preparation of bacterial 6-phytase (IPA Phytase (L)), batch PPQ 28432 was used in this study, manufactured by Novozymes A/S, Bagsvaerd, Denmark

Author(s): Raffaella Aureli[1], Petra Philipps[1], Jiri Broz[2]

Department(s) and Adress(es) : [1]NRD/CA, DSM Nutritional Products France
[2]NRD/CA, DSM Nutritional Products Ltd, Switzerland

Title: Effect of graded levels of a bacterial 6-phytase on apparent ileal digestibility of phosphorus in laying hens fed a maize-based diet low in phosphorus content (H-01/09).

Abstract

An experiment was carried out to determine the effect of bacterial 6- phytase on the mineral digestibility at ileal level in laying hens. Graded levels of the test product (500, 1000 and 2000 U per kg feed) were added to a maize/soybean meal based, P deficient basal diet (negative control group) containing 2.9 g P total per kg feed. The basal diet was also supplemented with 1 g P from DCP per kg to obtain a positive control group. Each dietary treatment was assigned to 24 replicates; each replicate consisted of one cage with two hens of 23 weeks of age per cage. The laying hens were fed with the low phosphorus basal diet without enzyme supplementation until 25 weeks of age to induce a phosphorus deficiency. The apparent ileal digestibility of phosphorus was clearly improved by phytase supplementation in a dose-dependent manner compared to the negative control treatment. The effects were significant ($p<0.01$). Relative improvements from 19.8 % to 28.2 % were demonstrated with dietary inclusion levels of phytase of 500 to 2000 U per kg feed compared to the negative control. The response of the apparent ileal digestibility of phosphorus to the addition of the phytase could be described by a non-linear regression $y = 45.9 + 12.6 (1-e^{-0.0019x})$, R^2 =0.98. Under the conditions of the present trial, tibia strength responded to phytase supplementation in a linear manner (R^2=0.99) with numerical improvement in a range of 12 % to 38 %.

NTRODUCTION

The laying hens require phosphorus for the production of the egg itself and to maintain skeletal integrity. Lack of sufficient phosphorus in the diets causes rickets in young chicks and poor shell quality and osteoporosis in laying hens. The majority of phosphorus in the feedstuffs of poultry is present in the chemical structure of phytic acid. The availability of phytate phosphorus is very low in poultry due to the inability of the birds to produce sufficient amount of endogenous phytase. The additional phosphorus added to the diet to meet the requirement leads often to excess phosphorus excretion in manure. Then, increasing development of microbial phytase has been necessary to reduce phosphorus supply and to improve the availability of phytate P present in feedstuff

In the frame of the IPA Mash project, the bacterial 6-phytase has been developed to enhance *in vivo* bioavailability of phosphorus in mash feed. The aim of the present laying hens' trial was to generate some data for the registration of the microbial phytase

The response of the laying hens on phytase supplementation was evaluated in terms of phosphorus utilization, egg production, and bone mineralization .The laying hens were fed a maize diet low in phosphorus content and supplemented with graded levels of the test product which was applied by spraying it into mash diets. The bacterial 6-phytase was tested at 500, 1000 and 2000 U per kg feed.

MATERIALS AND METHODS

The effect of the bacterial 6-phytase on the apparent ileal digestibility of phosphorus in laying hens was studied in a 4-week digestibility trial. The trial (H-01/09) was performed at the Research Center for Animal Nutrition (CRNA, DSM Nutritional Products France, F-68305 Village-Neuf) according to the official French instructions for experiments with live animals.

240 laying hens (Isa Brown), 23 weeks of age, supplied by a commercial hatchery (Elevage Avicole du Sundgau SARL, Route de Chavannes -sur -l' Etang, F-68210 Bréchaumont, France), were divided into groups of two hens per cage. The 120 groups were randomly allocated to five treatments with 24 replicates per treatment. The laying hens were housed in battery cages in an environmentally controlled room at a room temperature of 16°C. Experimental diets and tap water were made available for *ad libitum* consumption. In a 14-day pre-experimental period the laying hens were fed the low phosphorus basal diet containing 62.5 µg/kg Vitamin D_3, and without enzyme supplementation.

The basal diet in mash form was formulated based on maize (65.0 %) and soybean meal (23.6 %) as main ingredients to contain 2.6 g P /kg diet, and 34.5 g Ca /kg diet. To facilitate the ileal digestibility measurements, titanium dioxide (TiO_2) was added to the feed as inert dietary marker at a concentration of 1000 mg per kg feed. Besides a low absorbable P content the supply of other nutrients, minerals and vitamins with the diet to the hens were adequate to meet the hen's requirement. The detailed composition of the basal diets , the analyzed nutrient contents and the metabolisable energy (ME), calculated on the basis of analyzed nutrients (EC-equation, EEC, 1986) are listed in **Table 1.**

Beside the control treatment without enzyme supplementation, graded levels of the phytase were added to a phosphorus deficient basal diet. The unsupplemented P-deficient basal diet was also fed as such (negative control diet) and was supplemented with 1.0 g P from dicalcium phosphate dehydrate (DCP) as positive control diet. The bacterial 6-phytase in liquid form (lot PPQ 28432 with analysed phytase activity of 24450 U/g) was added at 500, 1000 and 2000 U per kg feed.

The added amount of the test product was based on the analysed phytase activity. Appropriate amount of the liquid preparation of the phytase product was diluted with 240 ml water and sprayed onto the mash feed to get the final concentrations in the feed corresponding to the different treatments. For procedural balance of all treatments, 240 ml of water were also sprayed onto the mash of the negative and positive control diets.

Feed samples were taken for analysis of the phytase activities. The determination of the phytase activity in the experimental diets was performed by BIOPRACT GmbH, D-12489 Berlin (Germany) on behalf of DSM Nutritional Products. One unit (U) of phytase is defined as the activity that release 1µmol inorganic phosphate from 5.0 mM phytate per minute at pH 5.5 and 37 °C.

The groups of hens were weighed at the beginning and at the end of the experimental period of the trial. Feed consumption was determined for the experimental period of four weeks. The eggs were collected daily and the number of broken eggs was noted for each group. Once a week, the collected eggs were weighed per group. Total egg production, egg weight and rate of broken eggs were calculated per group.

Excreta from six cages from each treatment were collected by a total collection method after three weeks of feeding experimental diets. The excreta were quantitatively collected once per day. The excreta from three days were pooled per group and were stored frozen (at -20°C), each day directly after collection. After thawing the total excreta of each group were homogenized, representative samples were taken and the percentage of dry matter and ash, as the concentration of phosphorus were determined.

At 29 weeks of age blood samples from six selected groups of hen per treatment were taken from *Vena jugularis*. The concentrations of inorganic phosphate (Pi) and calcium (Ca) in the plasma were determined with a Cobas®6000 module C 501 automatic analyzer according to the method described by Henry (1974) and Gindler and King (1972), using Roche Diagnostic kits PHOS 03183793 122 and Ca 20763128 322.

At the end of the trial, the hens were euthanized by cervical dislocation and the content of the terminal part of the ileum, defined as from 17 to 2 cm before the ileo-caecal junction, were sampled, pooled for two hens per cage, freeze-dried, and ground for chemical analysis. The contents of Ca and P as well as the concentration of TiO_2 as indigestible marker were determined in the digesta samples and in the feed.

The bone quality was assessed by measuring tibia strength and tibia ash percentage. The right tibiotarsuses were taken from six selected groups of hens per treatment. Tibiae were defleshed, and cartilaginous caps were removed after collection. They were kept frozen in plastics bags at -20°C to maintain wetness until analysis of ash content and breaking strength.
A segment of the central portion of the bone shaft (about 2 cm long) was prepared for use in determining bone strength in which an LR10K compression machine with a XLC/10K/A1 force captor and a compression device TH23-196/AL (Lloyd Instruments, Fareham, UK) was used to determine the force (in Newton) necessary to break the bone. Broken bones were pooled per cage, defatted with ethanol and ether, dried and incinerated at 550°C. Tibia ash was expressed as a percentage of dry bone weight.

In the same time, toe samples were obtained by severing the left middle toe through the joint between the second and the third tarsal bones from the distal end. The toes of the two hens within a cage were pooled. The composite samples were dried and then ashed in a muffle furnace at 550°C to determine toe ash as a percentage of dry weight.

The analyses of the nutrient content in the feed samples were performed according to standard methods (VDLUFA 1976) (Table 1). TiO_2, Ca and total P in feed, excreta and ileum content were determined by ICP according to DIN EN ISO 11885:1997 (DIN EN ISO 1998) after HNO_3 / H_4FN micro-onde mineralization. Phytate in feed was determined colorimetrically as released P after extraction, elution and wet digestion with HNO_3/H_2SO_4 (AOAC 1990).
The apparent ileal digestibility of phosphorus were calculated as follows:

$$\text{Value of apparent ileal digestibility for a nutrient X in \%} = 100 - 100 \times \left(\frac{[TiO_{2\,D}]}{[TiO_{2\,IC}]} \times \left(\frac{[X_{IC}]}{[X_D]} \right) \right)$$

where $[TiO_{2\,D}]$ = titanium dioxide content in the diet; $[TiO_{2\,IC}]$ = titanium dioxide content in the ileal content; $[X_D]$ = nutrient X content in the diet and $[X_{IC}]$ = nutrient X content in the ileal content (all parameters in g / kg DM).

For the statistical evaluation of the performance data, a one-factorial analysis of variance (factor: treatment) was carried out, using the software "Stat Box Pro", version 5.0 (Grimmer soft 1995). Where significant treatment effects ($p < 0.05$) were indicated, the differences among treatment means were analyzed with the Newman-Keuls test.

Non-linear regression analysis was performed with the program Origin 7.0. An exponential model of the following type was fitted to the data:

$$y = a+b\,(1-\exp(-kx))$$

with a: response (y-value) at zero phytase supplementation
b: maximum response to supplemented phytase (a+b = upper asymptote)
k: parameter describing the steepness of the curve
x: supplemented phytase (U/kg)
y: response (P utilization or utilized P concentration in the diet)

RESULTS AND DISCUSSION

The detailed composition of the basal diet, the analyzed nutrient contents and the metabolisable energy (ME), calculated on the basis of analyzed nutrients (EC-equation, EEC, 1986) are listed in **Table 1.** Analyzed nutrient contents were close to the calculated values. The protein content of the feed was 169 g and the basal diet contained 12.2 MJ ME per kg diet. Phosphorus content was nearly as expected. The laying hens were fed a diet containing 2.9 g total phosphorus per kg feed (mean of the diets A to D, as all diets were obtained from the same basal diet). The difference between P content in the positive and the negative control was 1.0 $g.kg^{-1}$. The content of non-phytic acid phosphorus in the basal diet was 0.70 g per kg feed, calculated as the difference between total phosphorus content and content of phytic phosphorus per kg feed. The content of calcium (23.1 $g.kg^{-1}$) was lower than intended.

Table 2 shows the determined product contents in the feed. The analyses were performed on the basis of phytase-activity. As intended, the native phytase activity in the basal diet was under the limit of quantification (LOQ). The analyzed phytase activities of the experimental diets used throughout the experiment were according to the target dosages.

The effects of phytase on performance parameters are shown in **Table 3** from day 15 to day 42. No significant differences among treatments were observed in egg weight, egg production and percentage of broken eggs. Nevertheless the egg production was improved by 2.7 % and 5.7 % with the addition of 500 and 1000 U of bacterial 6-phytase per kg feed, respectively, over the

negative control. Body weight at the beginning and at the end of the experiment is shown in **Table 4**. There were no significant differences among treatments in body weight at the beginning and at the end of the experiment. The lack of response of phytase supplementation to a low available phosphorus diet on performance parameters might suggest that four weeks of P depletion might be not enough to show signs of deficiency. Because of a short experimental period, performance results are indicative parameters.

The results of the apparent ileal digestibility of phosphorus are shown in **Table 5**. For analytical reason (laps in quantifying feed consumption), four cages in the treatment A (negative control) and one in the treatment C were eliminated from the statistical analysis and presentation of the results. The apparent ileal digestibility of phosphorus was clearly improved by phytase supplementation in a dose-dependent manner compared to the negative control treatment. The effects were significant ($p<0.01$). A relative improvement from 19.8 % to 28.2 % was demonstrated with dietary inclusion level of phytase of 500 to 2000 U per kg feed compared to the negative control.

In addition, the response of the apparent ileal digestibility of phosphorus to the addition of the phytase can be described by a non-linear regression. The regression curve clearly demonstrates a dose-dependent effect of the phytase on the apparent ileal P-digestibility with $y = 45.9 + 12.6\ (1-e^{-0.0019x})$, $R^2 = 0.98$ (**Figure 1**).

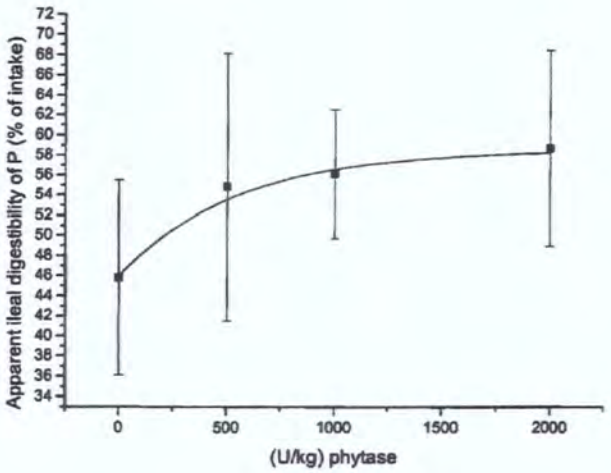


Figure 1: Effect of the supplementation of bacterial 6- phytase on apparent ileal P-digestibility in laying hens (mean ± stdv)

Moreover the effect of the phytase supplementation on apparent P digestibility for all supplementary levels was confirmed by the reduction of the P excretion (**Figure 2**).
The phosphorus concentration in excreta was numerically affected by phytase supplementation with a tendency to be significant ($p = 0.120$). The lowest P concentration in excreta was measured in birds fed 2000 U phytase per kg feed.

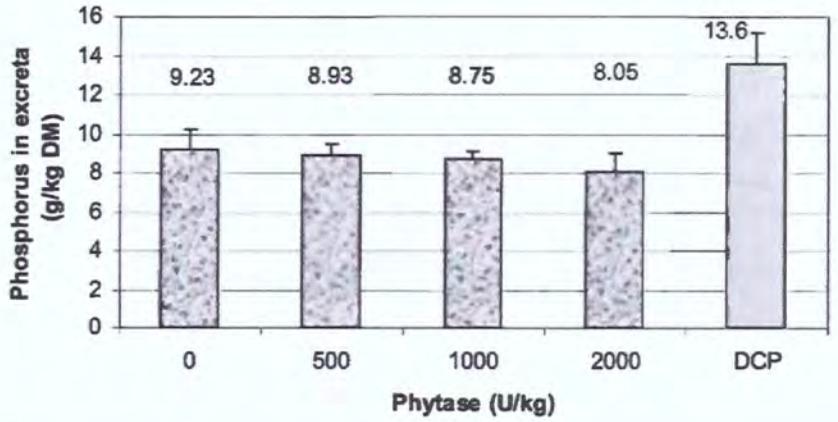


Figure 2: Effect of the supplementation of bacterial 6-phytase on Phosphorus in excreta (mean ± stdv)

The concentrations of inorganic phosphorus (Pi) and calcium (Ca) in the plasma are presented in **Table 6**. Mineral concentrations in plasma were not significantly affected by the inclusion of the phytase over the negative control. However, numerical increase of the concentration of Pi and Ca better than the positive control was recorded at 500 U phytase per kg feed.

Table 7 shows the effect of the phytase supplementation on the bone parameters. Tibia strength responded to phytase supplementation in a linear manner (R^2=0.99) up to 2000 U per kg feed but not significantly. Numerical improvement in a range of 12 % to 38 % was recorded. The effects on tibia strength obtained with the phytase were higher than this obtained with the positive control.
The addition of extra phosphorus from DCP or phytase to the diet had no effect on the tibia ash content. A numerical improvement of 1.8 % was only noted with the inclusion of 500 U phytase per kg feed over the negative control.

Toe ash measurements are shown in **Table 8**. Toe ash has been shown to be a good measurement of P status and accurate in determining P availability for poultry (Potter, 1988). In this experiment, phytase supplementation was not effective in improving toe ash. Only the inclusion of additional phosphorus showed a non-significant improvement of 5.2 % of toe ash over the negative control.

The results of the present trial with laying hens demonstrated that the supplementation of bacterial 6-phytase to a laying hen diet was effective improving apparent ileal phosphorus digestibility over the tested dose range from 500 to 2000 U per kg feed. A clear dose response on apparent ileal digestibility was found.
Even at the lowest level of the phytase preparation of 500 U per kg feed, beneficial effects on P utilisation were recorded compared to the P-deficient negative control group.

References

AOAC (1990)
Official Methods of Analysis of the AOAC [Association of Official Analytical Chemists], Arlington, Virginia 22201 USA

DIN EN ISO (1998)

Bestimmung von 33 Elementen durch induktiv gekoppelte Plasma-Atom-Emissionsspektrometrie (ISO 11885:1997; E22, 1998-04; Deutsche Fassung EN ISO 11885:1997), Beuth Verlag GmbH

EEC (1986)
Directive de la Commission du 9 avril 1986 fixant la méthode de calcul de la valeur énergétique des aliments composés destinés à la volaille, Journal Officiel des Communautés Européennes, L 130, 53-54

GINDLER, E.M. and J.D. KING (1972)
Determination of calcium concentration in human serum.AM.J.Clin.Pathol.58, 376-382

GRIMMERSOFT (1995)
StatBoxPro, Version 5.0, Manuel d'utilisation

HENRY R. (Editor) (1974) Clinical Chemistry
Principles and Techniques, 2nd Edition New York,
NY: Harper & Row, 723

ORIGINLAB CORPORATION (2002)
Origin Version 7, Programming Guide

VDLUFA (1976)
[Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, Hrsg.] Handbuch der landwirtschaftlichen Versuchs- und Untersuchungsmethodik (Methodenbuch). Band III: Die chemische Untersuchung von Futtermitteln, 3. Auflage 1976 mit 1. Erg. Lief. 1983, 2. Erg. Lief. 1993, 3. Erg. Lief. 1997, VDLUFA-Verlag, Darmstadt

Table 1: Feed composition of the experimental diets

Ingredients (%)	Negative control diet	Positive control diet
Maize	65.0	65.0
Soybean meal (50 % CP)	23.6	23.6
Soybean oil	1.65	1.65
DL-Methionine	0.20	0.20
Sand	0.20	
$CaCO_3$	8.20	7.85
DCP	-	0.55
NaCl	0.10	0.10
Premix	1.00	1.00
Titanium dioxyde	0.10	0.10
Calculated content:		
Crude protein (g/kg)	161	161
Calcium (g/kg)	34.5	34.5
Total P (g/kg)	2.6	3.9
ME_N[1] (MJ/kg)	11.9	11.9
Lysine (%)	0.83	0.83
Methionine + Cystine (%)	0.73	0.73
Analyzed content:		
Crude protein (g/kg)	169	169
Calcium (g/kg)	23.1	23.1
Total P (g/kg)	2.85	3.9
Phytate P (g/kg)	2.11	2.15
Non -phytic acid P (g/kg)	0.74	1.75

[1] Metabolisable Energy $_{N\text{-corr.}}$, calculated on the basis of the analyzed crude nutrients

Table 2: **Analysed content of enzyme activity in samples of the experimental diets**

Treatment	Product	Dose ($U.kg^{-1}$ feed)	Analysed phytase activity ($U.kg^{-1}$ feed)	Total P ($g.kg^{-1}$ feed)
A	Negative control	-	LOQ	2.5
B	IPA Phytase (L)	500	562	3.0
C	IPA Phytase (L)	1000	1114	3.0
D	IPA Phytase (L)	2000	2097	2.9
E	Positive control	-	LOQ	3.9

Table 3: Production performance of laying hens from week 28 to 31 of age mean ± stdev

Product	Negative control	IPA Phytase (L)			Positive control
Treatment	A	B	C	D	E
Dose (U. kg^{-1})	2.6 g P.kg^{-1}	500	1000	2000	3.9 g P.kg^{-1}
Cages x hens	24 x 2	24 x 2	24 x 2	24 x 2	24 x 2
Daily feed intake (per g/hen)	109.0 ^{AB}	113.3 ^{A}	112.4 ^{AB}	108.0 ^{B}	110.6 ^{AB}
	± 7.8	± 4.1	± 7.7	± 5.2	± 6.5
%	*100.0*	*104.0*	*103.2*	*99.1*	*101.5*
Egg weight (g)	57.4 ^{A}	57.6 ^{A}	58.2 ^{A}	58.1 ^{A}	57.4 ^{A}
	± 2.5	± 3.2	± 2.1	± 2.2	± 2.1
%	*100.0*	*100.3*	*101.4*	*101.2*	*99.9*
Egg production (%) total	90.1 ^{A}	92.6 ^{A}	95.2 ^{A}	90.3 ^{A}	80.3 ^{B}
	± 11.8	± 11.0	± 4.4	± 6.6	± 16.7
%	*100.0*	*102.7*	*105.7*	*100.1*	*89.1*
Broken eggs (%)	0.1 ^{A}	0 ^{A}	0.1 ^{A}	0 ^{A}	0.0 ^{A}
	± 0.5	± 0	± 0.4	± 0	± 0.7

Newman Keuls test: Means within a row, not sharing a common superscript, are significantly different ($p<0.05$).

Table 4: Effect of phytase on body weight at the beginning and at the end of experiment

	Product	Dose ($U.kg^{-1}$)	Body weight Beginning (g/hen)	SE	Body weight End (g/hen)	SE
A	Negative control	-	1683	16.2	1721	20.5
B	IPA Phytase (L)	500	1706	19.3	1779	19.3
C	IPA Phytase (L)	1000	1703	19.9	1750	23.3
D	IPA Phytase (L)	2000	1681	15.4	1704	16.8
E	Positive control	-	1673	22.9	1744	23.9
p			NS		NS	

NS = non significant (p>0.10)

Table 5: Apparent ileal digestibility of phosphorus in laying hens mean ± stdev

Product	Negative control	IPA Phytase (L)			Positive control
Treatment	A	B	C	D	E
Dose (U.kg^{-1})	2.6 g P.kg^{-1}	500	1000	2000	3.9 g P.kg^{-1}
Cages x hens	20 x 2	24 x 2	23 x 2	24 x 2	24 x 2
Phosphorus					
Apparent ileal P digestibility	45.8 ^{B}	54.8 ^{A}	56.1 ^{A}	58.7 ^{A}	35.8 ^{C}
% of intake	± 9.7	± 13.3	± 6.4	± 9.8	± 7.7
%	*100.0*	*119.8*	*122.6*	*128.2*	*78.2*

Newman Keuls test: Means within a row, not sharing a common superscript, are significantly different ($p<0.05$).

Table 6: **Concentration of inorganic phosphorus (P_i) and calcium (Ca) in the plasma of laying hens mean ± stdev**

Product	Negative control	IPA Phytase (L)			Positive control
Treatment	A	B	C	D	E
Dose ($U.kg^{-1}$)	2.6 g $P.kg^{-1}$	500	1000	2000	3.9 g $P.kg^{-1}$
Cages x hens	6 x 2	6 x 2	6 x 2	6 x 2	6 x 2
Pi (mmol/L)	1.23 ^{A}	1.50 ^{A}	1.36 ^{A}	1.20 ^{A}	1.41 ^{A}
	± 0.22	± 0.45	± 0.31	± 0.17	± 0.37
%	*100.0*	*122.0*	*110.6*	*97.6*	*114.6*
Ca (mmol/L)	6.17 ^{A}	6.63 ^{A}	6.14 ^{A}	5.73 ^{A}	6.36 ^{A}
	± 0.77	± 0.73	± 0.53	± 0.64	± 0.44
%	*100.0*	*107.5*	*99.5*	*92.9*	*103.1*

Newman Keuls test: Means within a row, not sharing a common superscript, are significantly different ($p<0.05$).

Table 7: Tibia strength and tibia ash percentage mean ± stdev

Product	Negative control	IPA Phytase (L)			Positive control
Treatment	A	B	C	D	E
Dose (U.kg^{-1})	2.6 g P.kg^{-1}	500	1000	2000	3.9 g P.kg^{-1}
Cages x hens	6 x 2	6 x 2	6 x 2	6 x 2	6 x 2
Tibia strength (N)	39 $^{A\,1}$	44 $^{A\,2}$	48 ^{A}	54 ^{A}	42 ^{A}
	± 17.5	± 7.3	± 13.0	± 20.3	± 21.2
%	*100.0*	*111.9*	*122.1*	*137.7*	*105.7*
Tibia ash (%)	48.4 ^{A}	49.2 ^{A}	48.6 ^{A}	48.1 ^{A}	46.5 ^{A}
	± 2.32	± 1.52	± 1.85	± 2.01	± 1.33
%	*100.0*	*101.8*	*100.5*	*99.5*	*96.2*

1 bone strength of ten samples out of twelve

2 bone strength of eleven samples out of twelve

Newman Keuls test: Means within a row, not sharing a common superscript, are significantly different ($p<0.05$).

Table 8: Toe ash percentage mean ± stdev

Product	Negative control	IPA Phytase (L)			Positive control
Treatment	A	B	C	D	E
Dose (U.kg^{-1})	2.6 g P.kg^{-1}	500	1000	2000	3.9 g P.kg^{-1}
Cages x hens	6 x 2	6 x 2	6 x 2	6 x 2	6 x 2
Toe ash (%)	31.7 ^{A}	31.6 ^{A}	30.1 ^{A}	31.3 ^{A}	33.4 ^{A}
	± 3.8	± 4.0	± 2.0	± 2.0	± 1.1
%	*100.0*	*99.7*	*95.0*	*98.9*	*105.2*

Newman Keuls test: Means within a row, not sharing a common superscript, are significantly different ($p<0.05$).

efsa

European Food Safety Authority

FEEDAP UNIT

ANNEX C [1]

TRIAL PROTOCOL DATA SHEET: FOR TERRESTRIAL ANIMALS

Identification of the additive: **6 bacterial phytase**	Batch number: **PPQ 28432**
Trial ID: **H-01/09**	Location: **Research Center for Animal Nutrition (DSM Nutritional Products France,F-68128 Village-Neuf**
Start date and exact duration of the study: **February-19-2009 to April-2-2009, 6 weeks**	
Number of treatment groups (+ control(s)): **3 (+2)**	Replicates per group: **24**
Total number of animals: **240**	Animals per replicate: **2**
Dose(s) of the additive/active substance(s)/agent(s) (mg/Units of activity/CFU kg^{-1} complete feed/L^{-1} water)	
Intended: **0/500/1000/2000 U/kg**	Analysed: **<0.01/562/1114/2097 U/kg**
Substances used for comparative purposes:	
Intended dose:	Analysed:
Animal species/category: **Laying hens**	
Breed: **Isa Brown**	Identification procedure: **per cage number**
Sex: **Females** Age at start: **23 wks**	Body weight at start: **1689 g**
Physiological stage: **Laying**	General health: **normal**
Additional information for field trials:	
Location and size of herd or flock:	
Feeding and rearing conditions:	
Method of feeding: **ad libitum**	
Diets (type(s)): **low phosphorus basal diet**	
Presentation of the diet: Mash ☒ Pellet ☐ Extruded ☐ Other	
Composition (main feedingstuffs): **65.0% maize/23.6% SBM**	
Nutrient content (relevant nutrients and energy content)	
Intended values: **11.9MJ/ME, 161 g Crude Protein (CP) , 2.6 g total P, 34.5 g Calcium**	
Analysed values: **12.2 MJ/ME, 169 g CP, 2.9 g total P, 0.74 g Non-Phytate-P, 23.1 g Calcium**	
Date and nature of the examinations performed: **laying performance, ileal digestibility, bone quality, excreta, plasma**	
Method(s) of statistical evaluation used: **one-factorial analysis of variance (factor:treatment), Newman-Keuls test**	
Therapeutic/preventive treatments (reason, timing, kind, duration): **nothing to report**	
Timing and prevalence of any undesirable consequences of treatment: **nothing to report**	

[1] Please submit this form using a common word processing format (e.g. MS Word).

FEEDAP UNIT

Date **12.02.2010**	Signature Study Director
	Pedro Pluijgers

† In case the concentration of the additive in complete feed/water may reflect insufficient accuracy, the dose of the additive can be given per animal day^{-1} or mg kg^{-1} body weight or as concentration in complementary feed.

Raw data of Trial H-01/09

I. INTRODUCTION

The following documentation summarizes supplementary raw data concerning the trial H-01/09 performed March –April 2009 at the Research Center for Animal Nutrition (CRNA, DSM Nutritional Products France, F-68305 Village-Neuf). This trial was reported under the following title: Effect of graded levels of bacterial 6-phytase on apparent ileal digestibility of phosphorus in laying hens fed a maize-based diet low in phosphorus content (H-01/09) (Aureli et al.2009)

REFERENCES

AURELI, R., PHILIPPS, P. and BROZ, J. (2009):
Effect of graded levels of bacterial 6- phytase on apparent ileal digestibility of phosphorus in laying hens fed a maize-based diet low in phosphorus content (H-01/09), DSM Report No.00000099, Regulatory Report, 09-June-2009

II. Raw data of Trial H-01/09

Raffaella Aureli, Petra Philipps and Jiri Broz

Effect of graded levels of bacterial 6- phytase on apparent ileal digestibility of phosphorus in laying hens fed a maize-based diet low in phosphorus content (H-01/09), DSM Report No.00000099, Regulatory Report, 09-June-2009

RDR 00000099

Analytical data on feed
Animal performance data
Data on ileal utilization of phosphorus
Data on calcium and inorganic phosphorus in plasma
Data on tibia strength and tibia ash
Data on toe ash
Data on phosphorus in excreta

09-June-2009

(Raffaella Aureli)

DSM Nutritional Products
B.P.170
F-68305 Saint-Louis cedex
France

2.1 Analytical data on feed

(see also tables 1 & 2 of report 00000099)

2.1.1 Nutrient content in feed

2.1.2 Ca/P/TiO_2

2.1.3 Phytate in feed

2.1.4 Phytase activity in feed

Service Volaille
H-01/09 : Aliment A

Analyses d'aliments									
Echantillons	Matière sèche = MS en %	Cendres en %	100% MS	Fibres en %	100% MS	Graisses en %	100% MS	Protéines en %	100% MS
A	89.50	10.98	12.26	2.23	2.49	5.26	5.88	16.893	18.87

Echantillons	Matière sèche = MS en %	Amidon en %	100% MS	Sucre en %	100% MS
A	89.50	43.87	49.02	3.26	3.642

674

25/03/2009

SERVICE VOLAILLE
H-01/09
Aliments A à E

CALCIUM - PHOSPHORE - TITANE							
Echantillon	gCa/100g MS	gCa/100g MF	gP/100g MS	gP/100g MF	mgTiO_2/kgMS	mgTiO_2/kg MF	MS%
A	2.55	2.27	0.28	0.25	859	764	89.03
B	2.63	2.34	0.34	0.30	1101	980	89.00
C	2.57	2.29	0.34	0.30	1023	911	89.07
D	2.64	2.35	0.33	0.29	994	884	88.97
E	2.38	2.12	0.44	0.39	983	875	89.08

675

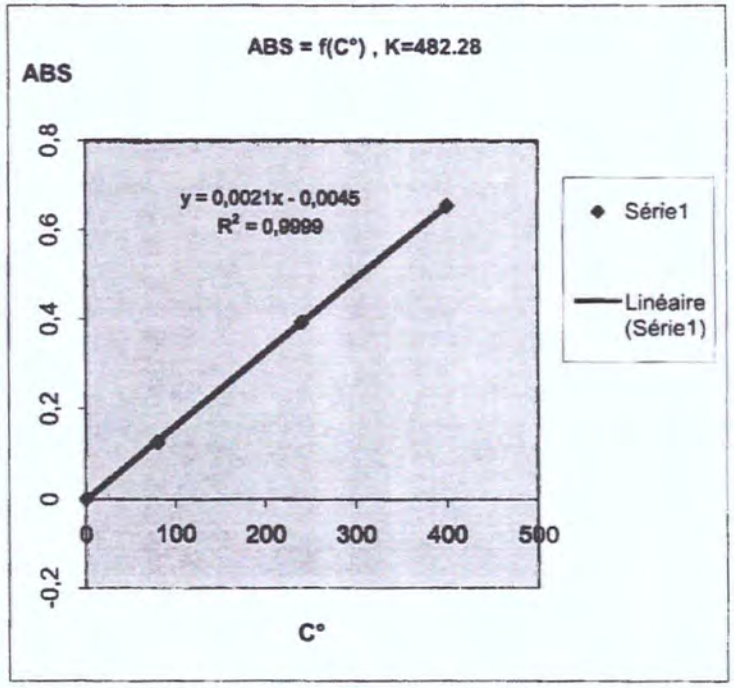
15/04/2009

Service Volaille
H-01/09 : Aliments A à E

COURBE STANDARD	0	0	Maïs, soja, aliments A à G	
	80	0,1255	637,45	619,63
	240	0,3929	610,84	
	400	0,6551	610,59	
	C°	Abs	K	Kmoyen

PHYTATES

ALIMENTS		ABS.	PHYTATES P (µg/g ech.)	Moyenne	PHYTATES (mg/g ech.)	Moyenne
Maïs (06/02/08)	1	0,1718	2129,045	2158,167	7,550	7,653
	2	0,1765	2187,290		7,756	
Soja (30/01/08)	3	0,3415	4232,065	4251,893	15,007	15,078
	4	0,3447	4271,721		15,148	
A	5	0,176	2181,093	2174,897	7,734	7,712
	6	0,175	2168,701		7,690	
B	7	0,1639	2031,143	2031,763	7,203	7,205
	8	0,1640	2032,383		7,207	
C	9	0,1737	2152,591	2186,670	7,633	7,754
	10	0,1792	2220,750		7,875	
D	11	0,1713	2122,848	2062,744	7,528	7,315
	12	0,1616	2002,640		7,102	
E	13	0,1728	2141,437	2145,155	7,594	7,607
	14	0,1734	2148,873		7,620	



676

DSM NUTRITINAL PRODUCTS
PHYTATE-P/NON PHYTATE-P
H-01/09

H-01/09	Composition de l'aliment	% P Phytique	g P Brut analysé	% P Phytique x g P Brut analysé	x % dans l'aliment
mais	0.65	**0.66**	2.20	**1.452**	**0.9438**
soja	0.2355	**0.6**	5.10	**3.06**	**0.72063**
P bicalcique		**0**		**0**	**0**
g/kg Phytate P					1.66443
µg/g Phytate P					1664.43
mg/g Phytate					**5.902**

	mg /g Phytate	
	calculé	analysé (AOAC)
ALIMENT	5.90	7.50

Valeurs mesurées

Phosphore total	Phytate-P (mg/g)	Non Phytate-P (mg/g)
2.90	2.12	0.78

BIOPRACT GmbH

Report of Analysis

18. Feb. 09

DSM Nutritional Products France
Dr. Petra Philipps
CRNA - BP170
F-68305 **Saint-Louis Cedex**
France

Request N°: H-01/09
Theme N°: 6106

Parameter: Phytase
Product
Batch used: PPQ 28432

Registration date: 13.02.2009 **Customer/Manufacturer:**Thema: 6106

Sample Number	Sample Label	Declaration U/kg	Found U/kg	Average	STDEV	CV
01	Treatment A - M	0				
				LOQ		
02	Treatment B - M	500	528			
			595	**562**	47	8%
03	Treatment C - M	1000	1154			
			1073	**1114**	57	5%
04	Treatment D - M	2000	2110			
			2083	**2097**	19	1%
05	Treatment E - M	0				
				LOQ		

M - mash
P - pellet
C - crumb
PM - premix
E - expanded
F - flour
TQ -Tel Quel

LOD - Limit of Detection
LOQ - Limit of Quantification

Responsible Analyst
J. König

2.2 Animal performance data

(see also tables 3 & 4 of report 00000099)

2.2.1 Raw data on laying performance and feed consumption on a weekly base

Nb oeufs 1:	number of eggs/cage week 1
pds oeufs 1:	weight of eggs/cage week 1
Prod 1-3:	egg production over whole experiment
pds tot 1-3:	total egg weight over whole experiment
casse:	broken eggs
Pro ajustée:	egg production including broken eggs
% prod oeufs	% egg production
pds oeufs:	mean egg weight per egg
masse oeuf:	total egg mass
aliment brut:	feed at the beginning
aliment rest:	feed at the end
consom 1-3:	feed consumption over whole experiment
poule-j:	feed intake per hen and day
cons/groupe:	feed consumption per group
cons/ouef:	feed consumption per egg
IC/mass	Feed conversion ratio

6 pages

2.2.2 Raw date on body weight of laying hens at the beginning and at the end of the trial

2.1.1 Raw data on laying performance and feed consumption on a weekly base

group	N°	Nb oeufs 1	Nb oeufs 2	Nb oeufs 3	Nb oeufs 4	pds oeufs 1	pds oeufs 2	pds oeufs 3	pds oeufs 4	Prod 1-3	pds tot 1-3	casse	Prod ajustée	% prod oeufs	pds oeuf	masse oeufs	aliment brut	aliment rest
A	1	14	14	13	14	822	796	736	790	55	3144	1	54	98.21	58.222	3202.2	9201	3012
A	2	3	5	4	1	165	255	193	59	13	672	0	13	23.21	51.692	672.0	9201	3145
A	3	13	13	13	13	736	740	744	745	52	2965	0	52	92.86	57.019	2965.0	9201	3104
A	4	13	13	13	13	726	677	683	696	52	2782	0	52	92.86	53.500	2782.0	9201	3801
A	5	13	13	14	14	775	749	812	863	54	3199	0	54	96.43	59.241	3199.0	9201	2704
A	6	12	13	14	13	749	775	848	796	52	3168	0	52	92.86	60.923	3168.0	9200	2249
A	7	14	13	14	12	793	720	769	640	53	2922	0	53	94.64	55.132	2922.0	9200	2588
A	8	14	14	14	13	824	870	874	817	55	3385	0	55	98.21	61.545	3385.0	9201	3164
A	9	14	14	14	14	791	784	787	804	56	3166	0	56	100.00	56.536	3166.0	9201	3592
A	10	13	13	14	13	805	754	827	765	53	3151	0	53	94.64	59.453	3151.0	9203	3313
A	11	9	7	8	7	514	390	451	403	31	1758	0	31	55.36	56.710	1758.0	9200	3518
A	12	13	14	14	13	745	788	810	743	54	3086	0	54	96.43	57.148	3086.0	9202	2884
A	13	14	12	13	14	847	706	782	889	53	3224	0	53	94.64	60.830	3224.0	9202	2876
A	14	14	14	13	14	760	777	748	829	55	3114	0	55	98.21	56.618	3114.0	9201	3001
A	15	9	14	14	13	499	799	769	734	50	2801	0	50	89.29	56.020	2801.0	9203	3666
A	16	11	8	11	12	657	457	675	748	42	2537	0	42	75.00	60.405	2537.0	9202	3219
A	17	13	14	13	14	719	787	716	801	54	3023	1	53	96.43	57.038	3080.0	9202	2949
A	18	14	14	14	12	796	787	816	687	54	3086	0	54	96.43	57.148	3086.0	9201	2624
A	19	8	10	11	12	454	545	633	725	41	2357	0	41	73.21	57.488	2357.0	9200	2832
A	20	14	14	13	13	767	738	680	706	54	2891	0	54	96.43	53.537	2891.0	9202	3038
A	21	14	14	13	14	794	770	709	764	55	3037	0	55	98.21	55.218	3037.0	9201	3215
A	22	9	10	8	10	508	603	476	609	37	2196	0	37	66.07	59.351	2196.0	9200	2922
A	23	14	14	14	14	811	796	816	865	56	3288	0	56	100.00	58.714	3288.0	9203	2707
A	24	13	14	8	8	755	869	448	466	43	2538	0	43	76.79	59.023	2538.0	7592	2646
group	N°	Nb oeufs 1	Nb oeufs 2	Nb oeufs 3	Nb oeufs 4	pds oeufs 1	pds oeufs 2	pds oeufs 3	pds oeufs 4	Prod 1-3	pds tot 1-3	casse	Prod ajustée	% prod oeufs	pds oeuf	masse oeufs	aliment brut	aliment rest
B	25	14	14	14	14	754	755	753	766	56	3028	0	56	100.00	54.071	3028.0	9101	2583
B	26	12	14	14	11	720	897	881	688	51	3186	0	51	91.07	62.471	3186.0	9101	2552
B	27	8	7	8	7	438	365	405	373	30	1581	0	30	53.57	52.700	1581.0	9102	3336
B	28	14	12	13	11	799	694	801	704	50	2998	0	50	89.29	59.960	2998.0	9101	2941
B	29	14	14	14	14	828	822	844	864	56	3358	0	56	100.00	59.964	3358.0	9100	2568
B	30	14	14	14	14	874	878	874	891	56	3517	0	56	100.00	62.804	3517.0	9102	2645
B	31	14	14	14	14	810	783	818	824	56	3235	0	56	100.00	57.768	3235.0	9102	2651
B	32	14	13	14	13	742	663	711	706	54	2822	0	54	96.43	52.259	2822.0	9101	3030
B	33	13	14	14	14	754	811	825	845	55	3235	0	55	98.21	58.818	3235.0	9103	2490
B	34	13	12	14	12	727	683	828	714	51	2952	0	51	91.07	57.882	2952.0	9100	3032
B	35	7	9	10	11	410	537	593	661	37	2201	0	37	66.07	59.486	2201.0	9099	2706
B	36	14	14	13	13	793	781	731	733	54	3038	0	54	96.43	56.259	3038.0	9102	2621
B	37	12	13	12	14	738	790	751	872	51	3151	0	51	91.07	61.784	3151.0	9100	2616
B	38	14	14	14	14	842	864	856	868	56	3430	0	56	100.00	61.250	3430.0	9099	2736
B	39	13	12	13	12	711	654	715	677	50	2757	0	50	89.29	55.140	2757.0	9103	2642
B	40	12	12	12	13	643	638	700	773	49	2754	0	49	87.50	56.204	2754.0	9100	2534
B	41	14	14	14	13	838	852	848	752	55	3290	0	55	98.21	59.818	3290.0	9103	2729
B	42	12	13	13	13	646	710	724	753	51	2833	0	51	91.07	55.549	2833.0	9100	3045
B	43	14	13	14	14	803	725	771	796	55	3095	0	55	98.21	56.273	3095.0	9104	2586
B	44	13	14	14	14	705	751	753	754	55	2963	0	55	98.21	53.873	2963.0	9101	3168
B	45	14	13	14	13	811	749	846	794	54	3200	0	54	96.43	59.259	3200.0	9099	2548
B	46	13	14	14	14	681	748	775	778	55	2982	0	55	98.21	54.218	2982.0	9100	2826
B	47	13	14	13	14	719	735	703	772	54	2929	0	54	96.43	54.241	2929.0	9100	2920
B	48	13	14	12	14	784	848	705	856	53	3193	0	53	94.64	60.245	3193.0	11236	4797

group	consom 1-2	poule-j	cons/jour	cons/groupe	cons/oeuf	IC/masse	% broken egg
A	6189	56	110.52	6189	112.53	1.9327	1.8
A	6056	56	108.14	6056	465.85	9.0119	0.0
A	6097	56	108.88	6097	117.25	2.0563	0.0
A	5400	56	96.43	5400	103.85	1.9410	0.0
A	6497	56	116.02	6497	120.31	2.0309	0.0
A	6951	56	124.13	6951	133.67	2.1941	0.0
A	6612	56	118.07	6612	124.75	2.2628	0.0
A	6037	56	107.80	6037	109.76	1.7835	0.0
A	5609	56	100.16	5609	100.16	1.7716	0.0
A	5890	56	105.18	5890	111.13	1.8692	0.0
A	5682	56	101.46	5682	183.29	3.2321	0.0
A	6318	56	112.82	6318	117.00	2.0473	0.0
A	6326	56	112.96	6326	119.36	1.9622	0.0
A	6200	56	110.71	6200	112.73	1.9910	0.0
A	5537	56	98.88	5537	110.74	1.9768	0.0
A	5983	56	106.84	5983	142.45	2.3583	0.0
A	6253	56	111.66	6253	115.80	2.0302	1.8
A	6577	56	117.45	6577	121.80	2.1312	0.0
A	6368	56	113.71	6368	155.32	2.7017	0.0
A	6164	56	110.07	6164	114.15	2.1321	0.0
A	5986	56	106.89	5986	108.84	1.9710	0.0
A	6278	56	112.11	6278	169.68	2.8588	0.0
A	6496	56	116.00	6496	116.00	1.9757	0.0
A	4946	56	88.32	4946	115.02	1.9488	0.0
group	consom 1-2	poule-j	cons/jour	cons/groupe	cons/oeuf	IC/masse	% broken egg
B	6518	56	116.39	6518	116.39	2.1526	0.0
B	6549	56	116.95	6549	128.41	2.0556	0.0
B	5766	56	102.96	5766	192.20	3.6471	0.0
B	6160	56	110.00	6160	123.20	2.0547	0.0
B	6532	56	116.64	6532	116.64	1.9452	0.0
B	6457	56	115.30	6457	115.30	1.8359	0.0
B	6451	56	115.20	6451	115.20	1.9941	0.0
B	6071	56	108.41	6071	112.43	2.1513	0.0
B	6613	56	118.09	6613	120.24	2.0442	0.0
B	6068	56	108.36	6068	118.98	2.0556	0.0
B	6393	56	114.16	6393	172.78	2.9046	0.0
B	6481	56	115.73	6481	120.02	2.1333	0.0
B	6484	56	115.79	6484	127.14	2.0578	0.0
B	6363	56	113.63	6363	113.63	1.8551	0.0
B	6461	56	115.38	6461	129.22	2.3435	0.0
B	6566	56	117.25	6566	134.00	2.3842	0.0
B	6374	56	113.82	6374	115.89	1.9374	0.0
B	6055	56	108.13	6055	118.73	2.1373	0.0
B	6518	56	116.39	6518	118.51	2.1060	0.0
B	5933	56	105.95	5933	107.87	2.0024	0.0
B	6551	56	116.98	6551	121.31	2.0472	0.0
B	6274	56	112.04	6274	114.07	2.1040	0.0
B	6180	56	110.36	6180	114.44	2.1099	0.0
B	6439	56	114.98	6439	121.49	2.0166	0.0

group	N°	Nb oeufs 1	Nb oeufs 2	Nb oeufs 3	Nb oeufs 4	pds oeufs 1	pds oeufs 2	pds oeufs 3	pds oeufs 4	Prod 1-3	pds tot 1-3	casse	Prod ajustée	% prod oeufs	pds oeuf	masse oeufs	aliment brut	aliment rest
C	49	14	14	13	13	836	826	734	759	54	3155	0	54	96.43	58.426	3155.0	9098	3083
C	50	11	13	10	13	654	740	592	765	47	2751	1	46	83.93	59.804	2810.8	9102	2409
C	51	14	14	14	14	816	827	854	863	56	3360	0	56	100.00	60.000	3360.0	9101	2299
C	52	13	14	12	13	798	873	750	828	52	3249	0	52	92.86	62.481	3249.0	9106	2894
C	53	13	12	13	13	728	658	704	717	51	2807	0	51	91.07	55.039	2807.0	9101	2776
C	54	13	14	12	14	755	790	711	854	53	3110	0	53	94.64	58.679	3110.0	9099	2653
C	55	14	14	14	12	811	788	789	679	54	3067	0	54	96.43	56.796	3067.0	9100	3299
C	56	13	13	14	13	785	810	839	774	53	3208	0	53	94.64	60.528	3208.0	9101	2664
C	57	12	14	13	14	711	836	781	816	53	3144	0	53	94.64	59.321	3144.0	9105	3206
C	58	12	14	13	14	696	787	764	815	53	3062	0	53	94.64	57.774	3062.0	9099	2304
C	59	13	14	14	14	761	822	815	835	55	3233	0	55	98.21	58.782	3233.0	9100	3305
C	60	14	13	14	12	815	707	807	711	53	3040	0	53	94.64	57.358	3040.0	9100	2738
C	61	14	13	14	13	772	687	745	695	54	2899	0	54	96.43	53.685	2899.0	9107	3447
C	62	14	14	14	14	758	761	766	780	56	3065	0	56	100.00	54.732	3065.0	9105	3286
C	63	13	14	14	14	791	832	831	831	55	3285	0	55	98.21	59.727	3285.0	9100	2750
C	64	14	14	13	14	833	818	750	816	55	3217	0	55	98.21	58.491	3217.0	9096	2733
C	65	14	14	14	14	793	817	835	843	56	3288	0	56	100.00	58.714	3288.0	9107	3098
C	66	13	14	13	13	699	731	712	744	53	2886	0	53	94.64	54.453	2886.0	9101	2859
C	67	12	13	11	13	671	751	633	755	49	2810	0	49	87.50	57.347	2810.0	9101	2937
C	68	13	12	14	9	746	706	814	526	48	2792	0	48	85.71	58.167	2792.0	9104	3336
C	69	14	13	14	13	801	751	844	802	54	3198	0	54	96.43	59.222	3198.0	9102	2525
C	70	13	14	14	14	756	825	836	843	55	3260	0	55	98.21	59.273	3260.0	9105	2337
C	71	14	13	14	14	832	769	875	877	55	3353	0	55	98.21	60.964	3353.0	9102	2851
C	72	14	14	14	14	796	798	821	824	56	3239	0	56	100.00	57.839	3239.0	10509	2991
group	N°	Nb oeufs 1	Nb oeufs 2	Nb oeufs 3	Nb oeufs 4	pds oeufs 1	pds oeufs 2	pds oeufs 3	pds oeufs 4	Prod 1-3	pds tot 1-3	casse	Prod ajustée	% prod oeufs	pds oeuf	masse oeufs	aliment brut	aliment rest
D	73	13	8	10	14	796	532	601	872	45	2801	0	45	80.36	62.244	2801.0	9103	3307
D	74	13	13	14	13	779	751	795	808	53	3133	0	53	94.64	59.113	3133.0	9101	2640
D	75	12	14	13	13	699	788	706	737	52	2930	0	52	92.86	56.346	2930.0	9103	3185
D	76	14	14	13	13	793	786	714	738	54	3031	0	54	96.43	56.130	3031.0	9103	2764
D	77	14	14	12	14	844	824	668	837	54	3173	0	54	96.43	58.759	3173.0	9103	2947
D	78	14	13	12	12	831	805	700	726	51	3062	0	51	91.07	60.039	3062.0	9101	2997
D	79	7	5	6	4	386	291	352	239	22	1268	0	22		57.636	1268.0	9104	3465
D	80	12	8	6	14	712	480	368	852	40	2412	0	40	71.43	60.300	2412.0	9099	3410
D	81	12	11	11	11	708	619	632	654	45	2613	0	45	80.36	58.067	2613.0	9100	3139
D	82	11	12	14	11	704	719	857	673	48	2953	0	48	85.71	61.521	2953.0	9099	3128
D	83	13	11	11	12	783	657	655	694	47	2789	0	47	83.93	59.340	2789.0	9099	3437
D	84	12	14	12	12	693	795	684	700	50	2872	0	50	89.29	57.440	2872.0	9102	2887
D	85	13	12	14	14	699	683	797	785	53	2964	0	53	94.64	55.925	2964.0	9101	3199
D	86	14	10	11	12	834	604	646	700	47	2784	0	47	83.93	59.234	2784.0	9102	2873
D	87	14	12	14	14	809	661	790	830	54	3090	0	54	96.43	57.222	3090.0	9100	2906
D	88	14	13	14	14	835	778	852	841	55	3306	0	55	98.21	60.109	3306.0	9102	2863
D	89	14	12	13	12	801	674	736	888	51	3099	0	51	91.07	60.765	3099.0	9104	3171
D	90	13	13	14	14	768	741	813	820	54	3142	0	54	96.43	58.185	3142.0	9099	2513
D	91	12	13	14	14	653	717	774	783	53	2927	0	53	94.64	55.226	2927.0	9101	2631
D	92	6	9	10	7	354	505	553	383	32	1795	0	32		56.094	1795.0	9103	3469
D	93	13	13	13	12	732	740	733	693	51	2898	0	51	91.07	56.824	2898.0	9102	2968
D	94	14	13	13	14	811	748	783	846	54	3188	0	54	96.43	59.037	3188.0	9100	2681
D	95	13	13	14	13	679	741	797	752	53	2969	0	53	94.64	56.019	2969.0	9100	3267
D	96	11	12	12	13	602	627	625	700	48	2554	0	48	85.71	53.208	2554.0	11496	5875

group	consom 1-2	poule-j	cons/jour	cons/groupe	cons/oeuf	IC/masse	% broken egg
C	6015	56	107.41	6015	111.39	1.9065	0.0
C	6693	56	119.52	6693	142.40	2.3812	1.8
C	6802	56	121.46	6802	121.46	2.0244	0.0
C	6212	56	110.93	6212	119.46	1.9120	0.0
C	6325	56	112.95	6325	124.02	2.2533	0.0
C	6446	56	115.11	6446	121.62	2.0727	0.0
C	5801	56	103.59	5801	107.43	1.8914	0.0
C	6437	56	114.95	6437	121.45	2.0065	0.0
C	5899	56	105.34	5899	111.30	1.8763	0.0
C	6795	56	121.34	6795	128.21	2.2191	0.0
C	5795	56	103.48	5795	105.36	1.7925	0.0
C	6362	56	113.61	6362	120.04	2.0928	0.0
C	5660	56	101.07	5660	104.81	1.9524	0.0
C	5819	56	103.91	5819	103.91	1.8985	0.0
C	6350	56	113.39	6350	115.45	1.9330	0.0
C	6363	56	113.63	6363	115.69	1.9779	0.0
C	6009	56	107.30	6009	107.30	1.8276	0.0
C	6242	56	111.46	6242	117.77	2.1629	0.0
C	6164	56	110.07	6164	125.80	2.1936	0.0
C	5768	56	103.00	5768	120.17	2.0659	0.0
C	6577	56	117.45	6577	121.80	2.0566	0.0
C	6768	56	120.86	6768	123.05	2.0761	0.0
C	6251	56	111.63	6251	113.65	1.8643	0.0
C	7518	56	134.25	7518	134.25	2.3211	0.0
group	**consom 1-2**	**poule-j**	**cons/jour**	**cons/groupe**	**cons/oeuf**	**IC/masse**	**% broken egg**
D	5796	56	103.50	5796	128.80	2.0693	0.0
D	6461	56	115.38	6461	121.91	2.0622	0.0
D	5918	56	105.68	5918	113.81	2.0198	0.0
D	6339	56	113.20	6339	117.39	2.0914	0.0
D	6156	56	109.93	6156	114.00	1.9401	0.0
D	6104	56	109.00	6104	119.69	1.9935	0.0
D	5639	56	100.70	5639	256.32	4.4472	0.0
D	5689	56	101.59	5689	142.23	2.3586	0.0
D	5961	56	106.45	5961	132.47	2.2813	0.0
D	5971	56	106.63	5971	124.40	2.0220	0.0
D	5662	56	101.11	5662	120.47	2.0301	0.0
D	6215	56	110.98	6215	124.30	2.1640	0.0
D	5902	56	105.39	5902	111.36	1.9912	0.0
D	6229	56	111.23	6229	132.53	2.2374	0.0
D	6194	56	110.61	6194	114.70	2.0045	0.0
D	6239	56	111.41	6239	113.44	1.8872	0.0
D	5933	56	105.95	5933	116.33	1.9145	0.0
D	6586	56	117.61	6586	121.96	2.0961	0.0
D	6470	56	115.54	6470	122.08	2.2105	0.0
D	5634	56	100.61	5634	176.06	3.1387	0.0
D	6134	56	109.54	6134	120.27	2.1166	0.0
D	6419	56	114.63	6419	118.87	2.0135	0.0
D	5833	56	104.16	5833	110.06	1.9646	0.0
D	5621	56	100.38	5621	117.10	2.2009	0.0

group	N°	Nb oeufs 1	Nb oeufs 2	Nb oeufs 3	Nb oeufs 4	pds oeufs 1	pds oeufs 2	pds oeufs 3	pds oeufs 4	Prod 1-3	pds tot 1-3	casse	Prod ajustée	% prod oeufs	pds oeuf	masse oeufs	aliment brut	aliment rest
E	97	14	11	14	12	835	630	820	715	51	3000	0	51	91.07	58.824	3000.0	9100	2264
E	98	13	9	8	13	748	523	467	818	43	2556	0	43	76.79	59.442	2556.0	9102	2884
E	99	14	13	12	13	863	755	742	814	52	3174	0	52	92.86	61.038	3174.0	9102	2675
E	100	10	8	7	8	551	440	391	443	33	1825	0	33	58.93	55.303	1825.0	9102	3618
E	101	13	13	13	14	717	746	751	808	53	3022	0	53	94.64	57.019	3022.0	9101	2505
E	102	14	12	14	13	744	646	747	713	53	2850	0	53	94.64	53.774	2850.0	9108	2869
E	103	14	14	14	14	842	813	818	839	56	3312	0	56	100.00	59.143	3312.0	9099	2459
E	104	12	11	14	14	684	632	791	796	51	2903	0	51	91.07	56.922	2903.0	9102	2784
E	105	8	7	7	7	464	403	403	462	29	1732	0	29	51.79	59.724	1732.0	9101	3045
E	106	13	14	14	14	729	795	786	820	55	3130	0	55	98.21	56.909	3130.0	9103	2831
E	107	14	13	11	12	765	729	629	704	50	2827	0	50	89.29	56.540	2827.0	9101	2492
E	108	9	8	8	8	534	474	453	481	33	1942	0	33	58.93	58.848	1942.0	9100	3459
E	109	12	13	14	13	611	688	748	708	52	2755	0	52	92.86	52.981	2755.0	9101	2534
E	110	14	12	13	13	823	687	730	746	52	2986	0	52	92.86	57.423	2986.0	9102	2474
E	111	8	8	8	8	468	464	462	472	32	1866	0	32	57.14	58.313	1866.0	9102	2894
E	112	13	13	12	9	739	753	711	542	47	2745	0	47	83.93	58.404	2745.0	9101	3295
E	113	10	13	12	10	586	760	694	583	45	2623	0	45	80.36	58.289	2623.0	9101	3161
E	114	13	13	13	11	755	746	725	620	50	2846	0	50	89.29	56.920	2846.0	9100	3173
E	115	7	7	9	7	396	398	526	463	30	1783	0	30	53.57	59.433	1783.0	9103	2979
E	116	7	7	8	7	414	406	462	419	29	1701	0	29	51.79	58.655	1701.0	9099	3086
E	117	13	13	11	14	696	699	591	794	51	2780	0	51	91.07	54.510	2780.0	9109	3129
E	118	6	8	13	14	339	436	718	829	41	2322	0	41	73.21	56.634	2322.0	9103	3049
E	119	13	10	7	7	690	530	391	397	37	2008	0	37	66.07	54.270	2008.0	9104	3519
E	120	14	13	13	14	835	748	738	836	54	3157	0	54	96.43	58.463	3157.0	9954	3411

group	consom 1-2	poule-j	cons/jour	cons/groupe	cons/oeuf	IC/masse	% broken egg
E	6836	56	122.07	6836	134.04	2.2787	0.0
E	6218	56	111.04	6218	144.60	2.4327	0.0
E	6427	56	114.77	6427	123.60	2.0249	0.0
E	5484	56	97.93	5484	166.18	3.0049	0.0
E	6596	56	117.79	6596	124.45	2.1827	0.0
E	6239	56	111.41	6239	117.72	2.1891	0.0
E	6640	56	118.57	6640	118.57	2.0048	0.0
E	6318	56	112.82	6318	123.88	2.1764	0.0
E	6056	56	108.14	6056	208.83	3.4965	0.0
E	6272	56	112.00	6272	114.04	2.0038	0.0
E	6609	56	118.02	6609	132.18	2.3378	0.0
E	5641	56	100.73	5641	170.94	2.9047	0.0
E	6567	56	117.27	6567	126.29	2.3837	0.0
E	6628	56	118.36	6628	127.46	2.2197	0.0
E	6208	56	110.86	6208	194.00	3.3269	0.0
E	5806	56	103.68	5806	123.53	2.1151	0.0
E	5940	56	106.07	5940	132.00	2.2646	0.0
E	5927	56	105.84	5927	118.54	2.0826	0.0
E	6124	56	109.36	6124	204.13	3.4347	0.0
E	6013	56	107.38	6013	207.34	3.5350	0.0
E	5980	56	106.79	5980	117.25	2.1511	0.0
E	6054	56	108.11	6054	147.66	2.6072	0.0
E	5585	56	99.73	5585	150.95	2.7814	0.0
E	6543	56	116.84	6543	121.17	2.0725	0.0

Body weight of laying hens
Beginning: 19.02.09
End: 02.04.09

H-01/09

A	Start	End	Weight gain/hen	B	Start	End	Weight gain/hen	C	Start	End	Weight gain/hen	D	Start	End	Weight gain/hen	E	Start	End	Weight gain/hen
N° cage	weight	weight		N° cage	weight	weight		N° cage	weight	weight		N° cage	weight	weight		N° cage	weight	weight	
1	3440	3636	98	1	3688	3759	35.5	1	3284	3242	-21	1	3418	3295	-61.5	1	3305	3289	-8
2	3584	3622	19	2	3456	3531	37.5	2	3746	4067	160.5	2	3451	3436	-7.5	2	3523	3776	126.5
3	3284	3476	96	3	3295	3451	78	3	3493	3459	-17	3	3229	3306	38.5	3	3284	3516	116
4	3074	2970	-52	4	3242	3564	161	4	3423	3494	35.5	4	3281	3456	87.5	4	3349	3334	-7.5
5	3669	3741	36	5	3618	3830	106	5	3655	3735	40	5	3421	3492	35.5	5	3584	3771	93.5
6	3237	3451	107	6	3626	3704	39	6	3383	3427	22	6	3512	3472	-20	6	3201	3293	46
7	3349	3359	5	7	3517	3544	13.5	7	3220	3130	-45	7	3389	3530	70.5	7	3529	3630	50.5
8	3386	3347	-19.5	8	3391	3478	43.5	8	3632	3738	53	8	3589	3725	68	8	3573	3587	7
9	3376	3549	86.5	9	3586	3675	44.5	9	2987	3150	81.5	9	3260	3136	-62	9	3296	3381	42.5
10	3212	3197	-7.5	10	3333	3461	64	10	3373	3464	45.5	10	3519	3413	-53	10	3164	3453	144.5
11	3417	3619	101	11	3060	3460	200	11	3359	3514	77.5	11	3238	3291	26.5	11	3154	3368	107
12	3536	3656	60	12	3394	3523	64.5	12	3707	3712	2.5	12	3533	3673	70	12	3643	3509	-67
13	3352	3390	19	13	3236	3355	59.5	13	3346	3449	51.5	13	3322	3361	19.5	13	3163	3327	82
14	3368	3453	42.5	14	3411	3623	106	14	3071	3227	78	14	3163	3260	48.5	14	3442	3644	101
15	3363	3212	-75.5	15	3724	3805	40.5	15	3556	3548	-4	15	3561	3505	-28	15	3951	4099	74
16	3313	3448	67.5	16	3664	3779	57.5	16	3326	3490	82	16	3430	3479	24.5	16	3139	3149	5
17	3485	3495	5	17	3473	3657	92	17	3400	3403	1.5	17	3149	3131	-9	17	3127	3289	81
18	3189	3413	112	18	3111	3239	64	18	3067	3159	46	18	3555	3548	-3.5	18	3022	3249	113.5
19	3631	3869	119	19	3264	3679	207.5	19	3415	3543	64	19	3458	3494	18	19	3397	3569	86
20	3016	3177	80.5	20	3187	3241	27	20	3456	3312	-72	20	3216	3259	21.5	20	3348	3811	231.5
21	3229	3248	9.5	21	3494	3716	111	21	3519	3632	56.5	21	3320	3378	29	21	3313	3473	80
22	3361	3442	40.5	22	3278	3310	16	22	3588	3763	87.5	22	3446	3679	116.5	22	3157	3285	64
23	3485	3545	30	23	3251	3223	-14	23	3308	3582	137	23	3106	3162	28	23	3050	3178	64
24	3396	3289	-53.5	24	3599	3787	94	24	3433	3724	145.5	24	3125	3280	77.5	24	3587	3719	66
Mean	3365	3442	39		3412	3558	73		3406	3499	46		3362	3407	22		3346	3487	71
Stdev	159	201	56		189	189	55		195	228	57		151	165	47		224	234	60

Mean Body weight (g/hens)	1682	1721		1706	1779		1703	1749		1681	1703		1673	1744
Stdev	80	101		95	94		97	114		75	83		112	117
SE	16.3	20.5		19.3	19.3		19.9	23.3		15.4	16.8		22.8	23.9

2.3 Data on ileal utilization of phosphorus

(see also table 5 of report 00000099)

3 pages

	Taux Phosphore en % de MS aliment	Taux Calcium en % de MS aliment	Taux de titane dans l'aliment en mg / kg de MS	Taux de titane dans le c. iléal en mg / kg de MS	Taux Phosphore en % de MS c. iléal	Taux Calcium en % de MS de c. iléal	Coefficient d'utilisation apparente du Phosphore en %
TT	XPFE	XLFE	TIFE	TIIL	XPIL	XLIL	RPHOP
A1	0.32	2.60	994.3	3639	0.68	3.00	41.94
A2	0.32	2.60	994.3	3699	0.52	4.66	56.32
A3	0.32	2.60	994.3	2655	0.45	3.46	47.33
A5	0.32	2.60	994.3	3579	0.53	4.00	53.98
A8	0.32	2.60	994.3	2522	0.42	0.91	48.26
A9	0.32	2.60	994.3	1959	0.29	4.45	54.00
A10	0.32	2.60	994.3	3956	0.55	3.10	56.80
A11	0.32	2.60	994.3	2419	0.56	5.14	28.07
A12	0.32	2.60	994.3	2946	0.50	8.80	47.26
A14	0.32	2.60	994.3	2342	0.46	2.14	38.96
A15	0.32	2.60	994.3	3653	0.63	4.45	46.42
A16	0.32	2.60	994.3	2934	0.45	2.81	52.34
A17	0.32	2.60	994.3	4146	0.57	2.39	57.29
A18	0.32	2.60	994.3	3005	0.69	5.85	28.65
A19	0.32	2.60	994.3	3419	0.71	5.22	35.48
A20	0.32	2.60	994.3	5160	0.95	2.31	42.80
A21	0.32	2.60	994.3	2387	0.43	1.42	44.02
A22	0.32	2.60	994.3	2458	0.44	7.60	44.37
A23	0.32	2.60	994.3	2436	0.31	2.19	60.46
A24	0.32	2.60	994.3	3356	0.75	7.06	30.55

45.77

9.71

	Taux Phosphore en % de MS aliment	Taux Calcium en % de MS aliment	Taux de titane dans l'aliment en mg / kg de MS	Taux de titane dans le c. iléal en mg / kg de MS	Taux Phosphore en % de MS c. iléal	Taux Calcium en % de MS de c. iléal	Coefficient d'utilisation apparente du Phosphore en %
TT	XPFE	XLFE	TIFE	TIIL	XPIL	XLIL	RPHOP
B1	0.32	2.60	994.3	4156	0.18	2.91	86.54
B2	0.32	2.60	994.3	3249	0.48	7.43	54.09
B3	0.32	2.60	994.3	3806	0.63	7.18	48.57
B4	0.32	2.60	994.3	4165	0.84	7.15	37.34
B5	0.32	2.60	994.3	2868	0.36	2.45	60.99
B6	0.32	2.60	994.3	4122	0.35	2.23	73.62
B7	0.32	2.60	994.3	2715	0.31	3.11	64.52
B8	0.32	2.60	994.3	4467	0.76	4.95	47.13
B9	0.32	2.60	994.3	2625	0.47	5.01	44.37
B10	0.32	2.60	994.3	3140	0.22	6.13	78.23
B11	0.32	2.60	994.3	3670	0.67	4.52	43.27
B12	0.32	2.60	994.3	3100	0.55	5.70	44.87
B13	0.32	2.60	994.3	3302	0.55	5.15	48.25
B14	0.32	2.60	994.3	2695	0.43	4.52	50.43
B15	0.32	2.60	994.3	2973	0.61	3.12	36.24
B16	0.32	2.60	994.3	3677	0.77	5.45	34.94
B17	0.32	2.60	994.3	2875	0.39	4.55	57.85
B18	0.32	2.60	994.3	3755	0.42	3.00	65.25
B19	0.32	2.60	994.3	3371	0.53	2.30	51.16
B20	0.32	2.60	994.3	3721	0.61	7.90	49.07
B21	0.32	2.60	994.3	3974	0.65	6.17	49.19
B22	0.32	2.60	994.3	3517	0.50	6.80	55.83
B23	0.32	2.60	994.3	3248	0.35	2.92	66.51
B24	0.32	2.60	994.3	3363	0.35	3.22	67.66

54.83

13.33

	Taux Phosphore en % de MS aliment	Taux Calcium en % de MS aliment	Taux de titane dans l'aliment en mg / kg de MS	Taux de titane dans le c. iléal en mg / kg de MS	Taux Phosphore en % de MS c. iléal	Taux Calcium en % de MS de c. iléal	Coefficient d'utilisation apparente du Phosphore en %
	XPFE	XLFE	TIFE	TIIL	XPIL	XLIL	RPHOP
C1	0.32	2.60	994.3	2504	0.30	3.07	62.77
C2	0.32	2.60	994.3	3129	0.52	8.32	48.36
C3	0.32	2.60	994.3	2455	0.35	4.90	55.71
C4	0.32	2.60	994.3	2678	0.44	3.71	48.96
C5	0.32	2.60	994.3	2784	0.32	2.72	64.28
C6	0.32	2.60	994.3	3053	0.46	6.14	53.18
C7	0.32	2.60	994.3	3234	0.38	5.31	63.49
C8	0.32	2.60	994.3	2069	0.19	2.99	71.47
C9	0.32	2.60	994.3	2837	0.43	4.73	52.91
C10	0.32	2.60	994.3	3142	0.41	8.41	59.45
C11	0.32	2.60	994.3	3646	0.63	4.97	46.31
C12	0.32	2.60	994.3	3106	0.48	3.07	51.99
C13	0.32	2.60	994.3	3363	0.45	3.02	58.43
C14	0.32	2.60	994.3	3031	0.43	6.80	55.92
C15	0.32	2.60	994.3	3908	0.58	3.86	53.89
C16	0.32	2.60	994.3	3514	0.55	5.94	51.37
C17	0.32	2.60	994.3	3530	0.54	3.75	52.47
C18	0.32	2.60	994.3	2989	0.52	8.96	45.95
C19	0.32	2.60	994.3	3103	0.44	3.69	55.94
C20	0.32	2.60	994.3	3510	0.45	7.14	60.16
C21	0.32	2.60	994.3	4172	0.57	4.90	57.55
C23	0.32	2.60	994.3	3131	0.35	5.28	65.26
C24	0.32	2.60	994.3	3850	0.56	5.56	54.81
						C	**56.11**
							6.43

	Taux Phosphore en % de MS aliment	Taux Calcium en % de MS aliment	Taux de titane dans l'aliment en mg / kg de MS	Taux de titane dans le c. iléal en mg / kg de MS	Taux Phosphore en % de MS c. iléal	Taux Calcium en % de MS de c. iléal	Coefficient d'utilisation apparente du Phosphore en %
TT	XPFE	XLFE	TIFE	TIIL	XPIL	XLIL	RPHOP
D1	0.32	2.60	994.3	3529	0.37	3.84	67.42
D2	0.32	2.60	994.3	2644	0.33	9.84	61.23
D3	0.32	2.60	994.3	3069	0.42	*4.45*	57.48
D4	0.32	2.60	994.3	3488	0.41	6.77	63.48
D5	0.32	2.60	994.3	3102	0.59	*3.36*	40.90
D6	0.32	2.60	994.3	3102	0.43	3.61	56.93
D7	0.32	2.60	994.3	3677	0.67	7.39	43.39
D8	0.32	2.60	994.3	3721	0.66	6.75	44.89
D9	0.32	2.60	994.3	2751	0.52	6.30	41.27
D10	0.32	2.60	994.3	4065	0.58	5.63	55.67
D11	0.32	2.60	994.3	3129	0.27	4.76	73.19
D12	0.32	2.60	994.3	3306	0.34	4.76	68.05
D13	0.32	2.60	994.3	3380	0.40	8.78	63.23
D14	0.32	2.60	994.3	4854	0.78	6.75	50.07
D15	0.32	2.60	994.3	2596	0.37	4.86	55.72
D16	0.32	2.60	994.3	3390	0.34	4.91	68.84
D17	0.32	2.60	994.3	5709	0.59	4.72	67.89
D18	0.32	2.60	994.3	3788	0.48	5.24	60.63
D19	0.32	2.60	994.3	3802	0.59	4.64	51.79
D20	0.32	2.60	994.3	3318	0.47	6.72	55.99
D21	0.32	2.60	994.3	2826	0.21	4.62	76.91
D22	0.32	2.60	994.3	3647	0.48	5.69	59.11
D23	0.32	2.60	994.3	3439	0.46	3.69	58.44
D24	0.32	2.60	994.3	4065	0.45	2.71	65.61
						D	**58.67**
							9.76

	Taux Phosphore en % de MS aliment	Taux Calcium en % de MS aliment	Taux de titane dans l'aliment en mg / kg de MS	Taux de titane dans le c. iléal en mg / kg de MS	Taux Phosphore en % de MS c. iléal	Taux Calcium en % de MS de c. iléal	Coefficient d'utilisation apparente du Phosphore en %
	XPFE	XLFE	TIFE	TIIL	XPIL	XLIL	RPHOP
E1	0.44	2.38	983.0	2682	0.69	4.40	42.51
E2	0.44	2.38	983.0	3724	1.01	5.47	39.41
E3	0.44	2.38	983.0	4559	1.15	4.48	43.64
E4	0.44	2.38	983.0	2577	0.89	**6.86**	22.84
E5	0.44	2.38	983.0	3429	1.04	5.95	32.23
E6	0.44	2.38	983.0	3439	0.94	7.33	38.94
E7	0.44	2.38	983.0	2568	0.75	**8.06**	34.75
E8	0.44	2.38	983.0	3591	0.98	7.45	39.03
E9	0.44	2.38	983.0	3347	1.03	8.62	31.24
E10	0.44	2.38	983.0	3328	1.12	**6.65**	24.82
E11	0.44	2.38	983.0	3766	1.02	5.15	39.49
E12	0.44	2.38	983.0	3789	1.08	6.73	36.33
E13	0.44	2.38	983.0	3227	0.92	**6.93**	36.31
E14	0.44	2.38	983.0	4895	0.93	2.32	57.56
E15	0.44	2.38	983.0	4045	1.16	6.20	35.94
E16	0.44	2.38	983.0	2845	0.95	10.20	25.39
E17	0.44	2.38	983.0	3194	1.01	6.89	29.36
E18	0.44	2.38	983.0	3553	1.00	4.65	37.12
E19	0.44	2.38	983.0	4131	1.23	6.49	33.49
E20	0.44	2.38	983.0	3461	1.06	8.36	31.57
E21	0.44	2.38	983.0	3009	0.89	5.40	33.91
E22	0.44	2.38	983.0	3790	1.01	5.01	40.46
E23	0.44	2.38	983.0	3442	1.14	8.33	26.01
E24	0.44	2.38	983.0	4148	0.99	4.81	46.68
						E	**35.79**
							7.70

2.4 Data on calcium and inorganic phosphorus in plasma

(see also table 6 of report 00000099)

3 pages

TRAITEMENTS	Taux de P plasmatique en mg/dl	Taux de Ca plasmatique en mg/dl	Taux de P plasmatique en mmol/L	Taux de Ca plasmatique en mmol/L
TT				
A2	3.42	24.26	1.10	6.05
A5	4.28	27.50	1.38	6.86
A8	3.84	21.88	1.24	5.46
A11	4.86	29.17	1.57	7.28
A14	3.58	24.28	1.16	6.06
A17	2.97	21.35	0.96	5.33
B2	3.28	25.19	1.06	6.28
B5	4.03	25.97	1.30	6.48
B8	6.87	31.39	2.22	7.83
B11	5.92	26.25	1.91	6.55
B14	4.11	27.87	1.33	6.95
B17	3.77	22.69	1.22	5.66
C2	4.78	26.94	1.54	6.72
C5	5.88	25.80	1.90	6.44
C8	3.32	21.44	1.07	5.35
C11	3.68	26.37	1.19	6.58
C14	3.68	23.79	1.19	5.94
C17	3.96	23.32	1.28	5.82
D2	4.46	20.70	1.44	5.16
D5	3.57	24.07	1.15	6.01
D8	4.07	25.17	1.31	6.28
D11	3.52	25.41	1.13	6.34
D14	3.84	23.49	1.24	5.86
D17	2.94	18.99	0.95	4.74
E2	4.69	28.07	1.51	7.00
E5	3.96	26.18	1.28	6.53
E8	3.19	23.99	1.03	5.98
E11	5.96	25.34	1.92	6.32
E14	3.13	23.14	1.01	5.77
E17	5.23	26.19	1.69	6.53
A	**3.82** *0.67*	**24.74** *3.08*	**1.23** *0.22*	**6.17** *0.77*
B	**4.66** *1.40*	**26.56** *2.91*	**1.50** *0.45*	**6.63** *0.73*
C	**4.22** *0.95*	**24.61** *2.11*	**1.36** *0.31*	**6.14** *0.53*
D	**3.73** *0.52*	**22.97** *2.58*	**1.20** *0.17*	**5.73** *0.64*
E	**4.36** *1.14*	**25.48** *1.76*	**1.41** *0.37*	**6.36** *0.44*

Calcium dans le Plasma

H1/09

N° échantillon	Portoir	Position	Calcium (mg/dl) Mesure 1	Calcium (mg/dl) Mesure 2	Calcium (mg/dl) Moyenne	% erreur maximale <2.5%	
1	0001	1	24.37	24.14	**24.26**	1.0	25.88
2	"	2	27.56	27.44	**27.50**	0.4	
3	"	3	21.92	21.84	**21.88**	0.4	25.52
4	"	4	29.30	29.03	**29.17**	0.9	
5	"	5	24.13	24.42	**24.28**	1.2	22.81
6	0002	1	21.45	21.24	**21.35**	1.0	
7	"	2	25.38	24.99	**25.19**	1.6	25.58
8	"	3	25.84	26.10	**25.97**	1.0	
9	"	4	31.37	31.40	**31.39**	0.1	28.82
10	"	5	26.34	26.15	**26.25**	0.7	
11	0003	1	27.99	27.75	**27.87**	0.9	25.28
12	"	2	22.74	22.63	**22.69**	0.5	
13	"	3	27.03	26.84	**26.94**	0.7	26.37
14	"	4	25.81	25.79	**25.80**	0.1	
15	"	5	21.55	21.33	**21.44**	1.0	23.90
16	0004	1	26.34	26.39	**26.37**	0.2	
17	"	2	23.69	23.89	**23.79**	0.8	23.55
18	"	3	23.18	23.45	**23.32**	1.2	
19	"	4	20.76	20.63	**20.70**	0.6	22.38
20	"	5	24.28	23.86	**24.07**	1.8	
21	0005	1	25.19	25.14	**25.17**	0.2	25.29
22	"	2	25.30	25.52	**25.41**	0.9	
23	"	3	23.46	23.52	**23.49**	0.3	21.24
24	"	4	18.77	19.21	**18.99**	2.3	
25	"	5	28.08	28.05	**28.07**	0.1	27.12
26	0006	1	26.14	26.21	**26.18**	0.3	
27	"	2	23.78	24.19	**23.99**	1.7	24.66
28	"	3	25.17	25.51	**25.34**	1.4	
29	"	4	23.20	23.07	**23.14**	0.6	24.66
30	"	5	26.18	26.20	**26.19**	0.1	

Phosphore dans le Plasma

H1/09

N° échantillon	Portoir	Position	Phosphore mg/dl Mesure 1	Phosphore mg/dl Mesure 2	Phosphore mg/dl Moyenne	% erreur maximale <2.5%	
1	0001	1	3.43	3.41	**3.42**	0.6	3.85
2	"	2	4.24	4.32	**4.28**	1.9	
3	"	3	3.85	3.83	**3.84**	0.5	4.35
4	"	4	4.90	4.81	**4.86**	1.9	
5	"	5	3.60	3.56	**3.58**	1.1	3.28
6	0002	1	2.94	3.00	**2.97**	2.0	
7	"	2	3.25	3.30	**3.28**	1.5	3.65
8	"	3	4.02	4.04	**4.03**	0.5	
9	"	4	6.82	6.91	**6.87**	1.3	6.39
10	"	5	5.91	5.92	**5.92**	0.2	
11	0003	1	4.13	4.08	**4.11**	1.2	3.94
12	"	2	3.78	3.75	**3.77**	0.8	
13	"	3	4.78	4.78	**4.78**	0.0	5.33
14	"	4	5.88	5.88	**5.88**	0.0	
15	"	5	3.30	3.34	**3.32**	1.2	3.50
16	0004	1	3.66	3.69	**3.68**	0.8	
17	"	2	3.64	3.71	**3.68**	1.9	3.82
18	"	3	3.94	3.98	**3.96**	1.0	
19	"	4	4.43	4.48	**4.46**	1.1	4.01
20	"	5	3.56	3.58	**3.57**	0.6	
21	0005	1	4.06	4.07	**4.07**	0.2	3.79
22	"	2	3.54	3.49	**3.52**	1.4	
23	"	3	3.87	3.81	**3.84**	1.6	3.39
24	"	4	2.92	2.95	**2.94**	1.0	
25	"	5	4.67	4.71	**4.69**	0.9	4.32
26	0006	1	3.96	3.95	**3.96**	0.3	
27	"	2	3.20	3.18	**3.19**	0.6	4.57
28	"	3	5.92	5.99	**5.96**	1.2	
29	"	4	3.10	3.16	**3.13**	1.9	4.18
30	"	5	5.21	5.25	**5.23**	0.8	

2.5 Data on tibia strength and tibia/toes ash

(see also table 7 and 8 of report 00000099)

2 pages

TRAITEMENTS	Résistance osseuse en N	Taux de cendres en %_tibias	Taux de cendres en %_toes
TT	R	CT	
A2	40.06	50.001	35.72
A5	39.26	48.558	34.34
A8	29.50	45.387	33.40
A11	72.32	51.873	28.71
A14	33.64	47.519	32.28
A17	21.51	46.847	25.69
B2	42.02	50.546	36.07
B5	41.26	46.777	28.69
B8	38.25	49.063	30.78
B11	57.58	49.811	36.69
B14	38.53	48.268	26.65
B17	46.85	50.798	30.61
C2	73.02	47.293	30.49
C5	45.55	50.616	32.07
C8	36.00	48.327	29.28
C11	41.56	51.069	32.66
C14	43.07	46.659	28.71
C17	49.26	47.419	27.42
D2	75.89	48.520	28.80
D5	53.58	48.660	29.97
D8	30.44	44.201	31.07
D11	72.59	50.134	34.14
D14	63.11	48.182	33.36
D17	29.70	48.725	30.72
E2	64.86	48.493	33.69
E5	23.45	46.875	34.25
E8	33.46	44.448	31.97
E11	28.25	46.172	32.39
E14	27.56	46.910	33.11
E17	72.23	46.018	34.66
A	**39.38**	**48.36**	**31.69**
	17.52	*2.32*	*3.78*
B	**44.08**	**49.21**	**31.58**
	7.31	*1.52*	*4.01*
C	**48.08**	**48.56**	**30.11**
	12.99	*1.85*	*2.02*
D	**54.22**	**48.07**	**31.34**
	20.26	*2.01*	*2.03*
E	**41.64**	**46.49**	**33.35**
	21.21	*1.33*	*1.05*

Traitement	Force à la rupture (N)	
A2	40.0584	**40.06**
A5	39.2592	**39.26**
A8	37.4288	**29.50**
A8	21.5808	
A11	54.551	**72.32**
A11	90.08	
A14	39.184	**33.64**
A14	28.093	
A17	20.29	**21.51**
A17	22.73	
B2	66.866	**42.02**
B2	17.172	
B5	32.155	**41.26**
B5	50.374	
B8	21.014	**38.25**
B8	55.491	
B11	57.58	**57.58**
B14	41.208	**38.53**
B14	35.851	
B17	53.593	**46.85**
B17	40.108	
C2	88.415	**73.02**
C2	57.621	
C5	44.055	**45.55**
C5	47.044	
C8	49.505	**36.00**
C8	22.493	
C11	55.959	**41.56**
C11	27.164	
C14	64.674	**43.07**
C14	21.473	
C17	46.8	**49.26**
C17	51.719	
D2	98.549	**75.89**
D2	53.227	
D5	54.483	**53.58**
D5	52.674	
D8	14.177	**30.44**
D8	46.707	
D11	107.39	**72.59**
D11	37.794	
D14	86.492	**63.11**
D14	39.727	
D17	22.947	**29.70**
D17	36.459	
E2	56.891	**64.86**
E2	72.824	
E5	34.843	**23.45**
E5	12.061	
E8	37.112	**33.46**
E8	29.805	
E11	22.935	**28.25**
E11	33.566	
E14	32.51	**27.56**
E14	22.618	
E17	91.542	**72.23**
E17	52.917	

2.6 Data on phosphorus in excreta

(see also figure 2 of report 00000099)

		Taux Phosphore en % de MS de fèces	Phosphore in excreta g/kg MS de fèces
	TT	**XPIL**	**XPIL**
1	**A2**	0.96	9.60
2	**A5**	0.88	8.80
3	**A8**	1.12	11.20
4	**A11**	0.85	8.50
5	**A14**	0.88	8.80
6	**A17**	0.85	8.50
7	**B2**	0.95	9.50
8	**B5**	0.84	8.40
9	**B8**	0.85	8.50
10	**B11**	0.95	9.50
11	**B14**	0.93	9.30
12	**B17**	0.84	8.40
13	**C2**	0.91	9.10
14	**C5**	0.80	8.00
15	**C8**	0.89	8.90
16	**C11**	0.88	8.80
17	**C14**	0.88	8.80
18	**C17**	0.89	8.90
19	**D2**	0.88	8.80
20	**D5**	0.93	9.30
21	**D8**	0.80	8.00
22	**D11**	0.75	7.50
23	**D14**	0.65	6.50
24	**D17**	0.82	8.20
25	**E2**	1.24	12.40
26	**E5**	1.42	14.20
27	**E8**	1.39	13.90
28	**E11**	1.28	12.80
29	**E14**	1.20	12.00
30	**E17**	1.63	16.30
	A	**0.92**	**9.23**
		0.10	*1.04*
	B	**0.89**	**8.93**
		0.06	*0.55*
	C	**0.88**	**8.75**
		0.04	*0.38*
	D	**0.81**	**8.05**
		0.10	*0.99*
	E	**1.36**	**13.60**
		0.16	*1.57*

III. Trial Protocol Data Sheet

Trial Protocol Data Sheet

According to EFSA Journal (2008) 778, 5-13 Technical guidance: Tolerance and efficacy studies in target animals
Data sheet to be filled out by the applicant and signed by the study director and then added to each trial report concerning safety and efficacy of the additive for the target animal

For terrestrial animals

Identification of the additive: 6 bacterial phytase Batch number: PPQ 28432 Trial ID: H-01/09 Location: Research Center for Animal Nutrition (DSM Nutritional Products France, F-68128 Village-Neuf) Start date and exact duration of the study:February-19-2009 to April-2-2009, 6 weeks
Number of treatment groups (+ control(s)): 3 (+2) Replicates per group: 24 Total number of animals: 240 Animals per replicate: 2
Dose(s) of the additive/active substance(s)/agent(s) (mg/Units of activity/CFU kg^{-1} complete feed/L^{-1} water) Intended:0/500/1000/2000 $U.kg^{-1}$ Analysed: <0.01/562/1114/2097 $U.kg^{-1}$ Substances used for comparative purposes: Intended dose: Analysed:
Animal species/category: Laying hens Breed: Isa Brown Identification procedure: per cage number Sex: Females Age at start: 23 wks Body weight at start: 1689 g Physiological stage: Laying General health: normal
Additional Information for field trials: Location and size of herd or flock: Feeding and rearing conditions: Method of feeding:
Diets (type(s)): low phosphorus basal diet Presentation of the diet: Mash ☒ Pellet ☐ Extruded ☐ Other Composition (main feedingstuffs): 65.0% maize / 23.6% SBM Nutrient content (relevant nutrients and energy content) Intended values: per kg: 11.9 MJ/ME, 161 g Crude protein, 2.6 g total P, 34.5 g Calcium Analysed values: per kg: 12.2 MJ/ME, 169 g Crude protein, 2.9 g total P, 0.74 g Non Phytate-P, 23.1 g Calcium
Date and nature of the examinations performed: laying performance, ileal digestibility, bone quality, excreta, plasma Method(s) of statistical evaluation used: one-factorial analysis of variance (factor: treatment), Newman-Keuls test Therapeutic/preventive treatments (reason, timing, kind, duration): nothing to report Timing and prevalence of any undesirable consequences of treatment: nothing to report

Date	Signature Study Director
09- June- 2009	Petra Pluijgers

efsa

European Food Safety Authority

FEEDAP UNIT

ANNEX I

TRIAL PROTOCOL DATA SHEET: FOR TERRESTRIAL ANIMALS

Identification of the additive: **6 bacterial phytase**	Batch number: **PPQ 28432**
Trial ID: **H-01/09**	Location: **Research Center for Animal Nutrition (DSM Nutritional Products France,F-68128 Village-Neuf**
Start date and exact duration of the study: **February-19-2009 to April-2-2009, 6 weeks**	
Number of treatment groups (+ control(s)): **3 (+2)**	Replicates per group: **24**
Total number of animals: **240**	Animals per replicate: **2**
Dose(s) of the additive/active substance(s)/agent(s) (mg/Units of activity/CFU kg^{-1} complete feed/L^{-1} water) Intended: **0/500/1000/2000 U/kg** Analysed: **<0.01/562/1114/2097 U/kg** † Substances used for comparative purposes: Intended dose: Analysed:	
Animal species/category: **Laying hens**	
Breed: **Isa Brown**	Identification procedure: **per cage number**
Sex: **Females** Age at start: **23 wks**	Body weight at start: **1689 g**
Physiological stage: **Laying**	General health: **normal**
Additional information for field trials:	
Location and size of herd or flock:	
Feeding and rearing conditions:	
Method of feeding: **ad libitum**	
Diets (type(s)): **low phosphorus basal diet**	
Presentation of the diet: Mash ☒ Pellet ☐ Extruded ☐ Other	
Composition (main feedingstuffs): **65.0% maize/23.6% SBM**	
Nutrient content (relevant nutrients and energy content) Intended values: **11.9MJ/ME, 161 g Crude Protein (CP) , 2.6 g total P, 34.5 g Calcium** Analysed values: **12.2 MJ/ME, 169 g CP, 2.9 g total P, 0.74 g Non-Phytate-P, 23.1 g Calcium**	
Date and nature of the examinations performed: **laying performance, ileal digestibility, bone quality, excreta, plasma**	
Method(s) of statistical evaluation used: **one-factorial analysis of variance (factor:treatment), Newman-Keuls test**	
Therapeutic/preventive treatments (reason, timing, kind, duration): **nothing to report**	
Timing and prevalence of any undesirable consequences of treatment: **nothing to report**	

[1] Please submit this form using a common word processing format (e.g. MS Word).

FEEDAP UNIT

Date **12.02.2010**	Signature Study Director
	Pedro Pluijgers

† In case the concentration of the additive in complete feed/water may reflect insufficient accuracy, the dose of the additive can be given per animal day^{-1} or mg kg^{-1} body weight or as concentration in complementary feed.

Trial Protocol Data Sheet

According to EFSA Journal (2008) 778, 5-13 Technical guidance: Tolerance and efficacy studies in target animals
Data sheet to be filled out by the applicant and signed by the study director and then added to each trial report concerning safety and efficacy of the additive for the target animal

For terrestrial animals

Identification of the additive: 6 bacterial phytase Batch number: PPQ 28432 Trial ID: H-01/09 Location: Research Center for Animal Nutrition (DSM Nutritional Products France, F-68128 Village-Neuf) Start date and exact duration of the study:February-19-2009 to April-2-2009, 6 weeks
Number of treatment groups (+ control(s)): 3 (+2) Replicates per group: 24 Total number of animals: 240 Animals per replicate: 2
Dose(s) of the additive/active substance(s)/agent(s) (mg/Units of activity/CFU kg^{-1} complete feed/L^{-1} water) Intended:0/500/1000/2000 $U.kg^{-1}$ Analysed: <0.01/562/1114/2097 $U.kg^{-1}$ Substances used for comparative purposes: Intended dose: Analysed:
Animal species/category: Laying hens Breed: Isa Brown Identification procedure: per cage number Sex: Females Age at start: 23 wks Body weight at start: 1689 g Physiological stage: Laying General health: normal
Additional Information for field trials: Location and size of herd or flock: Feeding and rearing conditions: Method of feeding:
Diets (type(s)): low phosphorus basal diet Presentation of the diet: Mash ☒ Pellet ☐ Extruded ☐ Other Composition (main feedingstuffs): 65.0% maize / 23.6% SBM Nutrient content (relevant nutrients and energy content) Intended values: per kg: 11.9 MJ/ME, 161 g Crude protein, 2.6 g total P, 34.5 g Calcium Analysed values: per kg: 12.2 MJ/ME, 169 g Crude protein, 2.9 g total P, 0.74 g Non Phytate-P, 23.1 g Calcium
Date and nature of the examinations performed: laying performance, ileal digestibility, bone quality, excreta, plasma Method(s) of statistical evaluation used: one-factorial analysis of variance (factor: treatment), Newman-Keuls test Therapeutic/preventive treatments (reason, timing, kind, duration): nothing to report Timing and prevalence of any undesirable consequences of treatment: nothing to report

Date	Signature Study Director

TAB

ANNEX 28

Annex 28

Esteve, E. and Broz, J. (2009). Report No. 00001628: Efficacy of IPA PHYTASE (= RONOZYME® HiPhos) in Turkeys. 2009

REPORT No. 00001628
Regulatory Document

DSM

Document Date:	12 August, 2009
Author(s):	E. Esteve-Garcia[1] and J. Broz[2]
	[1] Department of Animal Nutrition, IRTA, Centre Mas de Bover, Constanti (Spain) [2] Animal Nutrition and Health R&D, DSM Nutritional Products Ltd, Basel
Title:	**Efficacy of IPA phytase in turkeys**
Project No.	6106

Summary

An experiment was conducted to evaluate the efficacy of IPA phytase (M) in turkeys when used at graded inclusion levels added to a low-P maize-soybean meal-based diet. A total of 216 day-old female turkeys (BUT 9 strain) were used in this study, divided into 72 replicate groups of 3 birds each. The following six dietary treatments were compared: T-1) negative control fed the basal diet containing 0.27% of non-phytate P; T-2) NC diet + IPA phytase at 500 U/kg; T-3) NC diet + IPA phytase at 1000 U/kg; T-4) NC diet + IPA phytase at 2000 U/kg; T-5) NC diet + IPA phytase at 4000 U/kg; T-6) positive control fed the diet containing 0.1% of additional non-phytate P in form of dicalcium phosphate. Each dietary treatment was assigned to 12 replicate groups. Performance, bone mineralization, blood Ca and P concentration, and apparent Ca and P retention were used as the efficacy parameters. Body weight showed a positive response to IPA phytase supplementation which was fitted to a quadratic polynomial. Tibia ash percentage showed a curvilinear response which was fitted to a quadratic polynomial as well, indicating a response to all phytase levels. Ca and P retention also showed curvilinear responses to graded phytase levels. IPA phytase addition at 500, 1000, 2000 and 4000 U/kg diet increased significantly the P retention from 58.2% (negative control) to 68.4, 72.8, 76.2 and 78.7%, respectively.

This report consists of Pages I – II and 1 - 35

Distribution

Dr. M. Eggersdorfer, NRD
Dr. F. Fru, NRD/PA
Mr. J.-F. Hecquet, NBD/RG
Dr. A.-M. Klünter, NRD/CA
Dr. J. Pheiffer, NRD/PA
Dr. P. Philipps, NRD/CA
Mr. J.-P. Ruckebusch, ANH/GM

Approved

Name	Signature	Date
Main Author	signed by	
Dr. J. Broz, NRD/CA	J. Broz	12.08.2009
Principal Scientist / Competence Mgr	signed by	
Dr. J. Broz, NRD/CA	J. Broz	12.08.2009
Research Center Head	signed by	
Dr. A.-M. Klünter, NRD/CA	A.-M. Klünter	13.08.2009
Project Manager	signed by	
Dr. F. Fru, NRD/PA	F. Fru	18.08.2009

Nomenclature and Structural Formula

IPA phytase (M), enzyme product containing bacterial 6-phytase (EC 3.1.3.26), produced by submerged fermentation of a genetically modified *Aspergillus oryzae* strain. Lot PPQ 28656 was used in this study, manufactured by Novozymes A/S, Bagsvaerd, Denmark.

FINAL REPORT OF THE CONTRACT SIGNED WITH:

Company: DSM Nutritional Products

Title: EFFICACY OF IPA PHYTASE IN TURKEYS

Experiment number: E-106

Contract Code:	*2 2 5 5 0*
Organic Code:	*0 6 0 2*

Author: **Enric Esteve-Garcia**

Center: IRTA - RECERCA I TECNOLOGIA AGROALIMENTÀRIES
Monogastric Nutrition
Mas de Bover
Ctra. Reus-El Morell km. 3.8, E-43120 Constantí (Tarragona), Spain
Tel. 977 32 84 24 Fax 977 34 40 55

Number of pages: 35

Date: 10/08/2009

TABLE OF CONTENTS

SUMMARY

An experiment was conducted to evaluate the efficacy of IPA phytase (M) in turkeys at different doses, as compared to a negative control containing low phosphorus, and a positive control containing supplemental phosphorus from dicalcium phosphate. Performance, bone mineralization, blood calcium and phosphorus concentration, and apparent calcium and phosphorus retention were used as criteria for bioefficacy. Two hundred and sixteen day-old female turkeys (BUT 9 strain) were placed into 72 cages at three per cage, and randomly assigned one of six experimental diets: T-1) a negative control fed the diet based on maize and soybean meal containing 0.27 % non-phytate phosphorus and 1.2 % calcium; T-2) negative control + IPA phytase at 500 U/kg feed; T-3) negative control + IPA phytase at 1000 U/kg feed; T-4) negative control + IPA phytase at 2000 U/kg feed; T-5) negative control + IPA phytase at 4000 U /kg feed; T-6) a positive control fed the diet containing 0.1 % additional non-phytate phosphorus in the form of dicalcium phosphate dehydrate. Birds and feed were weighed at 21 days of age and performance was calculated for the respective period. At 21 days birds were deprived of feed for 16 h and excreta were collected quantitatively for 3 days. Feed consumption was also measured during the period, and birds were again deprived of feed for 16 h prior the end of excreta collection. Blood samples were also obtained at the end, and blood calcium and inorganic phosphorus concentration was determined. The same bird was sacrificed and left tibia was excised for bone ash determination. Body weight showed a response to IPA phytase supplementation which was fitted to a quadratic polynomial, indicating a response to all levels of supplementation. Feed to gain ratio was greater for the negative control than for the other treatments, and significantly greater for the positive control than for the phytase supplementation at 4000 U/kg. Bone ash also showed a curvilinear response which was fitted to a quadratic polynomial, indicating a response to all levels of supplementation. Calcium and phosphorus retention also showed curvilinear responses which were also fitted to a quadratic polynomial, indicating responses to all levels of supplementation. Blood calcium increased with phytase supplementation, but there was no clear pattern for blood phosphorus, as there was large variability in the negative control. It is concluded that IPA phytase significantly improves the performance of turkeys in terms of body weight gain, bone ash and calcium and phosphorus retention in the range between 0 and 4000 U/kg feed in a curvilinear fashion, indicating a response to all levels of supplementation.

RESPONSIBILITIES

Study director

E. Esteve-Garcia
IRTA, Monogastric Nutrition
Ctra. Reus-El Morell km. 3.8, 43120 Constantí
Tel. +34 977 32 84 24
Fax +34 977 34 40 55
E-mail: enric.esteve@irta.es

Study monitor

Dr Jiri Broz
DSM Nutritional Products
P.O. Box 2676
CH-4002 Basel
Switzerland
Tel. +41 61 815 87 35
E-mail: jiri.broz@dsm.com

Daily monitor

Ll. Llauradó, A.Mejías
IRTA, Monogastric Nutrition
Ctra. Reus-El Morell km. 3.8, 43120 Constantí
Tel. 34 77 34 32 52
Fax. 34 77 34 40 55
E-mail: lluis.llaurado@irta.es

Stockworkers

Josep Rodríguez & Joan Martí
IRTA, Monogastric Nutrition
Ctra. Reus-El Morell km. 3.8, 43120 Constantí

Feed manufacturing

Lluís Padrell, Andreu Vilalta
IRTA, Monogastric Nutrition
Ctra. Reus-El Morell km. 3.8, 43120 Constantí

Laboratory analysis

Anna Mª Pérez Vendrell
IRTA, Monogastric Nutrition
Ctra. Reus-El Morell km. 3.8, 43120 Constantí

OBJECTIVE

The objective of the study was to evaluate the efficacy of IPA phytase in turkeys when used at different doses and compared to a negative control fed a low-P basal diet, as determined by performance, calcium and phosphorus retention and plasma concentration, as well as tibia mineralization. In addition, a positive control receiving a diet containing supplemental dicalcium phosphate was included.

METHODOLOGY

Site of the experiment

IRTA, Monogastric Nutrition, Mas de Bover

Dates and duration of the experiment

The experiment started on 17 November 2008 and lasted 25 days.

Test product

Name of the product tested: IPA phytase (M)
Active ingredient: 6-phytase expressed in *Aspergillus oryzae*
Activity: 60 700 U/g product (analyzed on 5 November 2008)
Lot number: PPQ 28656
Manufactured by: Novozymes A/S, Bagsvaerd, Denmark
Supplied by: DSM Nutritional Products Ltd, Basel, Switzerland
Level of inclusion in the diet: 500, 1000, 2000 and 4000 U/kg diet, corresponding to 8.24, 16.48, 32.96 and 65.92 mg/kg feed, respectively.

Location and housing

Birds were placed into 72 cages, each cage measuring 0.9m², where they remained till 25 days of age. The cages were located in the digestibility unit. Stocking density was 3 birds / 0.9m^2 during the first 21 days and 2 birds/0.9 m^2 during the last 4 days, which was the collection period. There were 12 replicates per treatment.

Birds were placed in a room provided with artificial lights, gas heating and ventilation by extraction. Temperature inside the houses on arrival was 33-35 °C and was reduced by 2 °C each week. The lighting program was 23 h till day 4, 20 h till 10 days and 18 h till end. Feed was placed in individual feeders, and water was administered by means of cup drinkers.

Animals

Two hundred and fifty (250) female medium size turkeys of the BUT number 9 strain were used, and distributed into 72 cages. All cages contained 3 birds. Only animals free of any clinical signs, e.g. no leg problems, eyes opened, active behavior, and no other problems, were included in the trial. At the end of the third week, birds were weighed. Birds showing signs of illness, runts, and smaller birds were discarded. At 21 days, birds were weighed and that with the lowest weight was eliminated for the balance.

There were 4 cages with 3 turkeys on the experimental diets in reserve which were used for replacement in case of mortality or runts during the first 7 days.

Feeding program

Two different diets were used in this experiment. A low-P basal diet contained 0.27 % non-phytate phosphorus. A positive control diet contained 0.37 % non-phytate phosphorus. The composition of the different basal experimental diets is shown in Table 1. Analytical composition of the test diets is shown in Table 2. Feed was provided in mash form.

During the first 3 days, feed was placed in feed trays which were filled daily to ensure *ad libitum* feeding of the birds. The premix and trial feed were manufactured at:

IRTA
Feed Mill of Department of Monogastric Nutrition
Centre de Mas Bové
Ctra. Reus al Morell, km. 3.8
43120 Constantí
SPAIN

All feed ingredients, except fat, salt, dicalcium phosphate, calcium carbonate, the vitamin and mineral premix, and the test product were ground through a 25 mm hammer mill until the particles passed through a 3 mm sieve. The mixer is a 500 kg capacity horizontal mixer, and the mixing time was 5 min. A single mix of 500 kg of the low-P diet was prepared and divided into 5 fractions of 75 kg. The test product was premixed with about 500 g of the basal diet and mixed in the laboratory, and this premix was mixed for another 5 min with about 10 kg of feed in a bakery mixer. The second premix was then added into the final mix and mixed in a concrete mixer for 5 min.

The feeds used in this trial did not contain any antibiotic growth promoter or any probiotic feed additive.

The feed was put into bags, clearly labeled with capital characters T-1, T-2, T-3, T-4, T-5 and T-6. One-kg samples from each type of feed and treatment were taken and divided into two sets. One of the sets of samples was dispatched to IRTA Laboratory.

Feed samples were analyzed there for dry matter, crude protein, crude fiber, ether extract, total phosphorus, calcium and chloride content. The other set of samples was sent to Biopract GmbH, Berlin, for phytase analysis.

Treatments and experimental design

Treatment	Name	Phytase level	Basal diet	Product addition	Dicalcium phosphate	Non-phytate phosphorus
		U/kg		mg/kg	%	%
T-1	Negative control	0	Low-P diet	0	0.68	0.27
T-2	IPA phytase 500	500	Low-P diet	8.24	0.68	0.27
T-3	IPA phytase 1000	1000	Low-P diet	16.48	0.68	0.27
T-4	IPA phytase 2000	2000	Low-P diet	32.96	0.68	0.27
T-5	IPA phytase 4000	4000	Low-P diet	65.92	0.68	0.27
T-6	Positive control	0	Positive control	0	1.25	0.37

Controls and parameters

Performance

Period 0 to 21 days: Weight gain, feed consumption and feed to gain, of each cage.

Phosphorus and calcium balance

Between 22 and 25 days of age, excreta were collected daily. Birds underwent a 16-h fast prior to the start and finish of the collection period. Feed consumption was also measured during this period. At the end of the experiment, excreta were freeze dried, and homogenized in a grinder for calcium and phosphorus determination. Feeds were also analyzed for these components. Phosphorus and calcium retention was calculated.

Bone ash, blood calcium and phosphorus

One blood sample was taken for analysis of calcium and inorganic phosphorus. The same bird per cage was used for bone ash determination at 25 days of age. Bone ash was determined in the left tibia following the procedure of Ravindran *et al.* (1995)[1].

RESULTS AND DISCUSSION

Performance till 21 days of age is shown in Table 3. Birds fed the negative control diet showed a clear phosphorus deficiency, as their growth was clearly impaired and feed to gain ratio was clearly different and greater than that of the other treatments ($P < 0.05$). In fact, 17 out of 36 birds of this treatment did not finish the experiment because they died or were removed due to very poor performance. It must be noted that the level of non-phytate P in the diet was calculated to be 0.27 %, which is only 45% of the NRC requirement. In contrast, only one bird per treatment was removed or died in the other treatments, except in T-4 (2000 U/kg) in which two birds did not finish the experiment. Average daily gain responded in a curvilinear fashion, as shown in Figure 1, although the response to the treatment with 2000 U/kg diet was a bit below that of 1000 U/kg level, as two of the cages showed poor values, and the response would be more smooth without these two values. It must be noted that the experiment was not so much designed to measure performance, as there were only three birds per pen, as to measure mineral balance, tibia ash and blood Ca and P. Therefore, the precision of the performance measurements is subject to fairly large variability. Feed to gain of the negative control was significantly different from that of the other treatments ($P < 0.05$), and no significant differences were found among all treatments containing IPA phytase. However, the positive control resulted in a significantly less efficient feed conversion than the highest inclusion level of IPA phytase ($P<0.05$).

Percentage of tibia ash is shown in Table 4. There was a significant quadratic response to IPA phytase supplementation. Interestingly, the positive control resulted in greater tibia ash percentage than the treatment with 500 U/kg, while in terms of performance the results were very similar in these two treatments, suggesting the response to phytase to be somewhat lower in terms of bone mineralization than in daily gain. Based on the quadratic equations shown in Figures 1 and 3, it can be calculated that 0.1 % non-

[1] Ravindran, V, E.T. Kornegay, D.M. Denbow, Z. Yi, and R.M. Hulet (1995) Response of turkey poults to tiered levels of Natuphos® phytase added to soybean meal-based semi-purified diets containing three levels of non-phytate phosphorus. Poult. Sci. 74: 1843-1854.

phytate (inorganic) P is equivalent to 856 U/kg diet if measured in terms of body weight, while when measured in tibia ash the equivalence is 947 U/kg diet.

Blood calcium and phosphorus data are shown in Table 5. For blood calcium there was a curvilinear response to IPA phytase supplementation as shown in Figures 6 and 7, although results could be also interpreted as a plateau which is reached at about 1000 U/kg diet. Blood phosphorus showed a different response, as shown in Figures 4 and 5. The negative controls showed a mean value which is greater than that of the 500 U/kg level, and then values increased till the 1000 U/kg level and remain in a plateau level. A closer examination of the individual results shows that there are no evident outliers, but values scattered, as half of the values are above the mean, and the other half on the line or below. This suggests that in case of a clear deficiency blood phosphorus is not well regulated. In the case of blood calcium, in the negative control there was one value clearly below the line, and another above, but most of the values were grouped around the line, suggesting a tighter regulation of blood calcium. Comparing the blood Ca values versus final body weight for the negative controls, there is no clear relationship (Figure 10) nor between blood P and final body weight for the negative controls. Looking at the results of all treatments, in the case of Ca (Figure 11) there seems to be a linear trend for greater blood Ca levels with greater body weight, but no relationship between body weight and blood P.

Values for calcium and phosphorus retention are shown in Table 6 and in Figures 8 and 9, respectively. Calcium retention increased in a dose dependent manner as the level of IPA phytase increased. The quadratic model shows a good fit (R-square = 0.8795), and the highest value for Ca retention to be at the 4000 U/kg level. Phosphorus retention also showed a curvilinear response, and a good fit to the quadratic model, with the highest value achieved at the 4000 U/kg level (although the quadratic fit assumes a peak slightly over the 3000 U/kg level).

Comparing results of the different parameters, it can be concluded that IPA phytase supplementation showed curvilinear responses in terms of final body weight (and the weight gain), tibia ash, and phosphorus and calcium digestibilities. Maximum responses were apparently observed at the highest levels of phytase supplementation, if the curvilinear fit is considered as representative of the response.

Feed to gain ratio showed a clear difference between the negative, unsupplemented control and all other treatments, and a difference between the positive control and the highest level of phytase. Blood calcium and phosphorus did not seem to be good indicators of calcium and phosphorus availability, although a response of calcium availability to phytase was observed.

It can be concluded that IPA phytase supplementation of the low-P diet in turkeys improved performance, tibia ash percentage and calcium and phosphorus digestibilities. Blood calcium level also responded to phytase supplementation.

Signatures:

Dr. Enric Esteve	Dr. Joaquim Brufau
Study researcher	Director
Director	Mas de Bover
Monogastric Nutrition	
Date: 10/8/2009	Date: 10/8/2009

ANNEX I: TABLES AND FIGURES

Table 1. Composition of the experimental diets (in %)

	Negative control %	Positive control %
Maize	44.38	43.77
Soya oil	2.22	2.54
Soybean meal (48% CP)	49.50	49.60
DL-methionine	0.19	0.19
L-lysine HCl	0.01	0.01
Calcium carbonate	2.21	1.83
Dicalcium phosphate	0.68	1.25
Salt	0.40	0.40
Minerals & vitamins[1]	0.40	0.40
Choline chloride	0.01	0.01
Estimated nutrient content		
ME, MJ/kg	11.7	11.7
Crude protein	28.0	28.0
Crude fibre	2.7	2.7
Ether extract	4.7	5.0
Crude ash	6.7	6.9
Lysine	1.60	1.60
Methionine	0.61	0.61
Methionine + cystine	1.05	1.05
Threonine	1.07	1.07
Tryptophan	0.34	0.34
Calcium	1.2	1.2
Total phosphorus	0.54	0.64
Non-phytate phosphorus	0.27	0.37

[1] One kg of feed from contains: Vitamin A: 15000 IU; Vitamin D_3: 5000 IU; Vitamin E: 25 mg; Vitamin K_3: 4 mg; Vitamin B_1: 2,2 mg; Vitamin B_2: 8 mg; Vitamin B_6: 8 mg; Vitamin B_{12}: 15 µg; Folic acid: 3 mg; Biotin: 200 µg; Calcium pantothenate: 20 mg; nicotinic acid: 75 mg; Mn: 80 mg; Zn: 60 mg; I: 0,4 mg; Fe: 50 mg; Cu: 8 mg; Se: 0,2 mg; Ethoxyquin: 150 mg.

Table 2. Analytical composition of the experimental diets

Ref. Lab.	Sample	Dry matter (%)	Crude protein (%)	Ether Extract (%)	Crude ash (%)	Phosphorus (%)	Calcium (%)	Chloride (% NaCl)	Phytase (U/kg)
081011	**T-1** 14-11-08	89.71	26.7	4.71	6.01	0.52	1.19	0.460	59
081012	**T-2** 14-11-08	89.62	26.3	4.59	6.20	0.53	1.16	0.459	522
081013	**T-3** 14-11-08	89.68	26.7	4.57	6.16	0.53	1.16	0.463	1040
081014	**T-4** 14-11-08	89.66	26.8	4.57	6.33	0.53	1.19	0.446	1966
081015	**T-5** 14-11-08	89.66	25.9	4.51	6.28	0.54	1.23	0.432	4397
081016	**T-6** 14-11-08	89.52	25.4	4.79	6.23	0.62	1.18	0.445	61

Table 3. Performance between 1 and 21 days of age

Treatment	Diet	IPA phytase	Initial weight	Final weight	Average daily gain	Average daily feed	Feed/ gain
		U/kg	g	g	g/d	g/d	g/g
T-1	negative control	0	63	368c	13.8c	23.6c	1.709a
T-2	negative control	500	63	442b	17.3b	25.4bc	1.468bc
T-3	negative control	1000	63	464b	18.2b	27.0b	1.485bc
T-4	negative control	2000	63	458b	17.9b	26.6b	1.492bc
T-5	negative control	4000	63	515a	20.4a	29.0a	1.419c
T-6	positive control		63	438b	17.0b	26.3b	1.541b
Pooled Std deviation				*38.7*	*1.74*	*2.30*	*0.0874*
Linear				***	***	***	***
Quadratic				*	*		**

Means of 12 replicates of 3 chickens

Values followed by different letters are significantly different; Duncan's multiple test ($P < 0.05$).

Table 4. Percentage of tibia ash

Treatment	Diet	IPA phytase	Tibia ash
		U/kg	%
T-1	negative control	0	37.2e
T-2	negative control	500	41.1d
T-3	negative control	1000	44.6c
T-4	negative control	2000	47.7b
T-5	negative control	4000	49.8a
T-6	positive control		43.8c
Pooled Std deviation			*1.93*
Linear			***
Quadratic			***

Means of 12 replicates

Values followed by different letters are significantly different, Duncan's multiple test ($P < 0.05$).

Table 5. Blood concentration of calcium and inorganic phosphorus

Treatment	Diet	IPA phytase	Blood calcium	Blood inorganic phosphorus
		U/kg	mg/100 ml	mg/100 ml
T-1	negative control	0	6.23d	7.69a
T-2	negative control	500	7.55c	6.15bc
T-3	negative control	1000	8.65ab	7.17ab
T-4	negative control	2000	8.78ab	7.27ab
T-5	negative control	4000	9.12a	7.10ab
T-6	positive control		7.81bc	5.23c
Pooled Std deviation			*1.246*	*1.497*
Linear			***	NS
Quadratic			**	NS

Means of 12 replicates

Values followed by different letters are significantly different, Duncan's multiple test ($P < 0.05$).

Table 6. Apparent calcium and phosphorus retention

Treatment	Diet	IPA phytase	Calcium	Phosphorus
		U/kg	%	%
T-1	negative control	0	56.4bc	58.2d
T-2	negative control	500	60.5ab	68.4c
T-3	negative control	1000	59.1abc	72.8b
T-4	negative control	2000	62.8a	76.2ab
T-5	negative control	4000	64.2a	78.7a
T-6	positive control		55.4c	60.7d
Pooled Std deviation			*5.35*	*4.60*
Linear			**	***
Quadratic			NS	***

Means of 12 replicates

Values followed by different letters are significantly different, Duncan's multiple test ($P < 0.05$).

Figure 1. Body weigth at 21 days

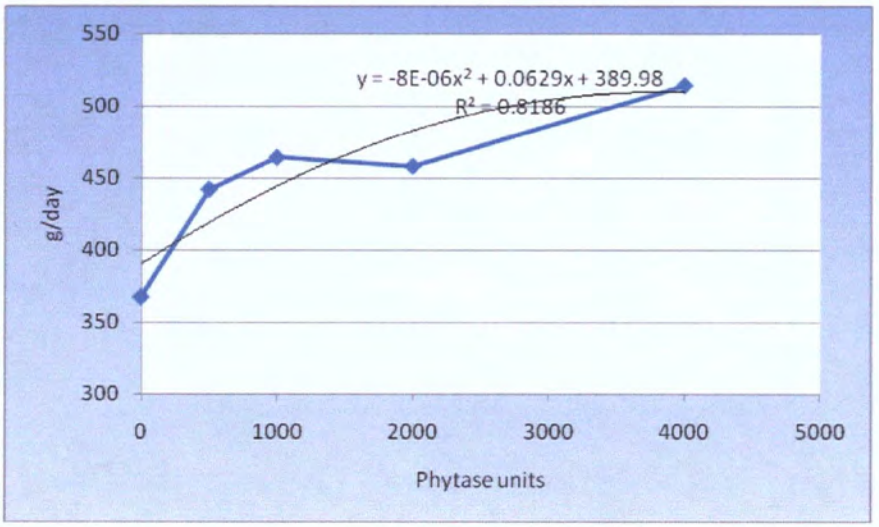


Figure 2. Feed to gain ratio

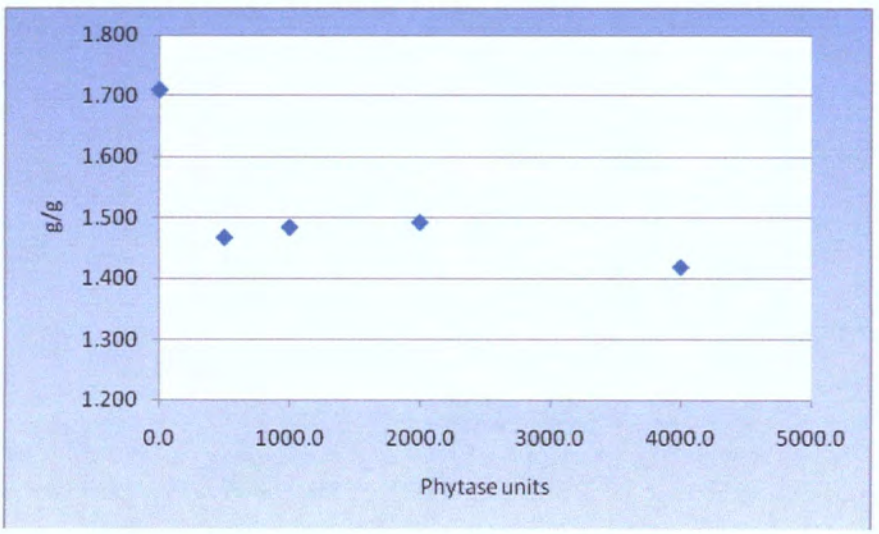


Figure 3. Tibia ash

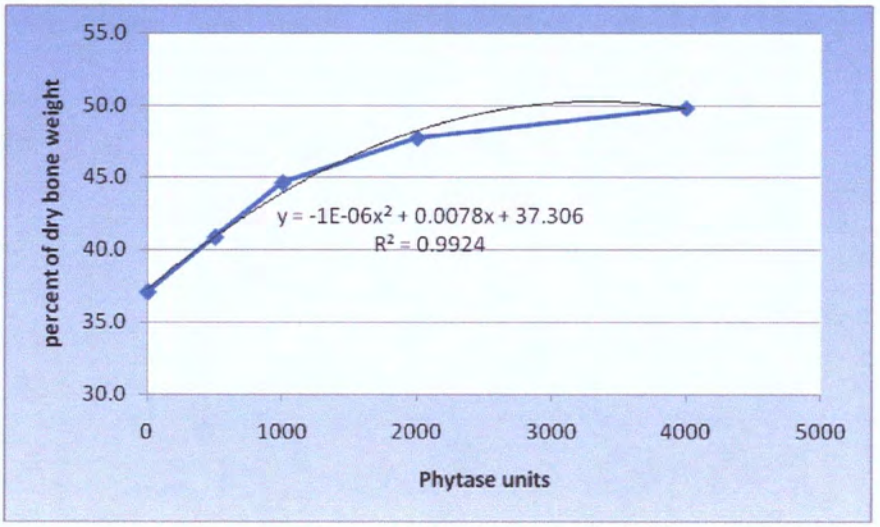


Figure 4. Blood phosphorus

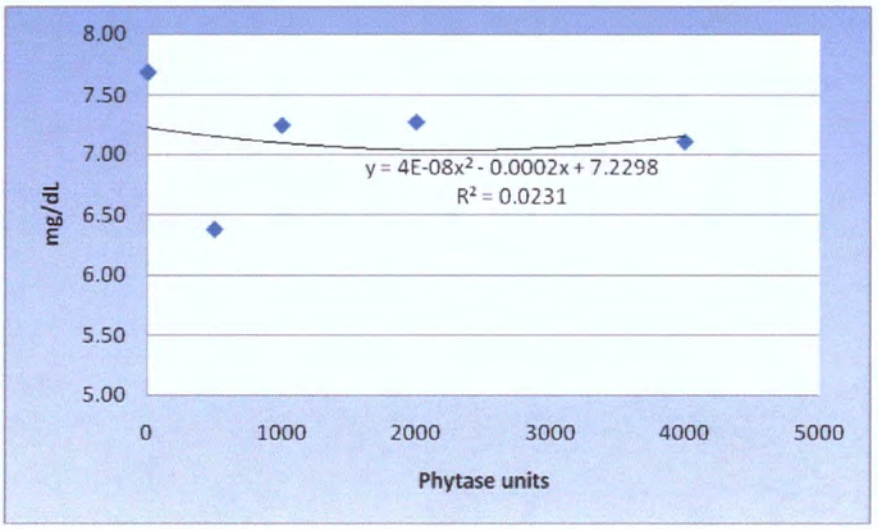


Figure 5. Blood phosphorus, individual observations

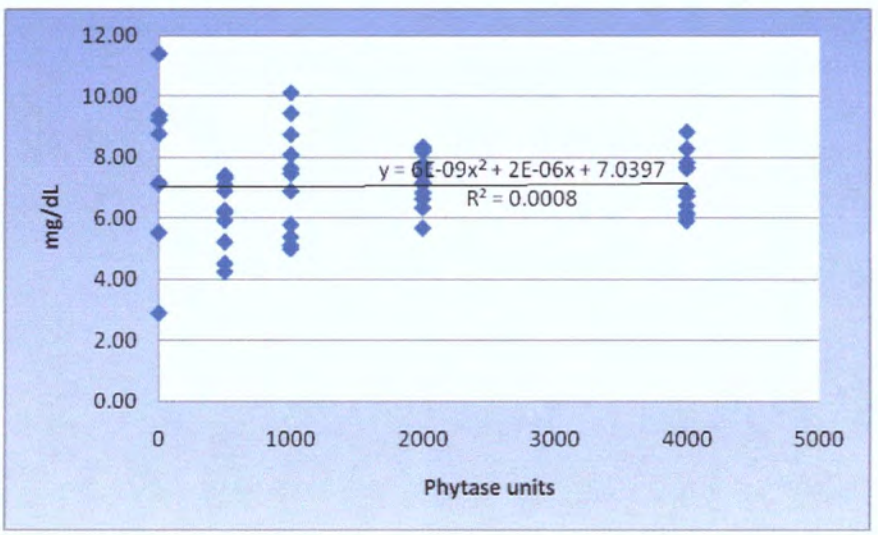


Figure 6. Blood calcium

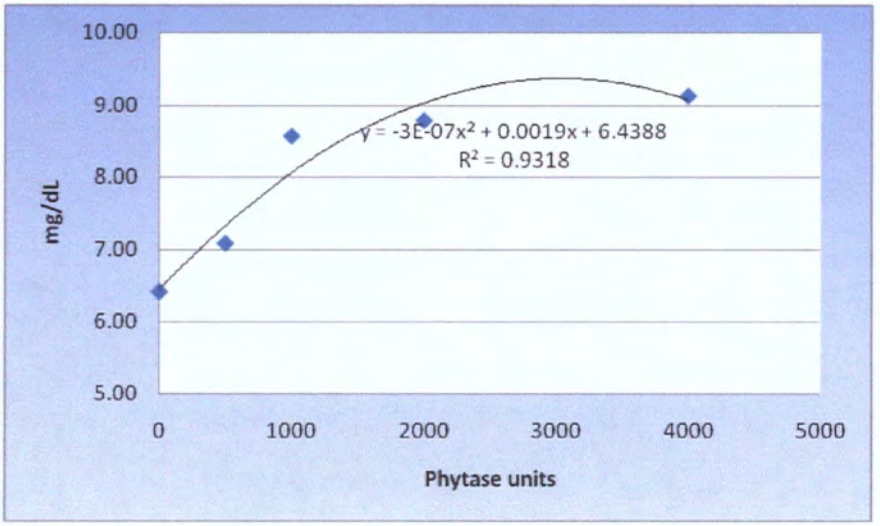


Figure 7. Blood calcium, individual observations

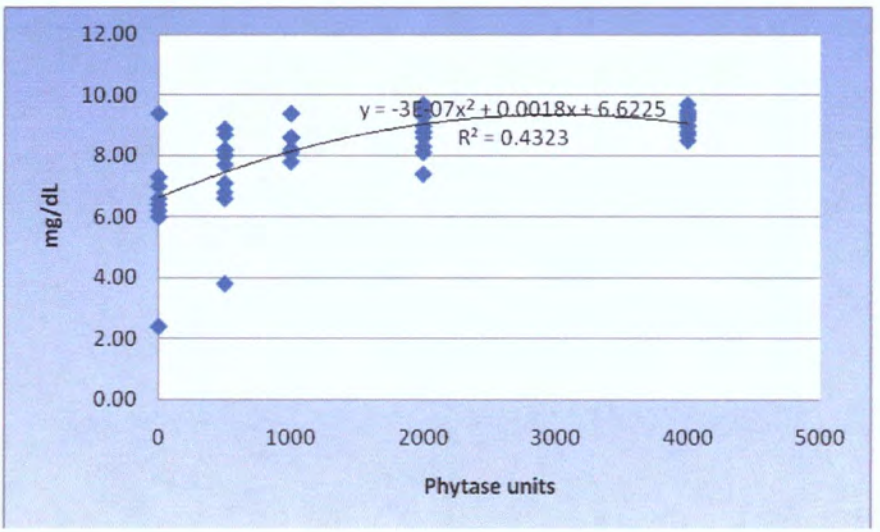


Figure 8. Calcium retention

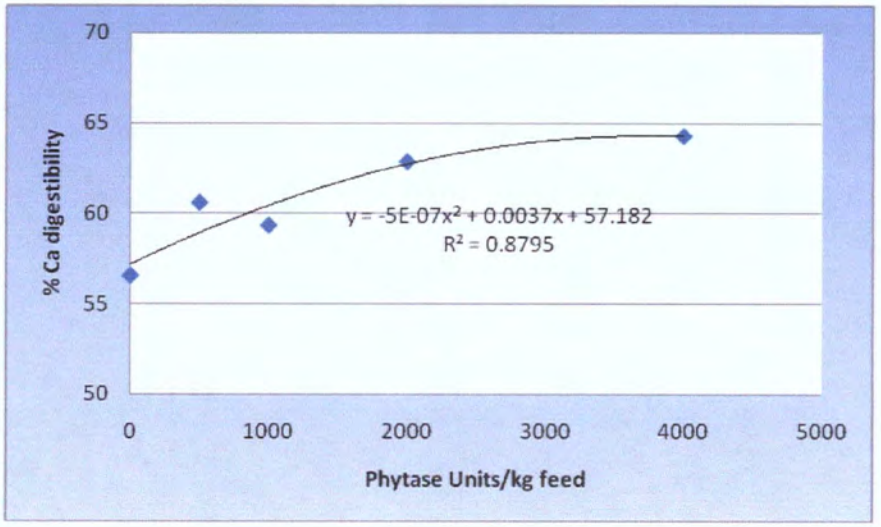


Figure 9. Phosphorus retention

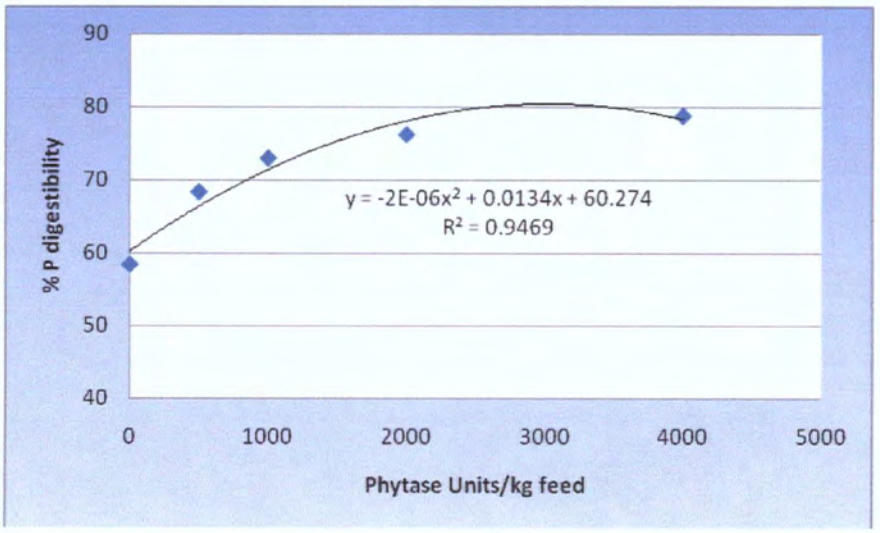


Figure 10. Bood calcium vs final body weight for the negative control

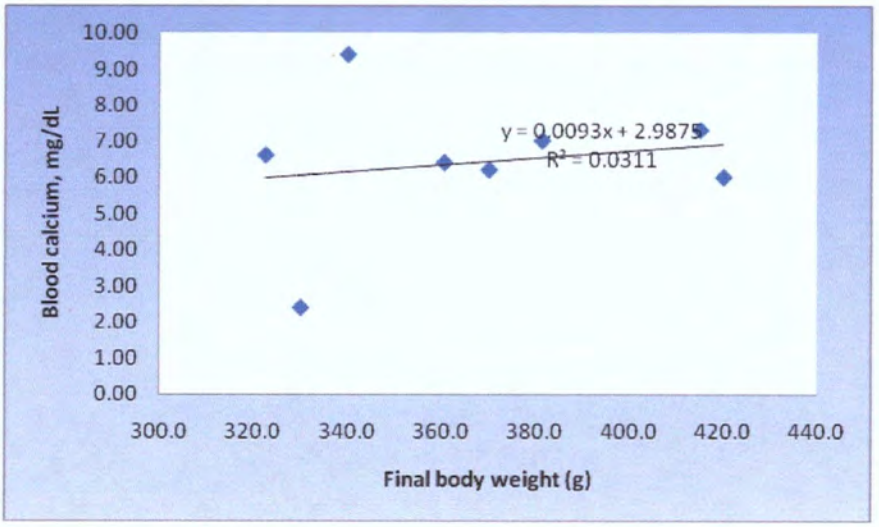


Figure 11. Blood calcium vs final body weight, all treatments

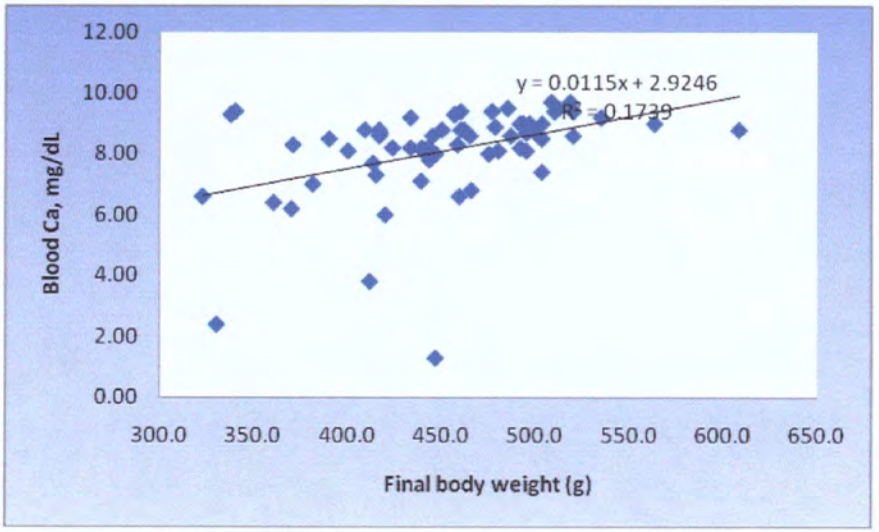


Figure 12. Blood phosphorus vs final body weight for the negative control

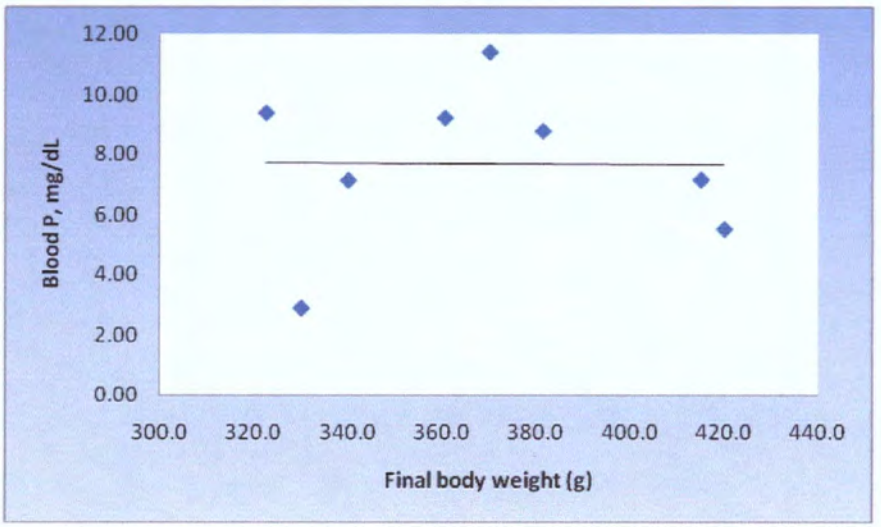
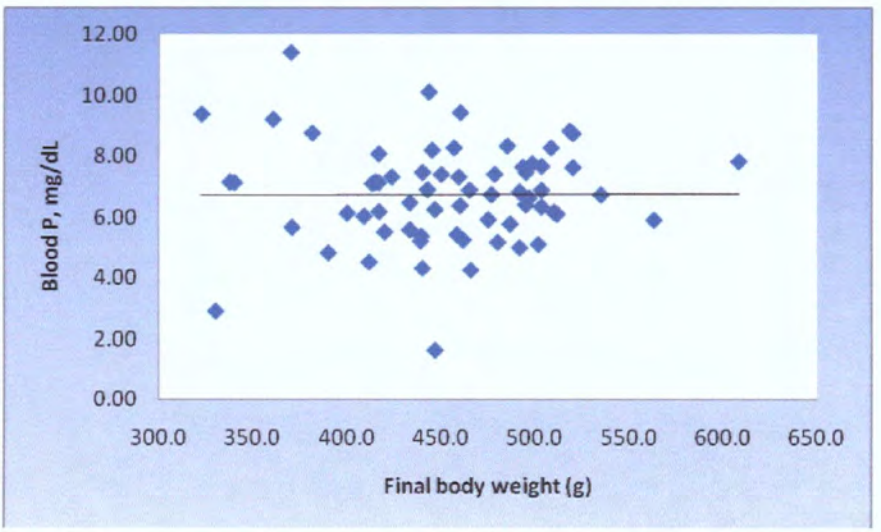


Figure 13. Blood phosphorus vs final body weight



ANNEX II: Annex C EFSA

efsa
European Food Safety Authority

FEEDAP UNIT

ANNEX C [1]

TRIAL PROTOCOL DATA SHEET: FOR TERRESTRIAL ANIMALS

Identification of the additive: **IPA Phytase (M)**	Batch number: **PPQ 28656**
Trial ID: **E-106**	Location: **IRTA- Mas de Bover**
Start date and exact duration of the study: **17 November 2008**	
Number of treatment groups (+ control(s)): **6**	Replicates per group: **12**
Total number of animals: **216**	Animals per replicate: **36**
Dose(s) of the additive/active substance(s)/agent(s) (mg/Units of activity/CFU kg^{-1} complete feed/L^{-1} water)	
Intended: **0, 500, 1000, 2000, 4000 and 0 U/kg** †	Analysed: **59, 522, 1040, 1966, 4397 and 61U/kg**
Substances used for comparative purposes: **Dicalcium Phosphate**	
Intended dose: **0.57 %**	Analysed: **0.57 %**
Animal species/category: **Growing turkeys**	
Breed: **BUT 9**	Identification procedure: **numbered cages**
Sex: **females** Age at start: **1 day**	Body weight at start: **63**
Physiological stage: **growing**	General health: **good**
Additional information for field trials:	
Location and size of herd or flock:	
Feeding and rearing conditions:	
Method of feeding:	
Diets (type(s)): **starter diet turkeys**	
Presentation of the diet: Mash ☒ Pellet ☐ Extruded ☐ Other	
Composition (main feedingstuffs): **maize, soybean meal**	
Nutrient content (relevant nutrients and energy content)	
Intended values: **11.7 MJ/kg ME, 0.54 total Phosphorus, 1.2 % calcium**	
Analysed values: **0.53 % total phosphorus, 1.18 % Ca**	
Date and nature of the examinations performed: **growth (21d), Ca P retention, bone ash (25 d)**	
Method(s) of statistical evaluation used: **ANOVA + Duncan, constrasts, covariaate analysis**	
Therapeutic/preventive treatments (reason, timing, kind, duration): **N/A**	
Timing and prevalence of any undesirable consequences of treatment: **N/A**	
Date **28 July 2009**	Signature Study Director

† In case the concentration of the additive in complete feed/water may reflect insufficient accuracy, the dose of the additive can be given per animal day^{-1} or mg kg^{-1} body weight or as concentration in complementary feed.

[1] Please submit this form using a common word processing format (e.g. MS Word).

ANNEX III: RAW DATA

E106 performance till 21 days

Obs	lot	trt	block	inwe	finwe	adg	adf	fg	level
1	1	1	1	65.0	415.0	15.9	27.4	1.723	0
2	7	1	2	63.3	340.0	12.6	23.6	1.874	0
3	10	1	3	65.0	420.0	16.1	26.7	1.652	0
4	33	1	4	65.0	381.3	14.4	23.0	1.601	0
5	34	1	5	61.7	322.5	11.9	21.2	1.786	0
6	35	1	6	60.0	360.5	13.7	23.1	1.694	0
7	50	1	7	63.3	330.0	12.1	22.8	1.878	0
8	61	1	8	61.7	.	.	.	.	0
9	71	1	9	64.3	370.0	13.9	24.5	1.761	0
10	83	1	10	63.3	350.0	13.0	22.7	1.740	0
11	93	1	11	66.7	398.3	15.1	23.5	1.556	0
12	94	1	12	58.3	368.3	14.1	21.7	1.540	0
13	18	2	1	60.0	417.0	16.2	24.8	1.526	500
14	19	2	2	65.0	436.3	16.9	25.7	1.524	500
15	21	2	3	66.7	478.3	18.7	27.5	1.469	500
16	29	2	4	60.0	465.7	18.4	27.3	1.483	500
17	38	2	5	58.3	475.0	18.9	27.1	1.433	500
18	39	2	6	57.3	442.7	17.5	23.8	1.359	500
19	51	2	7	61.7	411.7	15.9	24.6	1.544	500
20	66	2	8	61.7	423.7	16.5	25.7	1.563	500
21	69	2	9	60.0	439.0	17.2	24.7	1.434	500
22	73	2	10	63.3	459.3	18.0	25.4	1.413	500
23	78	2	11	58.3	446.7	17.7	24.2	1.370	500
24	87	2	12	60.0	413.3	16.1	24.1	1.499	500
25	2	3	1	60.0	491.7	19.6	28.5	1.453	1000
26	15	3	2	66.7	386.7	14.5	22.3	1.530	1000
27	17	3	3	63.3	460.0	18.0	26.7	1.478	1000
28	26	3	4	65.0	501.7	19.8	28.6	1.443	1000
29	27	3	5	63.3	439.3	17.1	25.0	1.463	1000
30	37	3	6	65.0	486.7	19.2	26.0	1.359	1000
31	49	3	7	63.3	520.0	20.8	32.6	1.568	1000
32	59	3	8	58.3	443.3	17.5	25.4	1.454	1000
33	62	3	9	62.7	465.0	18.3	27.4	1.500	1000
34	74	3	10	61.7	416.7	16.1	25.3	1.566	1000
35	75	3	11	63.3	520.0	20.8	27.7	1.336	1000
36	89	3	12	63.3	440.0	17.1	28.5	1.666	1000
37	9	4	1	63.3	445.0	17.3	26.8	1.543	2000
38	22	4	2	68.3	508.3	20.0	28.3	1.416	2000
39	23	4	3	68.3	370.7	13.7	22.1	1.607	2000
40	25	4	4	65.0	495.0	19.5	28.1	1.437	2000
41	30	4	5	58.3	491.7	19.7	27.9	1.417	2000
42	47	4	6	65.0	496.7	19.6	27.2	1.385	2000
43	54	4	7	63.3	498.3	19.8	28.6	1.448	2000
44	63	4	8	58.3	416.7	16.3	24.2	1.487	2000
45	65	4	9	66.7	503.3	19.8	29.7	1.498	2000
46	77	4	10	56.7	337.5	12.8	21.7	1.700	2000
47	82	4	11	61.7	485.0	19.2	28.5	1.480	2000
48	91	4	12	63.3	450.0	17.6	26.2	1.491	2000
49	6	5	1	63.3	608.3	24.8	34.9	1.407	4000
50	11	5	2	63.3	511.7	20.4	28.8	1.411	4000
51	13	5	3	70.0	518.3	20.4	30.1	1.478	4000
52	43	5	4	61.7	476.7	18.9	26.5	1.404	4000
53	45	5	5	68.3	503.3	19.8	28.1	1.421	4000
54	46	5	6	70.0	456.7	17.6	26.5	1.507	4000
55	53	5	7	65.0	563.3	22.7	31.5	1.389	4000
56	55	5	8	61.7	510.0	20.4	27.9	1.367	4000
57	70	5	9	61.7	493.3	19.6	27.5	1.402	4000
58	79	5	10	63.3	535.0	21.4	30.0	1.399	4000
59	81	5	11	65.0	503.3	19.9	27.7	1.392	4000
60	86	5	12	65.0	495.0	19.5	28.4	1.455	4000
61	3	6	1	56.7	461.7	18.4	27.4	1.489	.
62	5	6	2	60.0	433.3	17.0	26.5	1.559	.
63	14	6	3	65.0	440.0	17.0	25.4	1.489	.
64	31	6	4	60.0	390.0	15.0	23.4	1.561	.
65	41	6	5	63.3	400.0	15.3	23.9	1.561	.

Contract code: 2 2 5 5 0

66	42	6	6	61.7	409.0	15.8	23.9	1.512	.
67	57	6	7	65.0	446.7	17.3	25.6	1.473	.
68	58	6	8	63.3	460.0	18.0	26.7	1.482	.
69	67	6	9	66.7	480.0	18.8	35.3	1.881	.
70	85	6	10	65.0	447.3	17.4	25.9	1.493	.
71	90	6	11	68.3	458.3	17.7	26.4	1.491	.
72	95	6	12	61.7	433.3	16.9	25.3	1.500	.

E106 tibia ash

Obs	lot	trt	block	boneash	level
1	1	1	1	35.26	0
2	33	1	2	36.95	0
3	10	1	3	34.79	0
4	34	1	4	39.65	0
5	7	1	5	36.16	0
6	35	1	6	39.64	0
7	50	1	9	36.89	0
8	71	1	11	37.21	0
9	21	2	1	43.49	500
10	29	2	2	39.83	500
11	18	2	3	39.40	500
12	38	2	4	42.03	500
13	19	2	5	44.90	500
14	39	2	6	40.97	500
15	69	2	7	37.18	500
16	66	2	9	40.16	500
17	78	2	10	39.38	500
18	51	2	11	40.94	500
19	87	2	12	41.26	500
20	17	3	1	49.54	1000
21	37	3	2	40.84	1000
22	2	3	3	42.65	1000
23	26	3	4	43.08	1000
24	15	3	5	42.97	1000
25	27	3	6	44.68	1000
26	49	3	7	48.55	1000
27	89	3	8	49.09	1000
28	62	3	9	43.38	1000
29	74	3	10	41.74	1000
30	59	3	11	42.16	1000
31	75	3	12	46.73	1000
32	9	4	1	48.65	2000
33	25	4	2	45.22	2000
34	22	4	3	47.43	2000
35	30	4	4	47.63	2000
36	23	4	5	50.25	2000
37	47	4	6	47.04	2000
38	65	4	7	47.24	2000
39	77	4	8	50.88	2000
40	54	4	9	48.75	2000
41	82	4	10	47.10	2000
42	63	4	11	46.46	2000
43	91	4	12	45.71	2000
44	13	5	1	49.69	4000
45	45	5	2	49.25	4000
46	6	5	3	49.86	4000
47	46	5	4	51.03	4000
48	11	5	5	52.04	4000
49	43	5	6	46.91	4000
50	53	5	7	51.57	4000
51	81	5	8	50.75	4000
52	70	5	9	48.68	4000
53	86	5	10	48.62	4000
54	55	5	11	51.70	4000
55	79	5	12	47.79	4000
56	5	6	1	42.94	.
57	41	6	2	46.04	.

Contract code: *2 2 5 5 0*

58	14	6	3	42.41	.
59	42	6	4	44.37	.
60	3	6	5	45.64	.
61	31	6	6	41.16	.
62	57	6	7	42.99	.
63	85	6	8	44.28	.
64	58	6	9	44.99	.
65	90	6	10	43.58	.
66	67	6	11	43.22	.
67	95	6	12	43.95	.

Contract code: 2 2 5 5 0

E106 retention P and Ca

Obs	lot	trt	block	P	Ca	level
1	1	1	1	61.02	58.33	0
2	33	1	2	53.09	52.40	0
3	10	1	3	62.63	63.69	0
4	34	1	4	59.71	50.74	0
5	7	1	5	62.90	56.50	0
6	35	1	6	50.03	54.67	0
7	61	1	7	.	.	0
8	93	1	8	.	.	0
9	50	1	9	57.37	63.46	0
10	94	1	10	.	.	0
11	71	1	11	60.69	52.36	0
12	83	1	12	.	.	0
13	21	2	1	68.75	70.97	500
14	29	2	2	67.11	53.60	500
15	18	2	3	65.74	55.25	500
16	38	2	4	76.85	69.25	500
17	19	2	5	71.80	63.64	500
18	39	2	6	63.74	61.96	500
19	69	2	7	67.15	60.06	500
20	73	2	8	66.29	55.55	500
21	66	2	9	.	.	500
22	78	2	10	68.77	62.99	500
23	51	2	11	69.20	57.64	500
24	87	2	12	66.46	55.39	500
25	17	3	1	70.11	62.49	1000
26	37	3	2	.	.	1000
27	2	3	3	76.99	67.07	1000
28	26	3	4	69.26	57.77	1000
29	15	3	5	71.78	60.46	1000
30	27	3	6	72.71	73.70	1000
31	49	3	7	75.31	58.08	1000
32	89	3	8	64.16	51.72	1000
33	62	3	9	73.65	52.21	1000
34	74	3	10	70.99	50.30	1000
35	59	3	11	77.44	59.48	1000
36	75	3	12	79.97	59.33	1000
37	9	4	1	75.14	59.25	2000
38	25	4	2	76.50	60.38	2000
39	22	4	3	75.29	63.41	2000
40	30	4	4	83.32	61.42	2000
41	23	4	5	72.95	59.81	2000
42	47	4	6	74.07	64.42	2000
43	65	4	7	77.84	65.97	2000
44	77	4	8	72.15	68.16	2000
45	54	4	9	81.70	61.76	2000
46	82	4	10	75.57	64.21	2000
47	63	4	11	78.73	64.81	2000
48	91	4	12	70.85	60.04	2000
49	13	5	1	68.86	56.61	4000
50	45	5	2	78.96	66.26	4000
51	6	5	3	82.33	66.05	4000
52	46	5	4	76.54	60.32	4000
53	11	5	5	68.44	60.19	4000
54	43	5	6	85.42	64.75	4000
55	53	5	7	82.15	70.70	4000
56	81	5	8	82.24	60.59	4000
57	70	5	9	81.13	59.08	4000
58	86	5	10	87.26	76.75	4000
59	55	5	11	77.74	65.93	4000

Contract code: *2 2 5 5 0*

60	79	5	12	73.88	63.53	4000
61	5	6	1	60.86	60.32	.
62	41	6	2	57.28	52.03	.
63	14	6	3	56.40	48.68	.
64	42	6	4	71.10	58.51	.
65	3	6	5	59.48	56.72	.
66	31	6	6	65.87	55.68	.
67	57	6	7	64.93	53.94	.
68	85	6	8	58.17	60.72	.
69	58	6	9	57.85	56.42	.
70	90	6	10	54.62	50.79	.
71	67	6	11	62.67	55.44	.
72	95	6	12	59.68	52.49	.

E106 blood Ca and P

Obs	lot	pen	trt	block	level	Ca	P
1	1	1	1	1	0	7.3	7.15
2	33	33	1	2	0	7.0	8.77
3	10	10	1	3	0	6.0	5.52
4	34	34	1	4	0	6.6	9.39
5	7	7	1	5	0	9.4	7.13
6	35	35	1	6	0	6.4	9.22
7	50	50	1	9	0	2.4	2.89
8	71	71	1	11	0	6.2	11.41
9	21	21	2	1	500	8.9	7.41
10	29	29	2	2	500	6.8	4.25
11	18	18	2	3	500	8.7	6.18
12	38	38	2	4	500	8.0	5.92
13	19	19	2	5	500	.	.
14	39	39	2	6	500	8.2	6.90
15	69	69	2	7	500	7.1	5.22
16	73	73	2	8	500	6.6	7.32
17	66	66	2	9	500	8.2	7.32
18	78	78	2	10	500	8.0	6.25
19	51	51	2	11	500	3.8	4.52
20	87	87	2	12	500	7.7	7.10
21	17	17	3	1	1000	9.4	9.44
22	37	37	3	2	1000	8.6	5.78
23	2	2	3	3	1000	8.2	5.00
24	26	26	3	4	1000	8.6	5.11
25	15	15	3	5	1000	.	.
26	27	27	3	6	1000	8.2	5.38
27	49	49	3	7	1000	8.6	8.75
28	89	89	3	8	1000	8.1	7.47
29	62	62	3	9	1000	8.6	6.89
30	74	74	3	10	1000	8.6	8.08
31	59	59	3	11	1000	7.8	10.11
32	75	75	3	12	1000	9.4	7.63
33	9	9	4	1	2000	8.6	8.20
34	25	25	4	2	2000	8.1	7.47
35	22	22	4	3	2000	9.7	8.28
36	30	30	4	4	2000	9.0	6.83
37	23	23	4	5	2000	8.3	5.67
38	47	47	4	6	2000	9.0	6.63
39	65	65	4	7	2000	7.4	6.34
40	77	77	4	8	2000	9.3	7.15
41	54	54	4	9	2000	8.8	7.76
42	82	82	4	10	2000	9.5	8.34
43	63	63	4	11	2000	8.8	7.14
44	91	91	4	12	2000	8.8	7.40
45	13	13	5	1	4000	9.7	8.84
46	45	45	5	2	4000	8.5	7.67
47	6	6	5	3	4000	8.8	7.83
48	46	46	5	4	4000	9.3	8.28
49	11	11	5	5	4000	9.5	6.10
50	43	43	5	6	4000	9.4	6.74
51	53	53	5	7	4000	9.0	5.91
52	81	81	5	8	4000	9.0	6.88
53	70	70	5	9	4000	9.0	7.64
54	86	86	5	10	4000	8.7	6.41
55	55	55	5	11	4000	9.4	6.17
56	79	79	5	12	4000	9.2	6.73
57	5	5	6	1	0	9.2	5.59
58	41	41	6	2	0	8.1	6.13

Contract code: *2 2 5 5 0*

59	14	14	6	3	0	8.1	4.31
60	42	42	6	4	0	8.8	6.03
61	3	3	6	5	0	8.8	5.25
62	31	31	6	6	0	8.5	4.83
63	57	57	6	7	0	1.3	1.62
64	85	85	6	8	0	.	.
65	58	58	6	9	0	8.8	6.38
66	90	90	6	10	0	8.3	5.43
67	67	67	6	11	0	8.1	5.18
68	91	91	6	12	0	8.2	6.47

TAB

ANNEX 29

Annex 29

Ledoux, D.R. et *al.* (2009). Report No. 00002585: Efficacy of a novel phytase product (= RONOZYME® HiPhos) in young turkeys poults. 2009

REPORT No. 00002585
Regulatory Document

DSM

Document Date: 26 October, 2009

Author(s): D.R. Ledoux[1], R.E. Kutz[1], N.E. Ward[2] and J. Broz[3]

[1] ASRC, University of Missouri, Columbia MO (USA)
[2] DSM Nutritional Products, Inc., Parsippany (USA)
[3] Animal Nutrition and Health R&D, DSM Nutritional Products Ltd, Basel

Title: **Efficacy of a novel phytase product in young turkey poults**

Project No. 6106

Summary

An experiment was conducted involving a total of 150 day-old male turkey poults (Nicholas 88) in order to evaluate the efficacy of a novel microbial 6-phytase (IPA phytase). Dietary treatments included: (A) a negative control corn-soybean basal diet (BD) formulated to contain 1.00% Ca and 0,30% non-phytate phosphorus (npP); (B) the basal diet + IPA phytase (M) at 250 U/kg; (C) the basal diet + IPA phytase (M) at 500 U/kg; (D) the basal diet + IPA phytase (M) at 1000 U/kg; (E) the basal diet + IPA phytase (M) at 2000 U/kg. A completely randomized design was used, with 6 replicate pens of 5 poults allotted to dietary treatments from day 1 to day 21. Performance, Ca and P retention and tibia ash percentage were used as response parameters. Dietary supplementation with IPA phytase at 250, 500, 1000 and 2000 U/kg significantly improved P retention from 50.8% in the negative control to 64.2, 64.0, 71.8 and 74.3%, respectively. In addition, phytase supplementation improved body weight gain by an average of 16%, increased bone ash by an average of 24%, and also significantly increased Ca retention. These data demonstrate conclusively that the novel phytase was effective in improving phytate P utilization.

This report consists of Pages I – II and 1 – 13 & Annex C

Distribution

Dr. M. Eggersdorfer, NRD
Dr. F. Fru, NRD/PA
Mr. J.-F. Hecquet, NBD/RG
Dr. A.-M. Klünter, NRD/CA
Dr. J. Pheiffer, NRD/PA
Dr. P. Philipps, NRD/CA
Mr. J.-P. Ruckebusch, ANH/GM
Dr. N.E. Ward, DNP Parsipanny

Approved

Name	Signature	Date
Main Author	signed by	
Dr. J. Broz, NRD/CA	J. Broz	27.10.2009
Principal Scientist / Competence Mgr	signed by	
Dr. J. Broz, NRD/CA	J. Broz	27.10.2009
Research Center Head	signed by	
Dr. A.-M. Klünter, NRD/CA	A.-M. Klünter	28.10.2009
Project Manager	signed by	
Dr. F. Fru, NRD/PA	F. Fru	29.10.2009

Nomenclature and Structural Formula

IPA phytase (M), enzyme product containing bacterial 6-phytase (EC 3.1.3.26), produced by submerged fermentation of a genetically modified *Aspergillus oryzae* strain. Lot PPQ 28656 was used in this study, manufactured by Novozymes A/S, Bagsvaerd, Denmark.

EFFICACY OF A NOVEL PHYTASE PRODUCT IN YOUNG TURKEY POULTS

A FINAL REPORT

TO

DSM NUTRITIONAL PRODUCTS, INC.

March 18, 2009

INVESTIGATORS

D. R. Ledoux, PhD
R. E. Kutz, BS

INTRODUCTION

It has been conclusively demonstrated that microbial phytase is effective in degrading phytate and improving P availability to poultry fed rations consisting of cereal grains and oilseed meals (Nelson *et al.* 1971; Simons *et al.*, 1990; Schoner *et al.*, 1991, 1993; Zyla *et al.*, 1996). However, before new phytase products are approved for sale they will need to be evaluated both with respect to efficacy and safety. Therefore, the objectives of the present study were to determine the efficacy of a new phytase product.

MATERIALS AND METHODS

One hundred and fifty male Nicholas 88 turkey poults were purchased from a commercial hatchery, weighed, wing-banded, and randomly assigned to dietary treatments in chick batteries. Feed and water were provided for *ad libitum* consumption. A completely randomized design was used with 6 replicate pens of 5 poults allotted randomly to dietary treatments from day 1 to day 21.

Dietary treatments included: (A) a negative control corn-soybean meal basal diet (BD) formulated to contain 1.00% calcium (Ca) and 0.30% non- phytate phosphorus (npP) diet; (B) the basal diet plus 250 units of the novel phytase (IPA); (C) the basal diet plus 500 units of the novel phytase; (D) the basal diet plus 1000 units of the novel phytase; and (E) the basal diet plus 2000 units of the novel phytase. The novel phytase product was a microbial 6-phytase (EC 3.1.3.26) that was expressed in a genetically modified strain of *Aspergillus oryzae* and had a potency of 60,700 FYT/gram product (Lot No. PPQ 28656). Dietary treatments are outlined in Table 1.

With the exception of Ca and P, all diets met or exceeded the nutrient requirements of turkey poults (NRC, 1994), and were fed in mash form. Chromic oxide was used as an inert marker for determination of P and Ca retention.

Body weights were measured on a pen basis at the beginning and at day 21. Feed intake was also determined at day 21, and feed conversion calculated. Mortality was recorded as it occurs. In addition, poults were inspected twice daily and any health related problems recorded.

Samples of excreta were collected from each pen for three consecutive days beginning on day 17. The daily samples from each pen were dried in a forced air oven at 55 C, ground to pass a 1-mm screen, and composited. Sub samples were then collected from the composite samples for analysis of chromium, Ca, and P. Chromium, Ca, and P concentrations in feed and excreta samples were determined by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP).

On day 21, all poults in each pen were euthanized with carbon dioxide, and the middle toes from both feet were collected for determination of percent toe ash. Toes were dried at 100 C for 24 hours then ashed in a muffle furnace at 600 C overnight. Right tibiae were collected from 3 poults in each pen, stripped of adhering tissue following immersion in boiling water, ether extracted, dried at 100 C for 24 hours, weighed, and dry-ashed at 600 C overnight.

Data were analyzed by analysis of variance using the General Linear Models procedures of SAS (1984). Statistical significance was accepted at $P < 0.05$.

RESULTS

Diet Analyses

Dietary analysis of selected nutrients and phytase concentration in diets are summarized in Table 2. Calcium and P concentrations indicated that diets contained target concentrations. Dietary phytase concentrations ranged from a low of 80% of targeted values to a high of 107% of targeted values. Crude protein content of the diets averaged 26.32% instead of the targeted value of 28%.

Performance

Effects of dietary treatments on poult performance are summarized in Table 3. Feed intake (FI) increased with increasing dietary phytase inclusion but the difference in FI was only significantly higher in birds fed 1000 and 2000 U phytase/kg diet compared with birds fed the NC diet (715 and 698 g vs 598 g). Feed intake increases due to phytase supplementation of the NC diet ranged from a low of 4.5% to a high of 19.6%.

Body weight gain (BWG) also increased with increasing dietary phytase inclusion with BWG being significantly higher in birds fed 500, 1000 and 2000 U phytase/kg diet compared with birds fed the NC diet (510, 526 and 542 g vs 436 g). There were no differences ($P > 0.05$) in feed conversion among dietary treatments. Improvement in BWG due to phytase supplementation of the NC diet ranged from a low of 2.1% to a high of 24.3%.

Bone Mineralization

Effects of dietary treatments on bone ash are summarized in Table 4. Bone ash increased with increasing dietary phytase inclusion and was significantly higher in birds fed 500, 1000, and 2000 U/kg phytase compared with birds fed the NC diet (40.8, 41.9, and 48.0% vs 33.8%). Increase in percent bone ash due to phytase supplementation of the NC diet ranged from a low of 9.0% to a high of 41.8%.

Calcium and P Retention

Effects of dietary treatments on Ca and P retention are summarized in Table 4. Calcium retention increased with increasing dietary phytase concentration and was higher in all diets supplemented with phytase when compared with the NC diet that contained no phytase, averaging 61.7% for diets containing phytase compared with 44.1% for the NC diet that did not contain phytase. Increase in Ca retention due to phytase supplementation of the NC diet ranged from a low of 24.8% to a high of 51.6%.

Phosphorus retention also increased with increasing dietary phytase concentration and was higher in all diets supplemented with phytase when compared with the NC diet that contained no phytase, averaging 68.6% for diets containing phytase compared with 50.8% for the NC diet that did not contain phytase. The increase in P retention by birds fed phytase ranged from a low of 26% at 250 phytase units to a high of 46% at 2000 phytase units. Increase in P retention due to phytase supplementation of the NC diet ranged from a low of 26.2% to a high of 46.4%.

SUMMARY AND CONCLUSION

Phytase has been shown to increase the digestibility of phytate from around 25% to 50-70% in poultry (Schoner *et al.,* 1993; Kornegay *et al.,* 1996; Choct, 2006). In the current study, P retention improved from 50.8% in the NC diet to 74.3% in the diet containing 2000 U/kg phytase. In addition, phytase supplementation improved BWG by an average of 16%, increased bone ash by an average of

24%, increased Ca retention by an average of 40%, and P retention by an average of 35%. These data demonstrate conclusively that the novel phytase was effective in improving phytate P utilization.

LITERATURE CITED

Choct,M. 2006. Enzymes for the feed industry: past, present and future. World's Poultry Science Journal 62:5-16.

Kornegay, E. T., D. M. Denbow, Z. Yi, and, V. Ravindran 1996. Response of broilers to graded levels of microbial phytase added to maize-soybean-meal-based diets containing three levels of non-phytate phosphorus. Br. J. Nutr. 75:839-852.

Nelson, T. S., T. R. Shieh, R. J. Wodzinski, and J. H. Hare, 1971. Effects of supplemental phytase on the utilization of phytate phosphorus by chicks. J. Nutr. 101:1289.

NRC, 1994. Nutrient Requirements of Poultry, 9th revised edition. National Academy Press, Washington, DC.

SAS Institute, 1984. SAS User's Guide: Statistics, SAS Institute, Cary, NC.

Schoner, F. J., P. P. Hoppe, G. Schwarz, and H. Wiesche, 1991. Comparative effects of microbial phytase and inorganic phosphorus on performance and retention of phosphorus, calcium, and crude ash in broilers. J. Anim. Physiol. Anim. Nutr. 66:248.

Schoner, F. J., P. P. Hoppe, G. Schwarz, and H. Wiesche, 1993. Effects of microbial phytase and inorganic phosphate in broiler chicken: Performance and mineral retention at various calcium levels. J. Anim. Physiol. Anim. Nutr. 69:235.

Simons, P.C.M., H.A.J. Versteegh, A. W. Jongbloed, P. A. Kemme, P. Stump, K. D. Bos, M.G.E. Walters, R. F. Beudeker, and G. J. Verschoor, 1990. Improvements of phosphorus availability by microbial phytase in broilers and pigs. Brit. J. Nutr. 64:525.

Zyla, K., D. R. Ledoux, M. Kujawski, and T. L. Veum, 1996. The efficacy of an enzymic cocktail and a fungal mycelium in dephosphorylating corn-soybean meal-based feeds fed to growing turkeys. Poultry Sci. 75:381.

Table 1. Dietary Composition of the negative control basal diet used in the Experiment

Dietary Ingredients	---------- (Hatch - 3 week) ---------- NC (%)
Soybean Meal (48%)	49.490
Corn	45.378
Dicalcium Phosphate	0.819
Limestone	1.633
Corn Oil	0.728
Salt	0.384
DL-Methionine	0.203
L-Lysine.HCL	0.022
Trace Mineral	0.100
Vitamin Premix	0.060
Selenium Premix	0.080
CuSO4	0.004
Chromic oxide	0.100
Sand (Enzymes to be substituted for sand)	1.000
Nutrients, Calculated	
ME, kcal/kg	2800
Crude Protein, %	28.00
Available P, %	0.30
Ca, %	1.00
Na, %	0.17
Cl, %	0.27
Se, %	0.21
Cu, %	32
Zn, %	1.42
Lysine, %	1.60
Methionine, %	0.61
Met & Cyst, %	1.05
Threonine, %	1.06
Isoleucine, %	1.18
Leucine, %	2.30
Arginine, %	1.89
Glycine & Serine, %	2.56
Histidine, %	0.74
Tryptophan, %	0.39
Phenylalanine, %	1.33
Phenylalanine & Tyrosine, %	2.43
Valine, %	1.28

Table 2. Analyzed Selected Nutrient Content of Diets used in the Experiment

Diet	Moisture	CP	Fat	Fiber	Ash	Ca	P	Phytase (U/kg)
	(%)							
NC	11.98	26.30	3.20	3.61	7.52	1.06	0.63	63
NC + 250 FYT	12.01	26.80	3.32	3.15	7.44	1.02	0.61	216
NC + 500 FYT	11.61	26.30	3.31	2.86	7.76	1.09	0.63	448
NC + 1000 FYT	11.80	26.30	3.25	3.08	7.32	1.05	0.64	799
NC + 2000 FYT	12.21	25.90	3.24	2.98	7.09	1.08	0.67	2024
Mean[1]	11.92	26.32	3.26	3.14	7.43	1.06	0.64	-------

CP = Crude protein; NC = Negative control basal diet

Targets for NC and other diets were CP = 28%; Ca = 1.00%; P = 0.60%

[1]All NC diets prepared using a single basal diet

Table 3. Effects of dietary treatments on growth performance of turkey poults[1]

Treatment	Feed Intake (g)	Body Weight Gain (g)	Feed Conversion (g:g)
Negative Control (NC)[2]	598[c]	436[b]	1.387
NC+ 250 U/kg	631[bc]	445[b]	1.420
NC + 500 U/kg	625[bc]	510[a]	1.317
NC+1000 U/kg	715[a]	526[a]	1.366
NC+2000U/kg	687[ab]	542[a]	1.334
SEM	24	20	0.044
	----------Probability----------		
TRT	0.0132	0.0016	0.4955

[1]Data are means of 6 replicate pens of 5 poults each.
[2]NC diet contained 1.06% Ca and 0.63% total P by analysis
[a-c]Means in a column with no common superscript are significantly different ($P < 0.05$)

Table 4. Effects of dietary treatments on Ca and P retention and bone ash in turkey poults[1]

TRT	Ca Retention (%)	P Retention (%)	Bone Ash (%)
Negative Control (NC)[2]	44.08[c]	50.76[d]	33.83[c]
NC+250 U/kg	59.52[b]	64.16[c]	36.89[bc]
NC+500 U/kg	54.99[b]	64.04[c]	40.79[b]
NC+1000 U/kg	65.53[a]	71.76[b]	41.89[b]
NC+2000 U/kg	66.81[a]	74.32[a]	47.96[a]
SEM	1.70	0.61	1.76
	----------Probability----------		
TRT	<0.0001	<0.0001	0.0001

[1] Data are means of 6 replicate pens of 5 poults each.
[2]NC diet contained 1.06% Ca and 0.63% total P by analysis
[abc]Means within a column with no common superscript are significantly different ($P < 0.05$)

ANNEX

Exp 20-08 DSM Performance

Trt	Rep	AvGn	AFI	FC
A	1	433.8	535.8	1.332
A	2	433.4	618.4	1.427
A	3	446.6	637.4	1.427
A	4	413.2	571.2	1.382
A	5	408.4	560.8	1.373
A	6	480.8	664.6	1.382
B	1	401.6	537.4	1.338
B	2	466.8	671.4	1.438
B	3	487.2	714.2	1.466
B	4	455.6	617	1.354
B	5	461.8	653.4	1.415
B	6	394.8	594.8	1.507
C	1	468.7	602.5	1.378
C	2	478.2	669.6	1.4
C	3	624.7	578.1	1.181
C	4	561.5	616.3	1.171
C	5	425.8	593.6	1.394
C	6	502.4	692.6	1.379
D	1	598.8	759.2	1.268
D	2	500.8	673	1.344
D	3	538.6	674.2	1.252
D	4	495.6	751.4	1.516
D	5	491	792.2	1.613
D	6	528.8	637.2	1.205
E	1	567.8	744	1.31
E	2	619.2	797.8	1.288
E	3	569	695.4	1.222
E	4	485.4	628.2	1.294
E	5	519.8	652	1.326
E	6	488.9	605.2	1.564

A = NC
B = NC + 250 FYT
C = NC + 500 FYT
D = NC + 1000 FYT
E = NC + 2000 FYT

ANNEX

Exp 20-08 Excreta Calcium and Phosphorus Retention

Trt	Rep	ECaRet%	EPRet%
A	1	35.67753	52.2727273
A	2	43.39623	50.2717391
A	3	44.93997	49.2897727
A	4	49.0566	53.125
A	5	42.83019	48.4375
A	6	48.58491	51.171875
B	1	56.57895	63.0597015
B	2	57.8566	63.0110318
B	3	66.50718	63.7042062
B	4	53.42523	66.5955935
B	5	61.9883	63.0419332
B	6	60.73517	65.5294954
C	1	56.30215	65.013587
C	2	57.52984	64.4886364
C	3	48.80624	62.7130682
C	4	58.67769	63.4232955
C	5	53.22793	65.6929348
C	6	55.38721	62.890625
D	1	69.15064	72.9817708
D	2	66.13712	71.8070652
D	3	67.3913	72.826087
D	4	57.16783	69.4602273
D	5	67.3913	72.1467391
D	6	65.94551	71.3541667
E	1	61.03395	75.6410256
E	2	66.62809	74.3589744
E	3	69.81481	76.3076923
E	4	66.75084	73.0769231
E	5	70.2729	74.4939271
E	6	66.37427	72.0647773

A = NC
B = NC + 250 FYT
C = NC + 500 FYT
D = NC + 1000 FYT
E = NC + 2000 FYT

ANNEX

Exp 20-08 Bone Ash

Trt	Rep	Dry wt	Ash wt	Ash%
A	1	0.9184	0.3404	37.0645
A	1	1.0739	0.3782	35.2174
A	1	0.972	0.3515	36.1626
A	2	0.925	0.3375	36.4865
A	2	1.0376	0.3344	32.2282
A	2	1.1402	0.3839	33.6695
A	3	0.7783	0.2488	31.9671
A	3	0.8502	0.3172	37.3089
A	3	1.0236	0.3404	33.2552
A	4	1.0036	0.3417	34.0474
A	4	0.8689	0.304	34.9868
A	4	0.8932	0.2527	28.2915
A	5	0.8714	0.2866	32.8896
A	5	1.1543	0.3794	32.8684
A	5	0.9301	0.33	35.4801
A	6	1.0258	0.3309	32.2578
A	6	1.1251	0.3244	28.833
A	6	0.9477	0.341	35.9819
B	1	0.9848	0.3987	40.4854
B	1	0.5716	0.2243	39.2407
B	1	0.9617	0.3155	32.8065
B	2	0.9044	0.367	40.5794
B	2	0.9764	0.3882	39.7583
B	2	0.7482	0.3199	42.7559
B	3	1.1347	0.4319	38.0629
B	3	1.2475	0.4618	37.018
B	3	1.1981	0.4072	33.9871
B	4	1.0427	0.5034	48.2785
B	4	0.94	0.4547	48.3723
B	4	0.769	0.3705	48.1795

A = NC
B = NC + 250 FYT
C = NC + 500 FYT
D = NC + 1000 FYT
E = NC + 2000 FYT

B	5	1.4269	0.4475	31.3617
B	5	1.5128	0.4284	28.3184
B	5	1.1885	0.3423	28.801
B	6	1.6341	0.4522	27.6727
B	6	1.2567	0.3735	29.7207
B	6	1.4044	0.401	28.5531
C	1	0.8208	0.3643	44.3835
C	1	0.7892	0.3058	38.7481
C	1	1.2704	0.5489	43.2069
C	2	1.0341	0.4127	39.9091
C	2	0.9604	0.3757	39.1191
C	2	0.5579	0.2301	41.244
C	3	1.9562	1.0269	52.4946
C	3	1.3613	0.5273	38.735
C	3	0.9928	0.3781	38.0842
C	4	1.011	0.4098	40.5341
C	4	0.6419	0.2541	39.5856
C	4	0.783	0.2994	38.2375
C	5	0.8096	0.3601	44.4788
C	5	1.0287	0.4153	40.3713
C	5	1.1366	0.4727	41.5889
C	6	1.0184	0.3912	38.4132
C	6	1.0043	0.352	35.0493
C	6	1.433	0.574	40.0558
D	1	1.3675	0.6537	47.8026
D	1	1.2738	0.634	49.7723
D	1	1.2894	0.5218	40.4684
D	2	1.2972	0.663	51.1101
D	2	1.0111	0.5004	49.4907
D	2	0.9736	0.4538	46.6105
D	3	1.1561	0.4448	38.4742
D	3	1.4974	0.6027	40.2498
D	3	1.2392	0.5616	45.3196
D	4	1.9871	0.9868	49.6603

D	4	0.9629	0.3924	40.7519
D	4	1.0362	0.3809	36.7593
D	5	1.4081	0.5132	36.4463
D	5	1.4532	0.5078	34.9436
D	5	1.301	0.396	30.4381
D	6	1.4483	0.4918	33.9571
D	6	1.4495	0.5604	38.6616
D	6	1.355	0.5831	43.0332
E	1	1.1102	0.5632	50.7296
E	1	1.4984	0.7278	48.5718
E	1	1.3827	0.6825	49.3599
E	2	1.3015	0.6471	49.7196
E	2	1.2799	0.6472	50.5665
E	2	1.2233	0.6272	51.2712
E	3	1.2415	0.5693	45.8558
E	3	1.0778	0.4799	44.5259
E	3	1.4311	0.6262	43.7566
E	4	1.3548	0.6525	48.1621
E	4	1.1712	0.5611	47.9081
E	4	1.2339	0.5804	47.0378
E	5	1.5523	0.7003	45.1137
E	5	1.1242	0.5041	44.8408
E	5	1.0668	0.524	49.1189
E	6	0.9882	0.4747	48.0368
E	6	1.1895	0.5847	49.1551
E	6	0.8386	0.4151	49.4992

ANNEX C [1]

TRIAL PROTOCOL DATA SHEET: FOR TERRESTRIAL ANIMALS

Identification of the additive: **Microbial 6-Phytase**	Batch number: **Lot No. PPQ 28656**
Trial ID: **MU-EXP. 20-08**	Location: **Unit B, ASRC, University of Missouri, Columbia MO, USA**
Start date and exact duration of the study: **December 30, 2008 to January 20, 2009**	
Number of treatment groups (+ control(s)): **5**	Replicates per group: **6**
Total number of animals: **150**	Animals per replicate: **5**
Dose(s) of the additive/active substance(s)/agent(s) (mg/Units of activity/CFU kg^{-1} complete feed/L^{-1} water) Intended: **0, 250, 500, 1000, 2000 FYT/g** Analysed: **63, 216, 448, 799, 2024 FYT/g** † Substances used for comparative purposes: **NA** Intended dose: Analysed:	
Animal species/category: **Turkeys**	
Breed: **Nicholas 88**	Identification procedure: **Wing bands**
Sex: **male** Age at start: **day old**	Body weight at start: **54-56 g**
Physiological stage: **Starter**	General health: **Good**
Additional information for field trials:	
Location and size of herd or flock:	
Feeding and rearing conditions:	
Method of feeding:	
Diets (type(s)): **Corn-soybeanmeal basal diet** Presentation of the diet: Mash ☒ Pellet ☐ Extruded ☐ Other Composition (main feedingstuffs): **See Report Table 1** Nutrient content (relevant nutrients and energy content) Intended values: **See report Table 2** Analysed values:	
Date and nature of the examinations performed: **December 2008 to January 2009** Method(s) of statistical evaluation used: **One Way ANOVA, SAS 1984** Therapeutic/preventive treatments (reason, timing, kind, duration): **None** Timing and prevalence of any undesirable consequences of treatment: **None**	
Date 3/12/09	Signature Study Director David Fedon

† In case the concentration of the additive in complete feed/water may reflect insufficient accuracy, the dose of the additive can be given per animal day^{-1} or mg kg^{-1} body weight or as concentration in complementary feed.

[1] Please submit this form using a common word processing format (e.g. MS Word).

TAB

ANNEX 30

Annex 30

Juin, H. and Broz, J. (2009). Report No. 00003287 : Evaluation of IPA mash phytase (=RONOZYME® HiPhos) in turkeys. 2009

REPORT No. 00003287
Regulatory Document

DSM

Document Date:	17 December, 2009
Author(s):	H. Juin[1] and J. Broz[2]
	[1] INRA Le Magneraud, Surgères, France [2] Animal Nutrition and Health R&D, DSM Nutritional Products Ltd, Basel
Title:	**Evaluation of IPA Mash phytase in turkeys**
Project No.	6106

Summary

A 4-week performance and balance experiment involving 240 male turkeys (strain BUT T9) was conducted in order to evaluate the efficacy of a novel microbial 6-phytase (IPA Mash phytase) when added to a low-P, corn-based starter diet. Eight dietary treatments were compared in this study, as follows: R1 – negative control diet (0.20% available P); R2 – positive control diet 1 (0.25% available P); R3 – positive control diet 2 (0.30% available P); R4 – positive control diet 3 (0.35% available P); R5 – R1 + phytase at 500 U/kg; R6 – R1 + phytase at 1000 U/kg; R7 – R1 + phytase at 2000 U/kg; R8 – R1 + phytase at 4000 U/kg. Each dietary treatment was assigned to 15 replicates of 2 birds each. Body weight and feed conversion ratio were monitored as performance parameters. The apparent P utilization and tibia ash percentage were determined as main criteria of P availability. A positive and significant dose related response to phytase supplementation was noted in terms of live weight and feed conversion. Dietary supplementation with IPA Mash phytase at 500, 1000, 2000 and 4000 U/kg significantly improved the P utilization from 51.0% (negative control) to 61.0, 66.0, 72.3 and 77.0%, respectively. As the consequence of this effect, tibia ash percentage significantly increased from 30.1% (negative control) to 36.8, 40.4, 43.4 and 44.8%, respectively. Based on several response criteria the effects obtained with phytase addition at 1000 U/kg are fully comparable to that observed in positive control 3, which received 0.15% of additional available P in form of dicalcium phosphate.

This report consists of Pages I – II and 1 – 17, raw data & Annex C

Distribution

Dr. M. Eggersdorfer, NRD
Dr. F. Fru, NRD/PA
Mr. J.-F. Hecquet, NBD/RG
Dr. A.-M. Klünter, NRD/CA
Dr. J. Pheiffer, NRD/PA
Dr. P. Philipps, NRD/CA
Mr. J.-P. Ruckebusch, ANH/GM

Approved

Name	Signature	Date
Main Author	signed by	
Dr. J. Broz, NRD/CA	J. Broz	18.12.2009
Principal Scientist / Competence Mgr	signed by	
Dr. J. Broz, NRD/CA	J. Broz	18.12.2009
Research Center Head	signed by	
Dr. A.-M. Klünter, NRD/CA	A.-M. Klünter	18.12.2009
Project Manager	signed by	
Dr. F. Fru, NRD/PA	F. Fru	18.12.2009

Nomenclature and Structural Formula

IPA phytase (M), enzyme product containing bacterial 6-phytase (EC 3.1.3.26), produced by submerged fermentation of a generically modified *Aspergillus oryzae* strain. Lot PPQ 28656 was used in this study, manufactured by Novozymes A/S, Bagsvaerd, Denmark.

Trial report

Sponsor: **Jiri Broz**
DSM Nutritional Products Ltd
Animal Nutrition and Health R&D
CH 4002 Basel
SWITZERLAND

Investigator: **Hervé Juin**
INRA Le Magneraud
Surgéres
France

Trial site : INRA Le Magneraud
Surgères
France

Reference 09/HD3/DD/0107

Introduction

A novel bacterial 6-phytase, expressed in a genetically modified strain of *Aspergillus oryzae*, has been developed recently (IPA Mash phytase). The objective of this experiment was to evaluate the efficacy of this phytase product in young turkeys, using performance, P utilization and tibia mineralization as the main response criteria.

Material and methods

Animals

Day-old male turkey poults (BUT T9) were purchased for this experiment at the hatchery BOYE. St Hilaire de Loulay. France. They were kept in a floor-pen until day 9 of age and fed a commercial pre-starter diet. From day 9 until the termination of the experiment the birds were kept in wire-floored cages (2 per cage).

Experimental diets

Experimental starter diets contained corn and soybean meal as the main feed ingredients and were formulated to contain 25.0% crude protein. 1.15% methionine + cystine and 2950 kcal ME/kg. A basal, low-P diet was formulated to contain 0.20% of available P only. This basal diet was used in the negative control and all treatments supplemented with phytase. For comparative purposes three positive control diets were formulated to contain 0.25. 0.30 and 0.35% of available P. respectively, by increasing the dietary inclusion of dicalcium phosphate. Composition of the used diets and calculated nutrient contents are provided in Annex 1. All diets were manufactured by the experimental feed mill of INRA Le Magneraud and were fed as mash. Both feed and drinking water were available *ad libitum*.

Test product

IPA Mash phytase (M), lot PPQ 28656, containing 60700 U/g product, was used in this experiment.

Experimental treatments (D9/D29)

Treatment	Description	Inclusion of test product
R1	Low-P basal diet (0.20 % available P)	0
R2	Positive control diet 1 (0.25 % available P)	0
R3	Positive control diet 2 (0.30 % available P)	0
R4	Positive control diet 3 (0.35 % available P)	0
R5	R1 + IPA Mash phytase at 500 U/kg diet	8.24 ppm
R6	R1 + IPA Mash phytase at 1000 U/kg diet	16.48 ppm
R7	R1 + IPA Mash phytase at 2000 U/kg diet	32.96 ppm
R8	R1 + IPA Mash phytase at 4000 U/kg diet	65.92 ppm

Each dietary treatment was assigned to 15 replicate groups (cages).

Time schedule

Arrival of poults (D1): February 10th 2009
End of experiment (D29): March 10th 2009

		Operations
Floor-pen D1/D9	D1 D9	Poults were delivered to the pen. They were identified by a ring at the wing. Morning: Individual weighing. Control of feed consumption. Randomization into 8 groups of 30 poults.
Cages D9/D29	D9 D14 D22 D25 D29	Afternoon: Poults were transferred into the cages (2 per cage). Experimental diets were provided. Individual weighing. Control of feed and water consumption 9:00 am: Individual weighing. Beginning of excreta collection. Excreta were collected every day (till D25) and stored at –18°C. 9:00 am: Individual weighing. End of excreta collection. Control of feed and water consumption between D22 and D25. Individual weighing. Control of feed and water consumption. Blood samples were taken from 1 bird per cage. The same bird was slaughtered and left tibia was taken, then stored at –18°C. Samples of crop content and intestinal digesta (jejunum + ileum) were collected and stored at -18°C (for analysis of phytase activity).

Experimental parameters and measurements

Body weights of turkeys were recorded individually on day 9, day 14, day 22, day 25 and day 29 of age. Feed intake was registered on days 14. 25 and 29 per replicate group and calculated per bird in the respective period. For each replicate group and each period, feed conversion ratio was calculated as follows: feed consumption during the period / (live weight at the end + weight of dead animals – total live weight at the beginning).

The efficacy of IPA Mash phytase was evaluated on the basis of the following response parameters:

- Growth performance (weight gain. feed/gain ratio)
- Apparent phosphorus utilization (based on total excreta collection. days 22-25)
- Tibia ash percentage (day 29)
- Phosphorus concentration in excreta
- Serum concentration of inorganic P and Ca (day 29)

Excreta from each cage (replicate) were collected quantitatively from day 22 until day 25. During this period feed intake was also recorded. Excreta from each cage were stored in metallic plates at -18°C. After thawing, excreta were lyophilized and homogenized. On day 29, left tibia was taken from one bird per cage. Tibia bones were mechanically cleared of adhering tissue. and then dried at 103°C for 24 hours. Bones were incinerated at 550°C for 14 hours and ash weight was recorded. Samples of all diets and excreta were analysed for dry

matter, nitrogen and gross energy by standard INRA methods. Phosphorus in diets and excreta was determined by the INRA laboratory according to AFNOR method NF V08-106. Phytase in-feed analytics was conducted by DSM Biopract GmbH, Berlin (Germany). In addition, phytase activity in crop and ileum contents was analysed by the laboratory at INRA Le Magneraud using a standard method. Serum concentration of inorganic P and Ca were analysed by a specialized external laboratory.
The AMEn values of the diets were calculated according to the standard INRA procedure. Apparent utilization of P was calculated as percentage of its retention. compared to the total P intake during the balance period.

Statistical analysis
Individual values per bird were used for live weight and tibia ash measurements. Each replicate group was considered as one experimental unit for feed intake and feed conversion ratio. as well as for balance parameters. One-way analysis of variance was conducted using Minitab in order to determine the effects of experimental treatments. Significant differences among treatment means were determined by using Tukey test at $P<0.05$.

Results and discussion

During the whole experiment no disease or any abnormal behaviour due to the dietary treatments were noticed. Before the start of the experimental period (D9) the mean weight of birds was 155 ± 9 g.

The results of feed analyses are summarized in Annex 3. The in-feed determination of phytase activity confirmed the expected target values. Native phytase activity in all control diets (R1-R4) was below 50 U/kg diet. The analytical results for total P confirmed the graded addition of inorganic P in form of dicalcium phosphate.

Growth performance (Tables 1-4, Figure 1)
A positive and dose related response to phytase supplementation was observed, both in terms of live weight and feed conversion ratio. Also the inclusion of inorganic P in all 3 positive control diets (treatments R2, R3 and R4) resulted in a significant improvement of both parameters when compared to the negative control fed the low-P diet with 0.20% of available P. Turkeys receiving IPA Mash phytase at 2000 and 4000 U/kg diet, respectively, reached significantly higher final live weights (day 29) than positive control 3 (treatment R4). Phytase addition at 1000 U/kg diet (treatment R5) resulted in both final weight and feed conversion ratio comparable to that reached in positive control 3 (treatment R4). This observation clearly indicated that at 1000 U/kg diet IPA Mash phytase has the potency to replace 0.15% of available P in this particular starter diet.

Dietary metabolizable energy and apparent P utilization (Table 5, Figure 3)
The results regarding AMEn showed a negative influence of increasing inclusion of inorganic P, but there was no obvious explanation for this observation. Metabolizable energy determined for all diets supplemented with IPA Mash phytase was not significantly different when compared to the negative control.

Addition of inorganic P to the basal, low-P diet slightly improved the apparent utilization of P, but the observed difference was significant only for the highest dietary level of available P (R4 versus R1). In contrast, dietary supplementation with IPA Mash phytase resulted in significant and significant and strong improvements of P utilization. Phytase inclusion levels of 500, 1000, 2000 and 4000 U/kg improved the apparent P utilization from 51.0% (negative control) to 61.0, 66.0, 72.3 and 77.0%, respectively.

Tibia mineralization (Table 6, Figure 2)
As expected, tibia ash percentage was significantly improved by both dietary factors, i.e. the addition of inorganic P or microbial phytase. The response to IPA Mash phytase was dose-dependent and significant improvements were observed at all inclusion levels. Phytase addition at 500, 1000, 2000 and 4000 U/kg improved tibia ash percentage from 30.1% (negative control) to 36.8, 40.4, 43.4 and 44.8%, respectively. At 2000 and 4000 U/kg diet phytase addition resulted in a numerically higher tibia ash percentage when compared with positive control 3, which received 0.15% of additional available P.

Blood parameters (Table 7, Figure 4)
The results confirmed that both experimental factors, either addition of inorganic, available P or phytase supplementation, led to a dose related and significant increase of phosphorus concentration in blood serum. IPA Mash phytase at 4000 U/kg diet increased the level of inorganic P by 250% when compared to the negative control. Phytase supplementation at 1000 U/kg diet (treatment R6) resulted in the same serum level of inorganic P as positive control 3, which received 0.15% of additional available P. As expected, both experimental factors also slightly reduced the concentration of Ca in blood serum and thus normalized the ratio between these two minerals towards the physiological one.

Phytase activity in digestive tract (Table 8, Figure 5)
The results confirmed that phytase activity in crop samples is in line with its initial levels present in the respective diets. In contrast, only low phytase activity was detected in the ileal digesta and no significant differences among dietary treatments were noted anymore. This finding indicates that the majority of added microbial phytase was degraded during the passage in the gastro-intestinal tract.

Conclusion

In conclusion, the results of this performance and balance experiment confirmed the dose-related efficacy of IPA Mash phytase when used at graded inclusion levels in the started diet for turkeys. Growth rate, apparent P utilization and tibia ash percentage were shown as the most relevant response parameters.

Summary

A 4-week performance and balance experiment involving 240 male turkeys (strain BUT T9) was conducted in order to evaluate the efficacy of a novel microbial 6-phytase (IPA Mash phytase) when added to a low-P, corn-based starter diet. Eight dietary treatments were compared in this study, as follows: R1 – negative control diet (0.20% available P); R2 – positive control diet 1 (0.25% available P) ; R3 – positive control diet 2 (0.30% available P); R4 – positive control diet 3 (0.35% available P); R5 – R1 + phytase at 500 U/kg; R6 – R1 + phytase at 1000 U/kg; R7 – R1 + phytase at 2000 U/kg; R8 – R1 + phytase at 4000 U/kg. Each dietary treatment was assigned to 15 replicates of 2 birds each. Body weight and feed conversion ratio wer
e monitored as performance parameters. The apparent P utilization and tibia ash percentage were determined as main criteria of P availability. A positive and significant dose related response to phytase supplementation was noted in terms of live weight and feed conversion. Dietary supplementation with IPA Mash phytase at 500, 1000, 2000 and 4000 U/kg significantly improved the P utilization from 51.0% (negative control) to 61.0, 66.0, 72.3 and 77.0%, respectively. As the consequence of this effect, tibia ash percentage significantly increased from 30.1% (negative control) to 36.8, 40.4, 43.4 and 44.8%, respectively. Based on performance parameters, tibia mineralization and P concentration in blood plasma, the effects obtained with phytase addition at 1000 U/kg are fully comparable to that observed in positive control 3, which received 0.15% of additional available P in form of dicalcium phosphate.

Table 1: Growth performance before the balance period (D9/22)

Treatment		Weight D9 (g)	Weight D14 (g)	Weight D22 (g)
R1	0.20 % available P	153	247 ± 13 b	497 ± 33 d
R2	0.25 % available P	154	253 ± 19 ab	528 ± 42 cd
R3	0.30 % available P	155	260 ± 13 ab	558 ± 33 abc
R4	0.35 % available P	154	259 ± 28 ab	569 ± 53 ab
R5	R1 + IPA 500 U/kg	153	257 ± 16 ab	547 ± 40 bc
R6	R1 + IPA 1000 U/kg	157	262 ± 17 a	565 ± 41 ab
R7	R1 + IPA 2000 U/kg	154	264 ± 24 a	588 ± 59 a
R8	R1 + IPA 4000 U/kg	156	269 ± 24 a	594 ± 51 a
	Statistical significance	NS	P < 0.01	P < 0.01

a, b, c, d Means without a common letter are significantly different (P<0.05)

Table 2: Growth performance during the balance period (D25/29)

Treatment		No birds. D29	Weight D25 (g)	Weight D29 (g)
R1	0.20 % available P	30	617 ± 43 d	767 ± 84 e
R2	0.25 % available P	30	666 ± 53 c	860 ± 69 d
R3	0.30 % available P	30	705 ± 50 bc	918 ± 56 c
R4	0.35 % available P	30	729 ± 65 abc	955 ± 85 bc
R5	R1 + IPA 500 U/kg	30	696 ± 51 c	907 ± 72 cd
R6	R1 + IPA 1000 U/kg	30	720 ± 51 bc	949 ± 69 bc
R7	R1 + IPA 2000 U/kg	30	750 ± 75 ab	990 ± 100 ab
R8	R1 + IPA 4000 U/kg	30	769 ± 70 a	1013 ± 87 a
	Statistical significance		P < 0.01	P < 0.01

a, b, c, d, e Means without a common letter are significantly different (P<0.05)

Table 3: Feed intake during experimental period (D9/D29)

Treatment		Feed intake D9/D22 (g/bird)	Feed intake D22/D25 (g/bird)	Feed intake D25/D29 (g/bird)	Feed intake D9/D29 (g/bird)
R1	0.20 % available P	1127	404	530	2061
R2	0.25 % available P	1169	450	635	2253
R3	0.30 % available P	1261	498	707	2466
R4	0.35 % available P	1285	516	735	2536
R5	R1 + IPA 500 U/kg	1229	475	682	2386
R6	R1 + IPA 1000 U/kg	1257	503	717	2477
R7	R1 + IPA 2000 U/kg	1297	522	757	2576
R8	R1 + IPA 4000 U/kg	1304	537	762	2603

Table 4: Feed conversion ratio during experimental period D9/D29

Treatment		FCR D9/D22	FCR D22/D25	FCR D25/D29	FCR D9/D29
R1	0.20 % available P	1.644 ± 0.061 a	1.684 ± 0.119	2.123 ± 1.504	1.685 ± 0.082 a
R2	0.25 % available P	1.567 ± 0.064 b	1.628 ± 0.096	1.645 ± 0.136	1.598 ± 0.067 bc
R3	0.30 % available P	1.562 ± 0.036 b	1.741 ± 0.352	1.672 ± 0.144	1.616 ± 0.048 b
R4	0.35 % available P	1.552 ± 0.055 bc	1.617 ± 0.084	1.627 ± 0.080	1.584 ± 0.042 bc
R5	R1 + IPA 500 U/kg	1.563 ± 0.085 b	1.606 ± 0.117	1.620 ± 0.107	1.584 ± 0.059 bc
R6	R1 + IPA 1000 U/kg	1.539 ± 0.065 bc	1.632 ± 0.176	1.573 ± 0.113	1.563 ± 0.063 bcd
R7	R1 + IPA 2000 U/kg	1.497 ± 0.042 c	1.620 ± 0.130	1.581 ± 0.072	1.543 ± 0.050 cd
R8	R1 + IPA 4000 U/kg	1.491 ± 0.038 c	1.532 ± 0.083	1.567 ± 0.073	1.519 ± 0.029 d
	Stat. significance	P < 0.01	NS	NS	P < 0.01

a, b, c, d Means without a common letter are significantly different (P<0.05)

Table 5: Effects of dietary level of available P and IPA phytase addition on AMEn values and P utilization

Treatment		AMEn (Kcal/kg DM)	Apparent utilisation of P (% of intake)
R1	0.20 % available P	3181 ± 45 a	50.98 ± 1.72 f
R2	0.25 % available P	3167 ± 86 a	52.02 ± 1.54 f
R3	0.30 % available P	3074 ± 56 b	52.44 ± 2.09 ef
R4	0.35 % available P	3070 ± 68 b	54.34 ± 1.71 e
R5	R1 + IPA 500 U/kg	3143 ± 64 ab	60.95 ± 2.81 d
R6	R1 + IPA 1000 U/kg	3122 ± 104 ab	66.00 ± 2.56 c
R7	R1 + IPA 2000 U/kg	3097 ± 94 ab	72.26 ± 2.53 b
R8	R1 + IPA 4000 U/kg	3105 ± 77 ab	76.96 ± 2.18 a
	Statistical significance	P < 0.01	P < 0.01

a, b, c, d, e, f Means without a common letter are significantly different (P<0.05)

Table 6: Effects of dietary level of available P and IPA phytase addition on tibia ash concentration (day 29)

Treatment		Tibia dry matter (%)	Tibia ash (% of DM)
R1	0.20 % available P	30.43 e	30.13 ± 1.45 e
R2	0.25 % available P	32.18 d	34.40 ± 1.96 d
R3	0.30 % available P	34.64 c	38.53 ± 1.84 c
R4	0.35 % available P	36.10 b	41.27 ± 1.64 ab
R5	R1 + IPA 500 U/kg	33.60 c	36.84 ± 2.72 c
R6	R1 + IPA 1000 U/kg	35.58 bc	40.40 ± 2.48 b
R7	R1 + IPA 2000 U/kg	37.53 ab	43.37 ± 1.60 a
R8	R1 + IPA 4000 U/kg	38.53 a	44.78 ± 1.20 a
	Statistical significance	P < 0.01	P < 0.01

a, b, c, d, e Means without a common letter are significantly different (P<0.05)

Table 7: Serum concentration of Ca and inorganic P

Treatment		Ca (mg/L)	Inorganic P (mg/L)
R1	0.20 % available P	139 ± 11 a	32 ± 5 e
R2	0.25 % available P	136 ± 11 ab	33 ± 4 de
R3	0.30 % available P	131 ± 10 ab	41 ± 5 d
R4	0.35 % available P	126 ± 6 b	53 ± 9 c
R5	R1 + IPA 500 U/kg	137 ± 9 a	36 ± 6 de
R6	R1 + IPA 1000 U/kg	131 ± 10 ab	51 ± 12 c
R7	R1 + IPA 2000 U/kg	128 ± 8 ab	63 ± 9 b
R8	R1 + IPA 4000 U/kg	126 ± 5 b	80 ± 9 a
	Statistical significance	P < 0.01	P < 0.01

a, b, c, d, e Means without a common letter are significantly different (P<0.05)

Table 8: Phytase activity in diets and digestive tract (U/kg dry matter)

Treatment		Diet	Crop	Ileum
R1	0.20 % available P	101	111 ± 39 d	84 ± 72
R2	0.25 % available P	118	110 ± 41 d	124 ± 107
R3	0.30 % available P	47	125 ± 51 d	87 ± 49
R4	0.35 % available P	54	106 ± 53 d	86 ± 49
R5	R1 + IPA 500 U/kg	518	525 ± 313 cd	73 ± 34
R6	R1 + IPA 1000 U/kg	1223	990 ± 437 c	73 ± 34
R7	R1 + IPA 2000 U/kg	2845	1941 ± 701 b	143 ± 115
R8	R1 + IPA 4000 U/kg	4179	3189 ± 1082 a	100 ± 53
	Statistical significance	-	P < 0.01	NS

a, b, c, d Means without a common letter are significantly different (P<0.05)

Figure 1

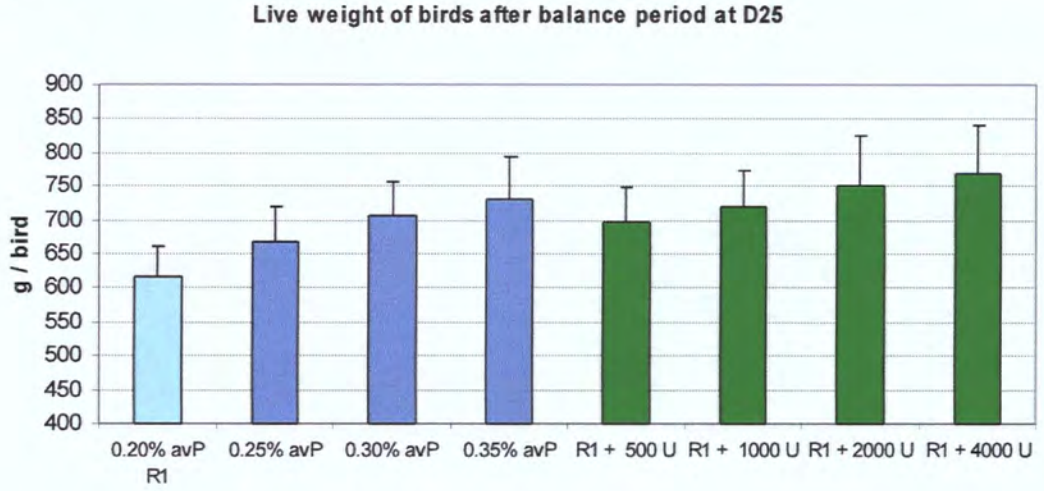


Figure 2

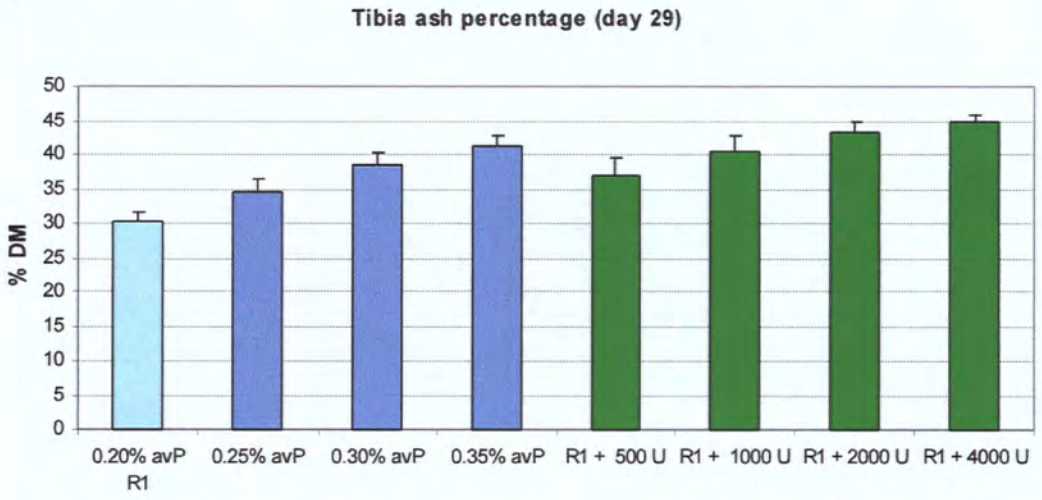


Figure 3

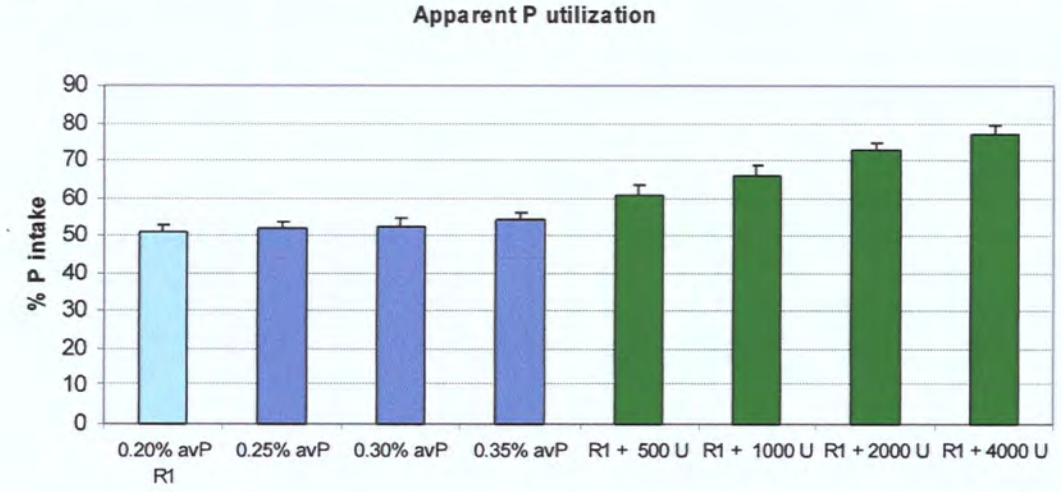


Figure 4

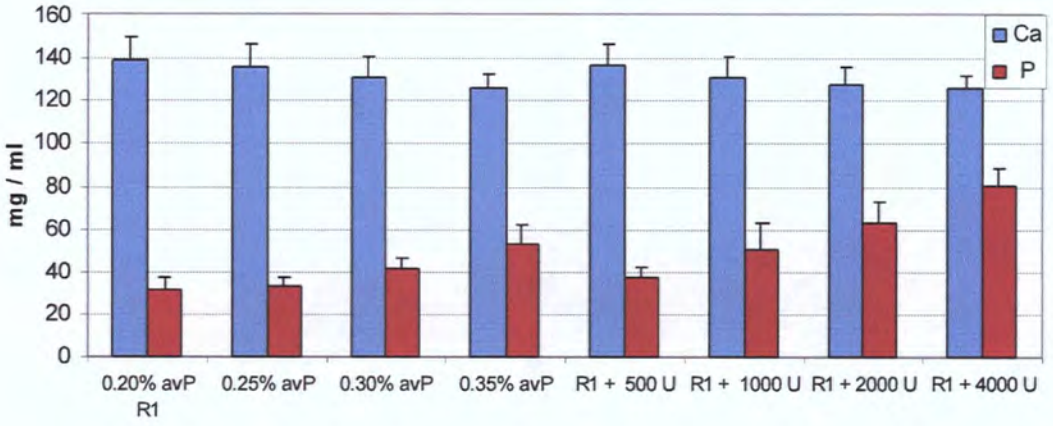
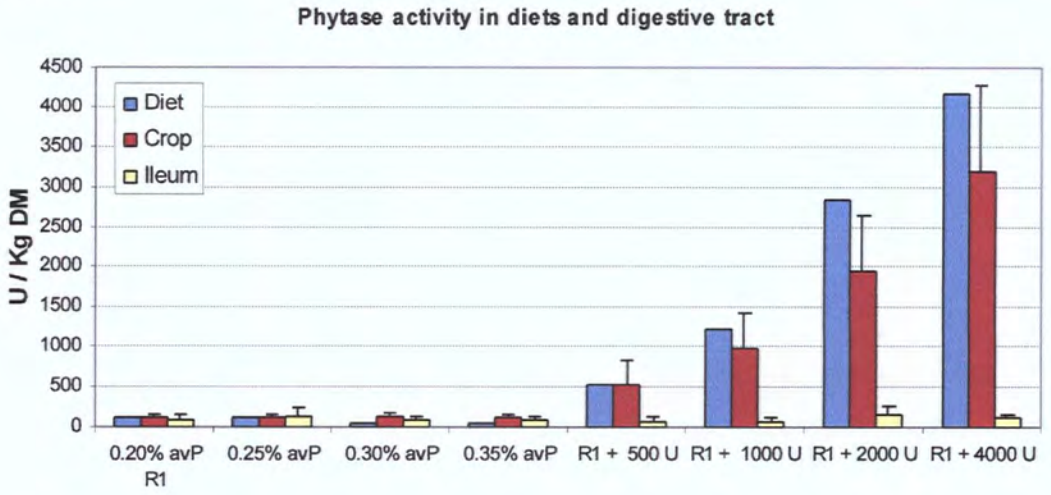


Figure 5



ANNEXES

Annex 1

Feed composition of basal diets (%):

	Pre-starter D1/D9	R1 0.2 Av P	R2 0.25 Av P	R3 0.3 Av P	R4 0.35 NPP
Corn	40.37	40.37	40.37	40.37	40.37
Wheat	5.34	5.78	5.78	5.75	5.78
Soybean meal. 48% CP	44.83	44.83	44.83	44.83	44.83
Vegetable oil	4.63	4.63	4.63	4.63	4.63
HCl Lysine	0.39	0.39	0.39	0.39	0.39
DL-Methionine	0.39	0.39	0.39	0.39	0.39
Calcium carbonate	0.90	2.00	1.76	1.52	1.26
Dicalcium phosphate	2.15	0.61	0.85	1.12	1.35
Salt	0.40	0.40	0.40	0.40	0.40
Premix(Vit/Trace min)	0.60	0.60	0.60	0.60	0.60

Calculated nutritional values (%)

	Pre-starter D1/D9	R1 0.2 Av P	R2 0.25 Av P	R3 0.3 Av P	R4 0.35 NPP
Metabolizable Energy (Kcal/kg)	2950	2950	2950	2950	2950
Crude protein	25.00	25.00	25.00	25.00	25.00
Lysine	1.67	1.67	1.67	1.67	1.67
Methionine + Cystine	1.15	1.15	1.15	1.15	1.15
Calcium	1.11	1.11	1.08	1.07	1.03
Available Phosphorus	0.50	0.20	0.25	0.30	0.35

Annex 2

Experimental design (cages)

Cage	1101	1102	1103	1104	1105	1106	1107	1108	1109	1110
Régime	R1	R7	R5	R2	R3	R4	R8	R6	R1	R6
Régime	R8	R7	R2	R4	R3	R5	R6	R1	R4	R5
Cage	1201	1202	1203	1204	1205	1206	1207	1208	1209	1210

Cage	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110
Régime	R8	R3	R2	R7	R1	R2	R8	R5	R6	R3
Régime	R4	R7	R3	R6	R2	R1	R4	R5	R8	R7
Cage	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210

Cage	3101	3102	3103	3104	3105	3106	3107	3108	3109	3110
Régime	R5	R4	R8	R2	R1	R7	R6	R3	R5	R1
Régime	R2	R4	R3	R6	R7	R8	R1	R6	R5	R4
Cage	3201	3202	3203	3204	3205	3206	3207	3208	3209	3210

Cage	3301	3302	3303	3304	3305	3306	3307	3308	3309	3310
Régime	R3	R2	R7	R8	R3	R8	R5	R2	R7	R6
Régime	R4	R1	R5	R1	R3	R7	R6	R8	R2	R4
Cage	3401	3402	3403	3404	3405	3406	3407	3408	3409	3410

Cage	4101	4102	4103	4104	4105	4106	4107	4108	4109	4110
Régime	R2	R4	R7	R1	R5	R6	R8	R3	R8	R3
Régime	R2	R7	R4	R1	R6	R5	R7	R2	R1	R8
Cage	4201	4202	4203	4204	4205	4206	4207	4208	4209	4210

Cage	4301	4302	4303	4304	4305	4306	4307	4308	4309	4310
Régime	R5	R4	R6	R3	R3	R4	R5	R6	R7	R2
Régime	R8	R1	R7	R1	R3	R5	R8	R4	R2	R6
Cage	4401	4402	4403	4404	4405	4406	4407	4408	4409	4410

Annex 3

Feed analyses

Treatment		Dry matter * (%)	Crude protein (% DM) *	Calcium (% DM) *	Total P (% DM) *	Phytase activity (U/kg) **
R1	0.20 % av P	89.03	27.66	1.40	0.58	44
R2	0.25 % av P	89.02	27.59	1.36	0.63	55
R3	0.30 % av P	89.46	27.67	1.36	0.69	44
R4	0.35 % av P	89.43	26.89	1.36	0.74	45
R5	R1+IPA 500 U/kg	89.19	27.65	1.42	0.60	581
R6	R1+IPA 1000 U/kg	89.36	27.98	1.38	0.59	919
R7	R1+IPA 2000 U/kg	89.53	27.69	1.45	0.58	2327
R8	R1+IPA 4000 U/kg	89.48	27.83	1.46	0.58	4075

* Analytics conducted by INRA

** Analytics conducted by DSM Biopract GmbH, Berlin

Individual weights

pen	Tr	N°	W 9	W 14	W 22	W 25	W 29
1101	R1	13869	139	232	488	616	794
1101	R1	13914	138	235	452	550	575
1102	R7	13775	138	250	582	774	999
1102	R7	13782	138	202	441	583	762
1103	R5	13858	158	271	622	807	1096
1103	R5	13887	158	257	563	720	925
1104	R2	13702	152	233	496	624	836
1104	R2	13971	151	240	494	605	793
1105	R3	13710	154	238	500	626	830
1105	R3	13839	154	263	576	744	990
1106	R4	13707	151	254	568	732	958
1106	R4	13762	151	254	543	699	955
1107	R8	13720	169	283	587	768	998
1107	R8	13776	169	291	650	828	1123
1108	R6	13738	148	252	527	677	890
1108	R6	13744	148	246	520	665	877
1109	R1	13834	156	248	510	632	818
1109	R1	13838	156	244	486	606	770
1110	R6	13906	162	266	546	691	895
1110	R6	13934	162	271	566	733	986
1201	R8	2932	145	250	564	697	944
1201	R8	13979	145	263	586	746	1013
1202	R7	13751	160	232	504	652	872
1202	R7	13836	160	243	575	752	977
1203	R2	13756	157	268	547	678	873
1203	R2	13807	157	270	578	712	936
1204	R4	13736	155	284	605	781	1033
1204	R4	13758	155	264	573	705	922
1205	R3	13810	148	241	514	662	876
1205	R3	13840	148	252	550	688	905
1206	R5	13757	158	271	556	727	962
1206	R5	13788	158	267	542	688	889
1207	R6	13719	149	248	532	680	900
1207	R6	13958	148	244	520	661	853
1208	R1	13801	161	274	569	728	927
1208	R1	13826	161	258	518	649	845
1209	R4	13764	155	283	605	756	982
1209	R4	13769	155	265	580	733	1000
1210	R5	13722	158	265	537	686	929
1210	R5	13725	158	254	531	643	837
2101	R8	13750	149	254	547	707	942
2101	R8	13879	150	222	513	666	920
2102	R3	13891	148	250	552	700	885
2102	R3	13929	148	254	506	673	886
2103	R2	13877	156	268	582	721	926
2103	R2	13895	156	250	542	698	906
2104	R7	13784	140	275	596	760	997
2104	R7	13789	140	250	532	681	879
2105	R1	13779	162	262	529	665	832

Individual weights

pen	Tr	N°	W 9	W 14	W 22	W 25	W 29
2105	R1	13999	161	249	512	645	826
2106	R2	13841	160	278	569	724	937
2106	R2	13942	160	261	571	710	936
2107	R8	13954	158	275	628	836	1067
2107	R8	13972	158	294	666	872	1155
2108	R5	13913	160	269	603	772	1021
2108	R5	13917	160	281	585	755	995
2109	R6	13923	156	265	566	712	909
2109	R6	13959	156	265	593	790	1044
2110	R3	13712	153	271	560	708	881
2110	R3	13787	153	265	599	745	991
2201	R4	13881	146	259	569	742	933
2201	R4	13940	146	232	564	736	968
2202	R7	13935	147	271	609	750	958
2202	R7	13952	147	239	530	662	874
2203	R3	13753	156	269	584	751	965
2203	R3	13761	156	262	549	701	903
2204	R6	13777	167	286	604	804	1041
2204	R6	13853	167	278	616	788	1017
2205	R2	13845	170	280	619	800	1020
2205	R2	13849	170	271	580	751	970
2206	R1	13790	136	243	523	629	783
2206	R1	13985	135	230	451	561	689
2207	R4	13983	156	283	616	781	1032
2207	R4	13988	156	284	622	795	1039
2208	R5	13856	157	271	592	744	957
2208	R5	13950	157	266	554	723	910
2209	R8	13926	159	267	572	730	957
2209	R8	13931	159	264	603	765	1011
2210	R7	13949	148	250	547	679	912
2210	R7	13955	148	258	621	803	1072
3101	R5	13919	139	221	484	621	819
3101	R5	13984	139	224	478	600	776
3102	R4	13859	141	225	515	687	950
3102	R4	13878	141	250	588	754	942
3103	R8	13882	144	233	551	721	942
3103	R8	13899	144	250	518	661	883
3104	R2	13828	136	227	471	588	758
3104	R2	13861	136	231	497	626	834
3105	R1	13921	164	266	531	668	858
3105	R1	13969	164	269	545	661	847
3106	R7	13815	153	266	582	734	950
3106	R7	13905	153	274	651	822	1073
3107	R6	13785	147	235	527	669	860
3107	R6	13973	146	246	510	654	857
3108	R3	13912	152	246	569	730	940
3108	R3	13933	152	257	589	753	952
3109	R5	13800	140	243	544	719	933
3109	R5	13814	140	237	518	670	880
3110	R1	13865	149	238	472	603	753
3110	R1	13870	149	236	470	573	640
3201	R2	13767	164	264	535	661	892

Individual weights

pen	Tr	N°	W 9	W 14	W 22	W 25	W 29
3201	R2	13997	163	270	566	712	909
3202	R4	13943	159	286	616	793	1033
3202	R4	13974	159	272	577	741	962
3203	R3	13844	152	246	519	606	892
3203	R3	13892	152	263	535	602	845
3204	R6	13960	153	257	566	713	933
3204	R6	13976	153	270	590	738	984
3205	R7	13850	154	292	642	850	1100
3205	R7	13884	154	258	571	744	1010
3206	R8	13957	161	274	622	816	1057
3206	R8	13962	161	290	655	835	1076
3207	R1	13851	151	240	520	641	819
3207	R1	13876	151	253	499	616	776
3208	R6	13808	141	259	525	697	943
3208	R6	13860	140	244	549	700	923
3209	R5	13964	155	254	558	710	880
3209	R5	13967	155	242	505	637	850
3210	R4	13711	155	259	598	752	1007
3210	R4	13904	154	245	528	650	847
3301	R3	13821	159	277	617	766	988
3301	R3	13898	159	273	579	719	924
3302	R2	13772	155	236	488	628	819
3302	R2	13842	155	255	551	690	885
3303	R7	13718	170	315	732	928	1214
3303	R7	13759	170	287	638	844	1138
3304	R8	13793	148	258	577	740	934
3304	R8	13795	148	248	546	714	947
3305	R3	13796	169	283	603	775	989
3305	R3	13894	169	280	617	785	1031
3306	R8	13867	136	240	548	702	921
3306	R8	13970	136	246	573	761	1026
3307	R5	13991	164	263	566	725	922
3307	R5	14000	164	273	564	728	940
3308	R2	13705	158	278	569	698	877
3308	R2	13713	158	273	547	703	884
3309	R7	13809	145	214	468	592	794
3309	R7	13941	145	248	562	701	932
3310	R6	13833	163	274	577	721	938
3310	R6	13902	163	261	561	711	966
3401	R4	13731	140	242	520	660	888
3401	R4	13765	140	242	529	667	898
3402	R1	13716	155	250	493	588	748
3402	R1	13723	155	251	530	626	817
3403	R5	13745	152	257	609	753	989
3403	R5	13803	152	258	583	718	931
3404	R1	13944	150	238	460	582	747
3404	R1	13963	149	234	474	591	733
3405	R3	13843	164	270	562	720	940
3405	R3	13852	164	282	616	788	1041
3406	R7	13915	158	253	566	714	957
3406	R7	13947	158	249	588	735	966
3407	R6	13717	161	275	576	721	940

Individual weights

pen	Tr	N°	W 9	W 14	W 22	W 25	W 29
3407	R6	13729	161	283	613	772	1028
3408	R8	13846	161	313	698	921	1152
3408	R8	13909	161	246	575	794	1085
3409	R2	13727	142	232	498	655	825
3409	R2	13822	142	251	507	656	842
3410	R4	13755	165	284	622	814	1029
3410	R4	13817	165	275	602	785	1023
4101	R2	13802	144	236	485	624	783
4101	R2	13864	143	226	446	570	737
4102	R4	13901	156	276	641	833	1118
4102	R4	13910	156	261	541	694	874
4103	R7	13874	165	290	660	823	1144
4103	R7	13928	165	297	650	817	1105
4104	R1	13951	162	251	501	616	772
4104	R1	13977	162	240	482	602	768
4105	R5	13886	166	276	525	688	903
4105	R5	13981	166	259	535	672	878
4106	R6	13766	161	262	573	750	1005
4106	R6	13783	161	274	621	811	1048
4107	R8	13742	160	293	597	764	971
4107	R8	13883	170	316	651	836	1097
4108	R3	13701	157	251	544	694	892
4108	R3	13749	157	271	566	707	893
4109	R8	13925	163	290	633	811	1103
4109	R8	13937	163	241	492	626	849
4110	R3	13735	163	276	569	758	954
4110	R3	13832	163	251	528	673	877
4201	R2	13728	166	249	501	639	819
4201	R2	13737	166	233	487	630	810
4202	R7	13848	155	269	610	757	991
4202	R7	13903	155	288	629	795	1042
4203	R4	13715	169	285	645	823	1071
4203	R4	13945	168	272	588	748	975
4204	R1	13880	141	238	466	580	747
4204	R1	13924	141	220	429	519	700
4205	R6	13706	166	296	656	804	1090
4205	R6	13953	165	287	587	737	943
4206	R5	13939	149	239	480	630	798
4206	R5	13948	149	258	551	715	936
4207	R7	13956	169	292	633	820	1061
4207	R7	13998	169	276	582	734	975
4208	R2	13830	143	254	526	670	873
4208	R2	13835	143	232	495	625	803
4209	R1	13741	159	245	482	606	765
4209	R1	13774	159	266	559	692	859
4210	R8	13812	158	268	575	752	975
4210	R8	13824	158	259	570	736	985
4301	R5	13721	138	231	476	619	789
4301	R5	13989	137	277	488	624	828
4302	R4	13965	147	240	487	632	812
4302	R4	13978	147	251	547	689	897
4303	R6	13703	148	256	600	786	980

Individual weights

pen	Tr	N°	W 9	W 14	W 22	W 25	W 29
4303	R6	13994	147	220	468	611	812
4304	R3	13930	151	260	553	697	892
4304	R3	13936	151	249	530	671	848
4305	R3	13740	149	245	555	718	928
4305	R3	13746	149	244	515	648	836
4306	R4	13732	159	145	388	539	723
4306	R4	13995	158	266	550	691	908
4307	R5	13888	151	240	545	646	851
4307	R5	13918	151	262	575	710	884
4308	R6	13794	164	250	521	668	891
4308	R6	13825	164	277	609	711	1026
4309	R7	13799	151	258	551	703	954
4309	R7	13813	151	268	605	791	1013
4310	R2	13730	145	241	515	639	829
4310	R2	13768	145	236	477	583	723
4401	R8	13818	162	297	689	915	1220
4401	R8	13855	162	278	610	783	1015
4402	R1	13872	162	226	506	647	798
4402	R1	13885	162	257	499	624	517
4403	R7	13932	160	274	609	760	986
4403	R7	13982	160	267	577	738	983
4404	R1	13868	156	247	472	589	726
4404	R1	13875	156	257	470	612	763
4405	R3	13760	146	252	535	655	865
4405	R3	13806	146	244	554	691	890
4406	R5	13827	157	246	550	690	929
4406	R5	13837	157	278	599	738	978
4407	R8	13771	166	288	622	807	1020
4407	R8	13819	166	273	593	772	996
4408	R4	13938	161	248	527	667	832
4408	R4	13946	161	282	617	786	1035
4409	R2	13752	168	285	567	702	898
4409	R2	13778	168	273	527	667	873
4410	R6	13908	167	255	544	685	898
4410	R6	13986	167	262	591	747	981

Data of balance study

cage	Tr	Excreta	Energy of Excreta	Protein of Excreta	P of Excreta	DM Intake	AMEn	P Utilisation
1101	R1	108,64	3550,5	27,6	0,8	337,7	3250,1	53,95
1109	R1	129,98	3698,9	28,1	0,8	389,3	3163,8	52,67
1208	R1	158,11	3696,8	29,1	0,8	442,8	3092,2	48,68
2105	R1	130,78	3664,8	27,8	0,8	390,2	3169,7	51,80
2206	R1	113,29	3594,8	31,6	0,9	351,5	3250,9	52,00
3105	R1	120,32	3704,1	30,7	0,9	375,6	3218,3	52,65
3110	R1	118,29	3691,1	27,7	0,8	351,3	3155,1	50,51
3207	R1	128,43	3638,5	29,1	0,9	378,7	3171,1	49,32
3402	R1	120,4	3628,5	29,0	0,9	347,3	3149,6	48,40
3404	R1	112,58	3616,1	29,2	0,8	337,6	3197,7	52,09
4104	R1	114,39	3650,5	29,2	0,9	359,8	3237,0	52,13
4204	R1	118,04	3686,2	31,4	0,8	338,4	3133,8	49,29
4209	R1	128,85	3583,9	29,2	0,9	381,3	3194,2	49,04
4402	R1	127,77	3608,4	27,8	0,9	387,0	3204,5	50,30
4404	R1	125,56	3660,1	30,3	0,8	366,4	3157,8	51,90
1104	R2	116,94	3693,8	28,8	0,9	359,8	3222,1	52,42
1203	R2	141,72	3627	29,8	0,9	424,1	3218,5	51,19
2103	R2	161,24	3696,5	27,6	0,9	449,1	3102,5	48,89
2106	R2	167,32	3802,4	28,8	0,8	465,4	3068,3	52,44
2205	R2	160,12	3733,9	29,3	0,9	471,2	3161,8	51,19
3104	R2	110,52	3729,4	30,3	0,9	376,9	3322,9	55,69
3201	R2	142,85	3688	28,1	0,9	414,4	3155,7	53,11
3302	R2	129,44	3676,2	29,2	0,9	397,8	3228,4	52,80
3308	R2	149,28	3736,9	28,9	0,9	423,9	3117,5	51,16
3409	R2	153,97	3739,9	27,7	0,9	425,4	3077,6	50,38
4101	R2	123,24	3676,6	27,7	0,9	358,0	3159,0	51,71
4201	R2	127,02	3691,3	27,7	0,9	385,3	3202,7	52,70
4208	R2	169,83	3778,1	28,6	0,7	439,6	2985,1	55,66
4310	R2	116,98	3593,7	27,9	0,9	355,6	3238,0	52,28
4409	R2	134,45	3624,2	29,9	0,9	409,0	3237,1	52,34
1105	R3	149,61	3758	29,1	0,9	437,4	3135,2	53,61
1205	R3	136,36	3653	30,5	0,9	409,4	3206,5	54,90
2102	R3	165,33	3786,8	27,7	0,9	448,8	3029,0	50,50
2110	R3	160,96	3737,4	28,1	0,9	460,4	3112,1	53,70
2203	R3	176,4	3737,9	29,4	0,9	486,2	3073,5	53,67
3108	R3	183,95	3762,4	28,2	0,9	488,0	3011,3	51,11
3203	R3	153,7	3692,1	29,3	0,9	415,0	3064,9	53,03
3301	R3	170,59	3765,2	28,4	0,9	447,8	2997,8	52,05
3305	R3	185,92	3800,9	28,6	0,9	498,3	3011,8	51,42
3405	R3	185,4	3690,1	29,9	0,9	494,7	3054,4	51,75
4108	R3	146,91	3672,1	27,4	0,9	417,4	3124,2	53,15
4110	R3	161,63	3734,5	28,5	1,0	450,2	3083,3	48,58
4304	R3	156,2	3678,2	26,0	0,8	442,0	3110,8	56,85
4305	R3	164,94	3715,3	26,8	0,9	447,2	3049,5	52,67
4405	R3	163,75	3671,3	29,2	0,9	434,0	3049,6	49,63
1106	R4	150,19	3786,2	28,3	0,9	442,8	3143,6	58,71
1204	R4	198,74	3880,6	30,1	0,9	499,7	2915,8	52,66
1209	R4	179,11	3760,3	28,5	1,0	490,8	3066,3	52,52
2201	R4	177,8	3758,3	28,5	0,9	488,7	3070,9	53,68
2207	R4	182,72	3736,4	29,1	0,9	499,5	3075,1	54,00
3102	R4	166,51	3765,4	29,6	0,9	475,2	3118,7	55,37

Data of balance study

cage	Tr	Excreta	Energy of Excreta	Protein of Excreta	P of Excreta	DM Intake	AMEn	P Utilisation
3202	R4	176,07	3713,3	28,0	1,0	492,1	3105,1	53,63
3210	R4	164,87	3694,1	28,4	0,9	439,6	3056,5	54,85
3401	R4	143,24	3670,3	28,4	0,9	404,4	3133,8	55,45
3410	R4	199,04	3843,2	27,0	0,9	531,5	2995,7	54,42
4102	R4	178,73	3797	31,4	1,0	524,8	3149,3	54,87
4203	R4	171,38	3799,6	28,8	1,0	485,0	3093,0	53,64
4302	R4	145,42	3719,6	27,1	0,9	417,4	3130,3	56,19
4306	R4	161,03	3733	26,5	0,9	426,7	3024,4	53,27
4408	R4	186,24	3755,2	28,4	0,9	474,4	2974,2	51,78
1103	R5	175,86	3719,6	29,7	0,7	495,2	3108,2	60,97
1206	R5	164,96	3711,3	28,4	0,7	474,2	3129,2	61,25
1210	R5	153,12	3739	27,3	0,7	428,4	3082,1	60,19
2108	R5	165,73	3748,3	27,5	0,6	487,5	3139,1	64,14
2208	R5	156,96	3631,7	29,5	0,7	476,2	3220,9	62,53
3101	R5	127,82	3749,2	28,8	0,7	385,3	3171,9	59,13
3109	R5	139,82	3771,7	28,6	0,6	438,4	3207,1	66,58
3209	R5	138,17	3711,1	29,1	0,7	411,7	3173,0	62,47
3307	R5	145,72	3746,9	27,2	0,7	446,6	3184,3	61,98
3403	R5	168,22	3766,9	29,9	0,7	462,4	3062,7	59,43
4105	R5	151,74	3704,9	29,2	0,7	421,2	3093,6	56,82
4206	R5	162,62	3756,1	27,8	0,7	445,5	3052,6	55,45
4301	R5	119,45	3640,6	28,3	0,7	389,4	3287,5	63,54
4307	R5	139,58	3733,2	28,7	0,7	400,4	3120,3	60,22
4406	R5	156,49	3679,2	28,2	0,7	441,7	3117,9	59,55
1108	R6	129,74	3599,5	29,0	0,6	421,1	3298,2	69,60
1110	R6	154,7	3763,9	28,1	0,6	456,9	3140,5	66,38
1207	R6	137,86	3679,5	28,1	0,6	404,0	3160,2	62,75
2109	R6	191,4	3795,4	27,8	0,6	516,3	3018,4	65,41
2204	R6	181,44	3774	28,8	0,6	506,1	3072,6	65,12
3107	R6	124,38	3743,8	27,3	0,6	393,0	3218,5	66,56
3204	R6	143,14	3708,7	27,6	0,6	438,5	3197,3	69,20
3208	R6	142,6	3697,2	29,3	0,7	437,0	3209,0	62,91
3310	R6	147,6	3817,4	28,0	0,6	423,1	3086,3	66,27
3407	R6	186,1	3744,5	29,8	0,5	503,5	3050,2	66,76
4106	R6	161,6	3666,1	28,1	0,6	493,3	3209,7	67,21
4205	R6	174,65	3728,5	27,9	0,6	493,5	3100,0	65,83
4303	R6	159	3708,6	27,4	0,5	452,4	3112,8	69,35
4308	R6	171,48	3734,6	27,2	0,6	480,5	3084,8	66,48
4410	R6	202,72	3748,1	26,7	0,6	483,5	2865,4	60,22
1102	R7	149,97	3781,9	27,4	0,4	456,7	3161,3	75,46
1202	R7	144,68	3762,2	29,6	0,5	458,7	3221,0	74,70
2104	R7	132,29	3693,1	28,9	0,5	427,0	3258,6	71,23
2202	R7	181,73	3834,3	28,9	0,5	487,4	2996,7	69,90
2210	R7	148,27	3730,8	28,6	0,5	443,0	3162,3	71,43
3106	R7	164,17	3875,3	28,3	0,4	476,8	3078,6	74,83
3205	R7	220,42	3787,8	27,1	0,5	552,8	2916,5	68,31
3303	R7	209,71	3846,5	27,8	0,5	574,0	3013,2	69,84
3309	R7	152,46	3749,8	28,1	0,4	414,5	3041,4	72,80
3406	R7	168,9	3784,6	27,7	0,5	466,4	3046,3	68,24
4103	R7	197,28	3754,3	27,9	0,5	546,8	3062,7	71,46
4202	R7	177,17	3820,4	29,7	0,4	488,1	3039,8	76,28

Data of balance study

cage	Tr	Excreta	Energy of Excreta	Protein of Excreta	P of Excreta	DM Intake	AMEn	P Utilisation
4207	R7	160,75	3765,5	29,0	0,5	498,2	3193,1	74,22
4309	R7	162,95	3704,8	25,8	0,4	461,7	3097,2	73,31
4403	R7	156,26	3731,4	28,2	0,5	469,2	3165,8	71,87
1107	R8	165,22	3668,9	27,0	0,4	517,7	3230,0	78,36
1201	R8	170,86	3776,2	28,8	0,4	466,2	3042,5	73,88
2101	R8	147,3	3766,2	27,5	0,3	422,2	3099,7	79,93
2107	R8	203,94	3829,8	28,5	0,4	569,8	3050,9	74,18
2209	R8	151,51	3759,8	28,5	0,4	443,3	3130,3	78,33
3103	R8	140,48	3783,7	30,0	0,4	423,7	3163,9	79,60
3206	R8	197,6	3872,3	30,4	0,4	558,6	3059,2	77,08
3304	R8	137,82	3769,9	28,4	0,4	436,9	3216,3	77,51
3306	R8	163,74	3789,4	28,0	0,4	461,2	3072,7	77,16
3408	R8	212,93	3864,5	30,0	0,4	565,5	2980,8	76,43
4107	R8	157,41	3778,8	28,3	0,4	493,4	3200,7	76,15
4109	R8	164,97	3851	29,5	0,4	453,6	3028,3	73,44
4210	R8	153,82	3759,7	28,7	0,4	467,0	3173,6	76,89
4401	R8	204,36	3831	27,9	0,4	563,2	3030,4	75,01
4407	R8	173,4	3782,9	27,6	0,3	499,2	3099,7	80,41

Phytase activity (U / DM)

Cage	Tr	Crop	Ileum	Cage	Tr	Crop	Ileum	Cage	Tr	Crop	Ileum
1101	R1	56	46	3202	R4		77	1102	R7		324
1109	R1	102	148	3210	R4	102	174	1202	R7	3085	
1208	R1	135	32	3401	R4	120	183	2104	R7	2231	381
2105	R1	103	66	3410	R4	33	25	2202	R7	2464	94
2206	R1	120	192	4102	R4	129	48	2210	R7	1305	41
3105	R1	95	48	4203	R4	91	122	3106	R7	2027	54
3110	R1		192	4302	R4	86	33	3205	R7	3014	72
3207	R1	143	103	4306	R4	152	56	3303	R7	2357	76
3402	R1	82	235	4408	R4	22	85	3309	R7	1630	55
3404	R1	66	50	1103	R5	421	15	3406	R7	929	97
4104	R1	104	17	1206	R5	680	27	4103	R7		135
4204	R1	80	41	1210	R5	363	114	4202	R7	1332	
4209	R1	104	24	2108	R5	862	121	4207	R7	1592	
4402	R1	184	30	2208	R5	291	58	4309	R7	1321	116
4404	R1	184	33	3101	R5	248	47	4403	R7		267
1104	R2	63	51	3109	R5	1127	36	1107	R8	3600	
1203	R2	93	119	3209	R5	311	136	1201	R8	2451	52
2103	R2	99	29	3307	R5	220	143	2101	R8	2831	22
2106	R2	147	73	3403	R5	330	50	2107	R8	1108	72
2205	R2	99	344	4105	R5	551	27	2209	R8	1777	79
3104	R2	85	41	4206	R5	256	10	3103	R8	2417	138
3201	R2	108	28	4301	R5	648	108	3206	R8	2968	148
3302	R2	180	344	4307	R5	390	121	3304	R8	5357	238
3308	R2	61	53	4406	R5	1174	119	3306	R8	4643	57
3409	R2	152	132	1108	R6	1573	30	3408	R8	3303	96
4101	R2	48	115	1110	R6	726		4107	R8	3329	100
4201	R2	79	233	1207	R6	559	79	4109	R8	4273	115
4208	R2	150	84	2109	R6	786	10	4210	R8	2771	79
4310	R2	172	83	2204	R6	1117	99	4401	R8	3809	76
4409	R2	111		3107	R6	993	124	4407	R8	3206	130
1105	R3	85	215	3204	R6	790	140				
1205	R3	135	48	3208	R6	704	78				
2102	R3	56	115	3310	R6	912	61				
2110	R3	130	108	3407	R6	1519	58				
2203	R3	106	134	4106	R6	588	88				
3108	R3	111	28	4205	R6	890	55				
3203	R3	129	73	4303	R6	2070	47				
3301	R3	193	87	4308	R6	1139	76				
3305	R3	75	55	4410	R6	484	81				
3405	R3	139	26								
4108	R3	260	75								
4110	R3	153	53								
4304	R3	106	60								
4305	R3	117	96								
4405	R3	75	128								
1106	R4	97	73								
1204	R4	95	38								
1209	R4	88	75								
2201	R4	86	77								
2207	R4	240	77								
3102	R4	141	150								

Data for bones

CAGE	Tr	DM %	Ash % DM	CAGE	Tr	DM %	Ash % DM	CAGE	Tr	DM %	Ash % DM
1101	1	28,24	28,04	3101	5	33,93	37,93	4101	2	33,88	34,87
1102	7	34,78	41,90	3102	4	38,66	44,80	4102	4	34,80	40,42
1103	5	31,77	35,32	3103	8	37,85	45,26	4103	7	39,74	46,00
1104	2	32,74	36,02	3104	2	31,94	33,82	4104	1	31,29	29,34
1105	3	32,85	37,12	3105	1	30,19	28,21	4105	5	32,89	34,53
1106	4	36,86	43,48	3106	7	38,18	43,97	4106	6	34,80	40,67
1107	8	39,03	46,48	3107	6	32,78	35,30	4107	8	38,92	44,00
1108	6	35,44	41,17	3108	3	33,58	36,00	4108	3	34,09	38,90
1109	1	28,48	29,15	3109	5	34,69	38,72	4109	8	38,37	44,06
1110	6	34,94	40,49	3110	1	29,78	28,25	4110	3	32,10	35,10
1201	8	39,91	46,19	3201	2	33,72	37,30	4201	2	32,50	35,65
1202	7	39,11	44,22	3202	4	35,22	41,14	4202	7	38,08	45,39
1203	2	33,72	37,99	3203	3	35,71	40,29	4203	4	36,12	40,89
1204	4	37,17	42,73	3204	6	38,59	42,54	4204	1	30,94	30,80
1205	3	35,75	39,47	3205	7	37,10	41,28	4205	6	36,82	41,78
1206	5	32,65	32,25	3206	8	40,57	46,57	4206	5	30,78	32,44
1207	6	31,85	35,53	3207	1	31,75	31,58	4207	7	36,96	43,60
1208	1	30,18	30,33	3208	6	36,39	42,31	4208	2	32,09	34,03
1209	4	35,75	40,75	3209	5	33,47	36,77	4209	1	30,56	31,17
1210	5	35,35	40,44	3210	4	35,27	39,73	4210	8	37,90	44,33
2101	8	37,61	43,26	3301	3	34,94	39,18	4301	5	31,87	34,71
2102	3	32,72	35,53	3302	2	31,22	32,95	4302	4	34,79	38,81
2103	2	30,46	30,67	3303	7	36,50	42,47	4303	6	37,17	43,54
2104	7	38,27	45,31	3304	8	38,61	44,92	4304	3	36,27	40,30
2105	1	29,23	29,61	3305	3	34,19	38,54	4305	3	35,64	39,52
2106	2	32,29	34,09	3306	8	38,47	43,63	4306	4	37,14	42,20
2107	8	38,64	43,83	3307	5	36,96	41,71	4307	5	35,15	37,27
2108	5	32,96	37,32	3308	2	30,88	32,13	4308	6	35,41	39,44
2109	6	36,89	42,19	3309	7	39,80	45,58	4309	7	35,78	41,64
2110	3	35,26	38,74	3310	6	34,48	37,71	4310	2	32,09	33,95
2201	4	36,45	42,44	3401	4	35,51	40,67	4401	8	36,68	44,31
2202	7	37,55	42,38	3402	1	31,27	30,15	4402	1	31,02	29,64
2203	3	36,34	40,85	3403	5	33,91	36,01	4403	7	36,35	42,36
2204	6	35,15	40,40	3404	1	31,69	32,30	4404	1	29,93	30,55
2205	2	31,12	32,91	3405	3	34,83	37,95	4405	3	35,27	40,50
2206	1	31,87	32,83	3406	7	37,41	42,08	4406	5	34,32	39,68
2207	4	35,06	39,29	3407	6	36,09	40,53	4407	8	39,21	45,64
2208	5	33,36	37,51	3408	8	40,35	46,16	4408	4	35,20	39,96
2209	8	35,89	43,00	3409	2	32,92	36,16	4409	2	31,20	33,47
2210	7	37,36	42,35	3410	4	37,54	41,72	4410	6	36,97	42,43

Data for serum

Cage	Tr	Ca mg/l	P mg/l	Cage	Tr	Ca mg/l	P mg/l	Cage	Tr	Ca mg/l	P mg/l
1101	R1	153	28	4108	R3	133	35	3107	R6	117	37
1109	R1	158	35	4110	R3	128	42	3204	R6	119	63
1208	R1	146	24	4304	R3	126	44	3208	R6	148	54
2105	R1	124	40	4305	R3	157	42	3310	R6	143	47
2206	R1	127	41	4405	R3	141	53	3407	R6	132	53
3105	R1	149	31	1106	R4	128	50	4106	R6	122	39
3110	R1	121	37	1204	R4	123	48	4205	R6	137	56
3207	R1	145	30	1209	R4	129	45	4303	R6	128	77
3402	R1	135	28	2201	R4	126	49	4308	R6	133	49
3404	R1	139	28	2207	R4	135	72	4410	R6	121	64
4104	R1	144	26	3102	R4	127	69	1102	R7	145	46
4204	R1	140	32	3202	R4	120	50	1202	R7	133	66
4209	R1	136	32	3210	R4	132	55	2104	R7	120	68
4402	R1	129	27	3401	R4	132	45	2202	R7	130	70
4404	R1	143	37	3410	R4	136	64	2210	R7	126	58
1104	R2	153	26	4102	R4	123	50	3106	R7	113	75
1203	R2	145	29	4203	R4	125	49	3205	R7	137	68
2103	R2	128	34	4302	R4	113	46	3303	R7	135	69
2106	R2	155	34	4306	R4	127	58	3309	R7	126	65
2205	R2	120	34	4408	R4	119	46	3406	R7	133	69
3104	R2	137	32	1103	R5	149	33	4103	R7	129	76
3201	R2	135	40	1206	R5	143	29	4202	R7	120	66
3302	R2	126	33	1210	R5	154	34	4207	R7	121	56
3308	R2	119	27	2108	R5	132	39	4309	R7	127	50
3409	R2	139	37	2208	R5	135	41	4403	R7	124	48
4101	R2	136	34	3101	R5	144	32	1107	R8	131	93
4201	R2	124	31	3109	R5	124	34	1201	R8	128	70
4208	R2	140	28	3209	R5	144	36	2101	R8	126	73
4310	R2	145	35	3307	R5	143	50	2107	R8	125	80
4409	R2	133	40	3403	R5	138	39	2209	R8	121	66
1105	R3	122	37	4105	R5	134	33	3103	R8	121	81
1205	R3	142	40	4206	R5	121	30	3206	R8	117	95
2102	R3	122	34	4301	R5	125	28	3304	R8	121	79
2110	R3	134	38	4307	R5	131	42	3306	R8	132	76
2203	R3	128	45	4406	R5	139	41	3408	R8	130	78
3108	R3	118	40	1108	R6	147	49	4107	R8	133	76
3203	R3	123	50	1110	R6	138	39	4109	R8	129	84
3301	R3	130	44	1207	R6	133	28	4210	R8	128	79
3305	R3	133	37	2109	R6	126	59	4401	R8	118	95
3405	R3	127	39	2204	R6	118	46	4407	R8	132	80

European Food Safety Authority

FEEDAP UNIT

ANNEX C [1]

TRIAL PROTOCOL DATA SHEET: FOR TERRESTRIAL ANIMALS

Identification of the additive: **IPA Mash phytase**	Batch number: **PPQ 28656**
Trial ID: **INRA Ref 09/HD3/DD/0107**	Location: **INRA Le Magneraud**
Start date and exact duration of the study: **10 February 2009, 29 days**	
Number of treatment groups (+ control(s)): **8**	Replicates per group: **15**
Total number of animals: **240**	Animals per replicate: **2**
Dose(s) of the additive/active substance(s)/agent(s) (mg/Units of activity/CFU kg^{-1} complete feed/L^{-1} water) Intended: **500, 1000, 2000, 4000 U/kg** Analysed: **581, 919, 2327, 4075 U/kg** † Substances used for comparative purposes: Intended dose: Analysed:	
Animal species/category: **Turkeys**	
Breed: **BUT T9**	Identification procedure: **ring at wing**
Sex: **male** Age at start: **day-old**	Body weight at start: **around 60 g**
Physiological stage: **growing birds**	General health: **excellent**
Additional information for field trials:	
Location and size of herd or flock:	
Feeding and rearing conditions:	
Method of feeding:	
Diets (type(s)): **Starter diet for turkeys**	
Presentation of the diet: Mash ☒ Pellet ☐ Extruded ☐ Other	
Composition (main feedingstuffs): **Corn, soybean meal, wheat, vegetable oil**	
Nutrient content (relevant nutrients and energy content) Intended values: **25% crude protein; 1.11- 1.03% Ca; 0.20 - 0.35% available P** Analysed values: **24.6% crude protein; 1.25 - 1.21%; 0.52 - 0.66% total P**	
Date and nature of the examinations performed: **Balance period (day 22-25), collection of excreta**	
Method(s) of statistical evaluation used: **Analysis of variance, Tukey test (at P<0.05)**	
Therapeutic/preventive treatments (reason, timing, kind, duration): **none**	
Timing and prevalence of any undesirable consequences of treatment: -	
Date **17 December 2009**	Signature Study Director

† In case the concentration of the additive in complete feed/water may reflect insufficient accuracy, the dose of the additive can be given per animal day^{-1} or mg kg^{-1} body weight or as concentration in complementary feed.

[1] Please submit this form using a common word processing format (e.g. MS Word).

Pages 830-1368 withheld in their entirety under (B)(4)

SUBMISSION

CONTINUED

IN

NEXT

VOLUME

SUBMISSION

CONTINUED

FROM

PREVIOUS

VOLUME

TAB

REFERENCES

Pages FDA/CVM1374-1465 have been removed in accordance with copyright laws.
Please page FDA/CVM109 for a list of references of copyrighted information.

Food

Agency Response Letter GRAS Notice No. GRN 000008

CFSAN/Office of Premarket Approval

March 2, 1999

Ms. Lori Gregg
Novo Nordisk BioChem North America, Inc.
77 Perry Chapel Church Road
Box 576
Franklinton, NC 27525-0576

Re: GRAS Notice No. GRN 000008

Dear Ms. Gregg:

The Food and Drug Administration (FDA) is responding to your notice, dated October 12, 1998, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). FDA received your notice on October 14, 1998, and designated your notice as GRAS Notice No. GRN 000008.

Your notice states that Novo Nordisk BioChem North America, Inc. (Novo) has determined, based on scientific procedures, that pectin esterase enzyme preparation from *Aspergillus oryzae* carrying the gene coding for pectin esterase from *Aspergillus aculeatus* is GRAS for use as a processing aid, primarily in fruit and vegetable products, at minimum levels necessary to achieve the desired effect in accordance with current good manufacturing practices. Your notice describes (1) generally available information about the technical effect of a pectin esterase enzyme preparation obtained by fermentation of a genetically modified strain of *A. oryzae* containing a gene encoding pectin esterase derived from *A. aculeatus*;(2) published information about the host microorganism *A. oryzae*; (3) information about the production microorganism, i.e., *A. oryzae* containing the gene encoding pectin esterase derived from *A. aculeatus*; (4) the manufacturing process, which includes standard methods for the fermentation, processing, and formulation of the enzyme preparation; (5) information about processing aids used in the manufacture of the enzyme preparation; and (6) a published article that describes toxicity studies conducted with the enzyme preparation. In your notice, you estimate dietary exposure to the enzyme preparation and consider the exposure to substances (e.g., methanol) that are a product of the enzyme reaction. Your notice states that the enzyme preparation meets the specifications for enzyme preparations provided in the Food Chemicals Codex (4th ed., 1996) and the specifications for enzyme preparations provided by the Joint FAO/WHO Expert Committee on Food Additives, including a specification that no mycotoxins are detected.

Based on the information provided by Novo, as well as other information available to FDA, the agency has no questions at this time regarding Novo's conclusion that pectin esterase enzyme preparation from *Aspergillus oryzae* carrying the gene coding for pectin esterase from *Aspergillus aculeatus* is GRAS under the proposed conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of this enzyme preparation. As always, it is your continuing responsibility to ensure that food ingredients that you market are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of this letter, as well as a copy of the information in your notice that conforms to the information in proposed § 170.36(c)(1), is available for public review and copying in the public reading room of the agency's Freedom of Information Staff.

Sincerely,

Alan M. Rulis, Ph.D.
Director
Office of Premarket Approval
Center for Food Safety and Applied Nutrition

Links on this page:

1413

Food

Agency Response Letter GRAS Notice No. GRN 000034

CFSAN/Office of Premarket Approval

April 19, 2000

Ms. Lori Gregg
Novo Nordisk BioChem North America, Inc.
77 Perry Chapel Church Road
Box 576
Franklinton, NC 27525

Re: GRAS Notice No. GRN 000034

Dear Ms. Gregg:

The Food and Drug Administration (FDA) is responding to the notice, dated November 16, 1999, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). FDA received your notice on November 17, 1999 and designated it as GRAS Notice No. GRN 000034.

The subject of the notice is aspartic proteinase enzyme preparation obtained from a strain of *Aspergillus oryzae* that contains a recombinant gene encoding an aspartic proteinase derived from *Rhizomucor miehei*. The notice informs FDA of the view of Novo Nordisk BioChem North America, Inc. (Novo Nordisk) that this aspartic proteinase enzyme preparation is GRAS, through scientific procedures, for use as a tenderizing agent in the meat industry when used at minimum levels necessary to accomplish the intended technical effect in accordance with current good manufacturing practices. Typical use levels range from 0.05 to 0.10 AU/kg meat.(1)

In your notice, you describe (1) a published review article about the safety of the host microorganism, *A. oryzae*; (2) scientific publications and recommendations issued by international organizations on the safety of enzymes used in food processing, including enzymes derived from genetically modified microorganisms; (3) published scientific articles that discuss the safety of the various components of the production organism, including the host organism, and the components of the genetic material that is introduced into the host organism; (4) the basis for your conclusion that the presence of a gene encoding resistance to the antibiotic ampicillin is not a concern; (5) chapters in several books that discuss the manufacturing process, which includes standard methods for the fermentation, processing, and formulation of the enzyme preparation; and (6) unpublished oral toxicity and genetic toxicity studies conducted with the subject aspartic proteinase enzyme preparation.

According to your notice, the enzyme component of the subject enzyme preparation is present in a milk-clotting enzyme preparation that is derived from *R. miehei*. The *R. miehei*-derived enzyme preparation has been used in food since 1969, and was approved for use in cheese production in 1972 (21 CFR 173.150(a)(4)). In addition, the specific enzyme preparation that is the subject of your notice was approved for use as a milk-clotting enzyme preparation in 1997 (21 CFR 173.150(a)(5)).

According to your notice, the enzyme preparation meets the specifications for enzyme preparations provided in the Food Chemicals Codex (4th ed., 1996). The enzyme preparation also meets the specifications for enzyme preparations provided by the Joint Expert Committee on Food Additives (JECFA; a joint committee of the Food and Agriculture Organization/World Health Organization).

Based on the information provided by Novo Nordisk, as well as other information available to FDA, the agency has no questions at this time regarding Novo Nordisk's conclusion that aspartic proteinase enzyme preparation obtained from a strain of *Aspergillus oryzae* that contains a recombinant gene encoding an aspartic proteinase derived from *Rhizomucor miehei* is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of this enzyme preparation. As always, it is your continuing responsibility to ensure that food ingredients that you market are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

FDA consulted with the Labeling and Additives Policy Division (LAPD) of the Food Safety and Inspection Service (FSIS), United States Department of Agriculture, regarding the use of aspartic proteinase enzyme preparation as a tenderizing agent in the meat industry. Based upon the information submitted by Novo Nordisk, FSIS concluded that the subject aspartic proteinase enzyme preparation would be suitable as a tenderizer for raw meat cuts, provided that the enzyme preparation is used as described in the notice, the use level does not exceed 0.1 percent, and water solutions of the enzyme preparation that are applied or injected into raw meat do not result in a weight gain of more than 5 percent. FSIS noted that 9 CFR Parts 317 and 381 describe labeling requirements that apply to the use of proteolytic enzymes in meat or poultry products. FSIS also pointed out that, in the past, the use of enzyme preparations as meat tenderizers has been the subject of rulemaking at FSIS, because such food ingredients change the characteristics of meat or poultry beyond the consumer's expectation of "meat" or "poultry." If you have any questions about whether the use of aspartic proteinase enzyme preparation as a tenderizing agent in the meat industry requires rulemaking under the statutes that FSIS implements, you should direct your inquiry to Dr. Robert Post, Director, LAPD, Office of Policy, Program Development and Evaluation, Food Safety and Inspection Service, 300 12th Street, SW, Room 602, Washington, DC 20250-3700. The telephone number for LAPD is (202) 205-0279 and the FAX number is (202) 205-3625.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the Office of Premarket Approval's homepage on the World Wide Web.

Sincerely,

Alan M. Rulis, Ph.D.
Director
Office of Premarket Approval
Center for Food Safety and Applied Nutrition

c: Dr. Robert Post, Director, LAPD
Office of Policy, Program Development and Evaluation
Food Safety and Inspection Service
300 12th Street, SW, Room 602
Washington, DC 20250-3700

1414

(1)AU = Anson Units. This is a measure of enzyme activity for a proteolytic enzyme.

Links on this page:

1415

Food

Agency Response Letter GRAS Notice No. GRN 000043

CFSAN/Office of Premarket Approval

September 22, 2000

Ms. Lori Gregg
Novo Nordisk BioChem North America, Inc.
77 Perry Chapel Church Road
Box 576
Franklinton, NC 27525

Re: GRAS Notice No. GRN 000043

Dear Ms. Gregg:

The Food and Drug Administration (FDA) is responding to the notice, dated April 25, 2000, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). FDA received your notice on April 28, 2000 and designated it as GRN No. 000043.

The subject of your notice is lipase enzyme preparation derived from *Aspergillus oryzae* carrying a gene encoding lipase from *Thermomyces lanuginosus*. The notice informs FDA of the view of Novo Nordisk that this lipase enzyme preparation is GRAS, through scientific procedures, for use in dough, baked goods, and the fats and oil industry at minimum levels necessary to achieve the desired effect. The lipase enzyme preparation would be used as a catalyst in the interesterification of glycerides and acidolysis between glycerides and fatty acids in fats and oils at a maximum level of one kilogram of lipase per ton of triglycerides. The lipase enzyme preparation would be used in the hydrolysis of primary ester bonds in triglycerides in dough and baked goods for the purpose of modifying lipid-gluten interactions at a maximum level of one to five grams per 100 kg of flour.

In your notice, you describe: (1) a published review article about the safety of the host microorganism, *A. oryzae;* (2) scientific publications and recommendations issued by international organizations on the safety of enzymes used in food processing, including enzymes derived from genetically modified microorganisms; (3) published scientific articles that discuss the safety of the various components of the production organism, including the host organism, and the components of the genetic material that is introduced into the host organism; (4) the basis for your conclusion that the presence of a gene encoding resistance to the antibiotic ampicillin is not a concern; (5) chapters in several books that discuss the manufacturing process, which includes standard methods for the fermentation, processing, and formulation of the enzyme preparation; and (6) a published review of oral toxicity and genetic toxicity studies conducted with the subject lipase enzyme preparation.

According to your notice, the enzyme preparation meets the specifications for enzyme preparations provided in the Food Chemicals Codex (4th ed., 1996). The enzyme preparation also meets the specifications for enzyme preparations provided by the Joint Expert Committee on Food Additives (JECFA; a joint committee of the Food and Agriculture Organization/World Health Organization).

Based on the information provided by Novo Nordisk, as well as other information available to FDA, the agency has no questions at this time regarding Novo Nordisk's conclusion that lipase enzyme preparation derived from a genetically modified strain of *A. oryzae* that contains a recombinant gene encoding *T. lanuginosus* lipase is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of this enzyme preparation. As always, it is your continuing responsibility to ensure that food ingredients that you market are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the Office of Premarket Approval's homepage on the Internet (at http://vm.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,

Alan M. Rulis, Ph.D.
Director
Office of Premarket Approval

Links on this page:

1416

Food

Agency Response Letter GRAS Notice No. GRN 000075

CFSAN/Office of Food Additive Safety

August 14, 2001

Novozymes North America, Inc.
77 Perry Chapel Church Road
P.O. Box 576
Franklinton, NC 27525

Re: GRAS Notice No. GRN 000075

Dear Ms. Gregg:

The Food and Drug Administration (FDA) is responding to the notice, dated April 11, 2001, that you submitted on behalf of Novozymes North America, Inc. (Novozymes) in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). FDA received your notice on April 13, 2001 and designated it as GRAS Notice No. GRN 000075.

The subject of the notice is lipase enzyme preparation obtained from *Aspergillus oryzae* (*A. oryzae*) carrying a recombinant gene encoding a lipase from *Fusarium oxysporum* (*F. oxysporum*). The notice informs FDA of the view of Novozymes that the lipase preparation is GRAS, through scientific procedures, for use as a processing aid in the modification of fats and oils and in baking applications. The enzyme catalyzes the hydrolysis of ester bonds of triglycerides and diacylphospholipids, and the lipase formulations would be used at the following levels: 1 kilogram (kg) to produce 1 ton of de-gummed oil, 1 kg to produce 400 kg modified lecithin, 1 kg to produce 500 kg modified egg yolk, and 5 grams (g) per 100 kg flour.

The notice describes scientific publications and recommendations issued by international organizations on the safety of enzymes used in food processing, including enzymes produced from bioengineered organisms. As discussed in these documents, the safety of an enzyme preparation depends on the safety of the enzyme itself, the host organism, the inserted genetic material, the production organism, and the manufacturing process used in producing the enzyme preparation. The notice includes a safety evaluation of each of these components in support of Novozymes' GRAS determination.

In assessing the safety of the enzyme itself, the notice discusses the history of safe use of lipases in food processing. The notice cites a published article and monograph reporting the use of microbial lipases in food production since 1952. Novozymes also notes that several lipases (animal and *Rhizopus niveus*) are affirmed as GRAS, lipase from *Mucor miehei* (now known as *Rhizomucor miehei*) is approved for use as a food additive, and a lipase preparation produced by *A. oryzae* expressing a *Thermomyces lanuginosus* lipase is the subject of GRAS Notice No. 000043 (GRN 000043). The notice includes published and unpublished structural and sequence information for several of these lipases as well as for the subject lipase (produced from *A. oryzae* containing the *F. oxysporum* lipase). Novozymes concludes that the subject lipase preparation is substantially equivalent to other known lipases used in food production.

In assessing the safety of the host organism, *A. oryzae*, the notice refers to a review article on *A. oryzae*, describing the organism as having a long history of safe industrial use and as being commonly used in the production of food processing enzymes. Novozymes states that *A. oryzae* is nontoxigenic and nonpathogenic based on criteria given in a published article and notes that *A. oryzae* is also considered nonpathogenic by JECFA (The Joint Food and Agriculture Organization/World Health Organization's (FAO/WHO) Expert Committee on Food Additives). The notice also describes the specific strain of *A. oryzae* used as the host organism: Jal228 is an amylase negative, alkaline protease negative, neutral metalloprotease I negative derivative of the fully-characterized, well-known industrial production strain of *A. oryzae* (Ahlburg) Cohn.

The notice provides information about the plasmid, pMStr20, used in the construction of the *A. oryzae* production strain. The plasmid contains defined fungal chromosomal DNA fragments and synthetic DNA linker sequences including a promoter sequence from an *A. niger* neutral amylase II (NA2) gene, the 5' non-translated leader sequence of an *A. nidulans* triose phosphate isomerase gene, the DNA sequence encoding the *F. oxysporum* lipase, a terminator sequence from an *A. niger* amyloglycosidase gene, an *A. nidulans* acetamidase selectable marker gene, and the *E. coli* cloning plasmid vector pUC19. The notice cites published scientific articles to support Novozymes' view that these DNA sequences are well-known, well-characterized, and commonly used.

At the request of OFAS, Novozymes provided additional information (dated June 18, 2001) on the safety of the donor strain for the lipase gene, *F. oxysporum*. Novozymes notes that this particular fungus is not generally regarded as a primary pathogen. However, specific strains of *F. oxysporum* produce toxic secondary metabolites, including fusaric acid, monoliformine, and zearalenone and have been associated with eye infections in humans. Novozymes concludes that the pathogenic and toxigenic potential of *F. oxysporum* is not a safety concern in the lipase preparation because only the coding sequence of the lipase enzyme is introduced into the production organism, *A. oryzae*. No genetic sequences involved in secondary metabolite production or pathogenic properties are introduced into *A. oryzae* that would increase its toxic potential.

The notice discusses the safety of the *A. oryzae* production strain, designated MStr115. This strain is a spontaneous mutant of strain MStr110, constructed by transformation of the host strain Jal228 with the lipase expression fragment, a purified DNA fragment from plasmid pMStr20. The production organism complies with the Organization for Economic Co-operation and Development criteria for Good Industrial Large Scale Practice microorganisms and meets the criteria for a safe production microorganism described in scientific publications and recommendations issued by international organizations. Using the Southern hybridization technique, Novozymes assessed the identity and stability of the introduced DNA and concluded that the DNA is integrated into the *A. oryzae* chromosome as expected and is not prone to genetic transfer to other organisms.

The notice describes the manufacturing process used to produce the lipase preparation as a two-step process: submerged fed-batch pure culture fermentation of the *A. oryzae* production strain and recovery (which includes purification and formulation). Novozymes follows standard industry practices and uses a quality management system that complies with the requirements of ISO 9001. In addition, the materials used in the fermentation and recovery processes are standard ingredients used by the enzyme industry; the notice cites several published articles to support this statement. The notice also lists specifications for the lipase enzyme preparation that comply with the specifications for enzyme preparations provided in the Food Chemicals Codex (4th ed., 1996) and the specifications provided by JECFA in Compendium of Food Additive Specifications, volume 2 (JECFA, 1992).

Novozymes' notice includes an unpublished summary of toxicology studies performed with the lipase preparation. These studies include a 13-week subchronic oral toxicity study in rats, an Ames mutagenicity test, and an in vitro cytogenetic test in cultured human lymphocytes. The studies showed no treatment related toxicity, induction of gene mutation, or chromosomal aberrations.

Based on the information provided by Novozymes, as well as other information available to FDA, the agency has no questions at this time regarding Novozymes' conclusion that lipase enzyme preparation obtained from *Aspergillus oryzae* carrying a recombinant gene encoding a *Fusarium oxysporum* lipase is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of this enzyme preparation. As always, it is Novozymes' continuing responsibility to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,
Alan M. Rulis, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

Links on this page:

Food

Agency Response Letter GRAS Notice No. GRN 000090

CFSAN/Office of Food Additive Safety

April 4, 2002

Jack Harris
Enzyme Technical Association
1800 Massachusetts Ave., N.W.
Second Floor
Washington, DC 20036

Re: GRAS Notice No. GRN 000090

Dear Mr. Harris:

The Food and Drug Administration (FDA) is responding to the notice, dated November 8, 2001, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on November 16, 2001, and designated it as GRAS Notice Nos. GRN 000088, GRN 000089 and GRN 000090 (as explained below).

The subject of the notice is a group of ten microbially derived enzyme preparations, i.e., carbohydrase, pectinase, protease, glucose oxidase and catalase enzyme preparations from *Aspergillus niger*, carbohydrase and protease enzyme preparations from *Aspergillus oryzae*, carbohydrase enzyme preparation from *Rhizopus oryzae*, invertase enzyme preparation from *Saccharomyces cerevisiae*, and lactase enzyme preparation from *Kluyveromyces marxianus*. The notice informs FDA of the view of the Enzyme Technical Association (ETA) that these enzyme preparations are GRAS, through common use in food, for use as enzymes in catalyzing specific reactions in the processing of food. Each of the enzyme preparations is used at levels not to exceed current good manufacturing practice. These enzyme preparations are also the subjects of a GRAS affirmation petition (GRP 3G0016) submitted by the Ad Hoc Enzyme Technical Committee (now known as ETA) to FDA in 1973 and amended a few times thereafter. In its notice, the ETA requested that FDA convert the filed GRAS affirmation petition GRP 3G0016 for these ten enzyme preparations to a GRAS notice in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)).

For administrative expediency, FDA divided ETA's GRAS notice into three separate GRAS notices. GRN 000088 includes invertase from *Saccharomyces cerevisiae*, and lactase from *Kluyveromyces marxianus*. GRN 000089 includes carbohydrase, pectinase, protease, glucose oxidase and catalase from *Aspergillus niger*. GRN 000090 includes carbohydrase and protease from *Aspergillus oryzae*, and carbohydrase from *Rhizopus oryzae*. In this letter, FDA responds to GRN 000090.

Carbohydrase enzyme preparation from *Rhizopus oryzae* is approved as a food additive for use in the production of dextrose from starch (21 CFR 173.130; 29 FR 14663; October 28, 1964). Because the agency considered that the safe use of carbohydrase enzyme preparation from *R.oryzae* was established by the food additive regulation, FDA's review of GRP 3G0016 did not include whether the data and information in GRP 3G0016 established that this safe use is generally recognized. Consistent with its view during the evaluation of GRP 3G0016, FDA did not evaluate, during its review of GRN 000090, whether carbohydrase enzyme preparation from *R. oryzae* is GRAS through experience based on common use in food.

Commercial enzyme preparations that are used in food processing typically contain an enzyme component, which catalyzes the chemical reaction that is responsible for the technical effect, as well as substances used as stabilizers, preservatives or diluents. Enzyme preparations may also contain constituents derived from the source organism and constituents derived from the manufacturing process, e.g., components of the fermentation media or the residues of processing aids. In GRP 3G0016, ETA includes data and information about the technical effect of each enzyme component, the source microorganism (i.e., *A. oryzae*), and the method of manufacture.

Identity and Technical Effect

Carbohydrase enzyme preparation from *A. oryzae* is an enzyme preparation obtained from the culture filtrate resulting from a pure culture fermentation of a non-pathogenic and non-toxicogenic strain of *A. oryzae*. The preparation contains alpha-amylase (EC 3.2.1.1) or glucoamylase (EC 3.2.1.3), which catalyzes the hydrolysis of starch or starch polysaccharides in food.

Protease enzyme preparation from *A. oryzae* is an enzyme preparation obtained from the culture filtrate resulting from a pure culture fermentation of a non-pathogenic and non-toxicogenic strain of *A. oryzae*. The preparation contains peptide hydrolases, such as alkaline proteinase (EC 3.4.21.14), aspartic proteinase (EC3.4.23.6), and neutral proteinase (EC 3.4.24.4), which catalyze the hydrolysis of proteins or polypeptides in food.

Source Microorganism and Method of Manufacture

The source microorganism for each of the enzyme preparations described in GRN 000090 is *A. oryzae*. The general taxonomy and characteristics of *A. oryzae* are described in standard compendia (Ref. 1). In GRP 3G0016, ETA includes publications that describe generally accepted microbiological techniques that are used in the manufacture of the enzyme preparations from *A. oryzae*. All microbial strains used in enzyme manufacture are started from a pure laboratory culture of *A. oryzae* and grown in a sterile liquid nutrient medium or sterile moistened semisolid medium. Generally accepted microbiological techniques are used to exclude contaminating organisms and to avoid development of substrains from within the culture itself. Although specific conditions of fermentation vary from manufacturer to manufacturer, common fermentation procedures, which have been described in the literature, are: (1) the submerged culture method, and (2) the semisolid culture method. During fermentation by either method, the pH, temperature, disappearance of certain ingredients, purity of culture, and level of enzyme activity are carefully controlled. The fermentation is harvested at the point where laboratory tests indicate that maximum production of enzyme activity has been attained.

In GRP 3G0016, ETA includes publications that show that carbohydrase and protease from *A. oryzae* are excreted into the fermentation medium. In the submerged culture method, the extracellular location of the enzyme means that no extraction step is needed, and the microorganism and other insoluble matter are removed from the fermentation medium by filtering or centrifuging. In the semisolid culture method, the enzyme is extracted either directly from the moist material, or later after the culture mass has been dried, followed by further processing steps such as clarification, evaporation, precipitation, drying and grinding.

Each of the enzyme preparations described in GRN 000090 meets the general and additional requirements in the monograph on enzyme preparations in the Food Chemicals Codex, 4th ed. (1996), pp. 128-135.

Evidence of Common Use in Food Before 1958

The statutory basis for ETA's determination that carbohydrase and protease enzyme preparations from *A. oryzae* are GRAS for their intended use is through experience based on common use in food before 1958. Under 21 CFR 170.30(c)(1), general recognition of safety through experience based on common use in food is based solely on food use of the substance prior to January 1, 1958, and ordinarily is based upon generally available data and information.

In GRP 3G0016, ETA includes an article, published in 1952 (Ref. 2), that states that fungal carbohdrase was used in brewing and baking and in the production of corn syrup. Although the article published in 1952 does not specify *A. oryzae* as the fungal source of carbohydrase, ETA also includes an article, published in 1957 (Ref. 3), that states that fungal carbohydrase was used in starch conversion, baking, brewing, syrup production and production of other food products and specifically identifies *A. oryzae* as the source fungus. ETA also includes another article, published in 1958 (Ref. 4), that states that fungal carbohydrase was used in starch conversion, baking, and processing cereal products, fruit juices and other food products and identifies the source fungus as *A. oryzae*.

In GRP 3G0016, ETA includes an article, published in 1952 (Ref. 2), that states that fungal protease was used in brewing and baking. ETA also includes an article, published in 1957 (Ref. 3), that states that fungal protease was used in baking and beer and ale production. Although these articles do not specify *A. oryzae* as the fungal source of protease, ETA also includes another article, published in 1957 (Ref. 5), that states that fungal protease was used in the chilling of beer, meat tenderizing, and baking and specifically identifies *A. oryzae* as the source fungus. ETA also includes an article, published in 1958 (Ref. 4), that states that fungal protease was used in baking, processing cereal products, manufacturing beer and ale, and meat tenderizing and identifies the source fungus for the protease enzyme preparation as *A. oryzae*.

Conclusions

Based on the information provided by ETA, as well as the information in GRP 3G0016 and other information available to FDA, the agency has no questions at this time regarding ETA's conclusion that carbohydrase and protease enzyme preparations from *A. oryzae* are GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of these enzyme preparations. As always, it is the continuing responsibility of each manufacturer to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

Consultation with the Food Safety and Inspection Service of the U. S. Dept. of Agriculture

Because the protease enzyme preparation from *Aspergillus oryzae* would be used to tenderize meat, FDA consulted with the Labeling and Consumer Protection Staff of the Food Safety and Inspection Service of the United States Department of Agriculture (FSIS) during its evaluation of GRN 000090. FSIS has determined that ETA has not provided any data to support the suitability of protease enzyme preparation from *A. oryzae* for use in meat and poultry products. Suitability relates to the effectiveness of an ingredient in performing the intended purpose of use and the assurance that the conditions of use will not result in an adulterated product or one that misleads consumers. FSIS concludes that ETA needs to provide data that establish that the protease enzyme preparation is being used at the lowest level necessary to achieve the intended technical effect in the specific meat and poultry products to which application is desired. FSIS requests that ETA be advised to seek regulatory guidance from FSIS about the use of protease enzyme preparation from *A. oryzae* in meat and poultry products. ETA should direct this inquiry to Dr. Robert Post, Director, Labeling and Consumer Protection Staff, Office of Policy, Program Development and Evaluation, Food Safety and Inspection Service, 300 12th Street, SW, Room 602, Washington, DC 20250-3700. The telephone number of his office is (202) 205-0279 and the telefax number is (202)205-3625[1].

In accordance with the interim policy discussed in the GRAS proposal (62 FR 18938 at 18954), FDA has not committed any resources to review of GRP 3G0016 since November 16, 2001, the date that we received your conversion request.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in ETA's notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,

Alan M. Rulis, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

cc: Dr. Robert Post, Director
Labeling and Consumer Protection Staff
Office of Policy, Program Development and Evaluation
Food Safety and Inspection Service
300 12th Street, SW, Room 602
Washington, DC 20250-3700

References

1. Monographs on "*Aspergillus niger* Group" and "*Aspergillus flavus* Group", in Raper, K. B. and Fennel, D. I., "The genus *Aspergillus*," Williams and Wilkins Company, Baltimore, Maryland, pp. 293-334 and 357-404 (1965).

2. Reed, G., "Industrial enzymes - Now speed natural processes," *Food Engineering*, **24**: pp. 105-109 (1952).

3. Underkofler, L.A. and W. J. Ferracone, "Commercial enzymes - Potent catalyzers that promote quality," *Food Engineering*, **29**: pp. 123-133 (1957).

4. Underkofler, L.A., R.R. Barton, and S.S. Rennet, "Microbiological process report - Production of microbial enzymes and their applications," *Applied Microbiology*, **6**: pp. 212-221 (1958).

5. Kirk, R.E. and Othmer, D.F. (eds.), "Enzymes, Industrial" in *Encyclopedia of Chemical Technology*, First Supplement Volume, Interstate Cyclopedia, Inc., New York, NY (1957).

1420

[1]FSIS also informed FDA that ETA has not provided any data to support the suitability of carbohydrase enzyme preparation from *A. oryzae* for use in meat and poultry products. In its notice, ETA does not describe any uses for this enzyme preparation in meat and poultry products.

Links on this page:

Food

Agency Response Letter GRAS Notice No. GRN 000103

OFAS/Office of Food Additive Safety
August 19, 2002

Lori Gregg
Novozymes North America, Inc.
77 Perry Chapel Church Road
Box 576
Franklinton, NC 27525

Re: GRAS Notice No. GRN 000103

Dear Ms. Gregg:

The Food and Drug Administration (FDA) is responding to the notice, dated March 25, 2002, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on March 27, 2002, filed it on April 24, 2002, and designated it as GRAS Notice No. GRN 000103.

The subject of the notice is a lipase enzyme preparation from *Aspergillus oryzae* carrying a gene constructed from a modified *Thermomyces lanuginosus* lipase gene and a portion of the *Fusarium oxysporum* lipase gene. The notice informs FDA of the view of Novozymes North America, Inc. (Novozymes) that the lipase enzyme preparation is GRAS, through scientific procedures, for use as a processing aid in bakery products, egg yolks, whole eggs, and fats and oils at minimum levels necessary in accordance with good manufacturing practice. Novozymes estimates that the lipase enzyme preparation would be used at the following levels: 0.5 grams (g) per kilogram (kg) flour, 1 kg to produce 250 kg of modified egg yolk/whole egg, 1 kg to produce 400 kg of modified lecithin, and 1 kg to produce 20 tons of de-gummed oil.

Commercial enzyme preparations that are used in food processing typically contain an enzyme component, which catalyzes the chemical reaction that is responsible for its technical effect, as well as substances used as stabilizers, preservatives or diluents. Enzyme preparations may also contain constituents derived from the production organism and constituents derived from the manufacturing process, e.g., components of the fermentation media or the residues of processing aids. Novozymes' notice provides information about each of these components of the lipase enzyme preparation from *A. oryzae.*

In assessing the safety of the enzyme itself, Novozymes discusses the history of safe use of lipases in food processing. Novozymes cites published articles reporting the use of microbial lipases in food production since 1952. Novozymes describes specific lipase enzyme preparations that have been used in food, including the following:

- Animal lipase, which FDA affirmed as GRAS for use as an enzyme to hydrolyze fatty acid glycerides (21 CFR 184.1415)
- Lipase enzyme preparation derived from *Rhizopus niveus*, which FDA affirmed as GRAS for use as an enzyme for the interesterification of fats and oils (21 CFR 184.1420)
- Esterase-lipase derived from *Mucor miehei* (now known as *Rhizomucor miehei*), which FDA approved for use as a flavor enhancer (21 CFR 173.140)
- Lipase enzyme preparation from *A. oryzae* carrying a gene encoding lipase from *T. lanuginosus*, which Novozymes determined to be GRAS for use in dough, baked goods, and the fats and oil industry (GRAS Notice No. GRN 000043)
- Lipase enzyme preparation from *A. oryzae* carrying a recombinant gene encoding lipase from *Fusarium oxysporum*, which Novozymes determined to be GRAS for use as a processing aid in the modification of fats and oils, and in baking applications (GRAS Notice No. GRN 000075)
- Lipase enzyme preparation from *Candida rugosa*, which Amano Enzymes, Inc. determined to be GRAS for use in the modification of fats and oils (GRAS Notice No. GRN 000081).

Novozymes describes the lipase enzyme that is the subject of its notice as a 339 amino acid hybrid protein derived from two other lipase enzymes that are used in food. The N-terminal 284 amino acid residues are derived from the *T. lanuginosus* lipase, with three amino acid substitutions, and the remainder of the hybrid protein (amino acids 285-339) is derived from the *F. oxysporum* lipase. Novozymes notes that the lipases used to construct the hybrid protein have been the subject of previous GRAS notices (i.e. GRN 000043 for the lipase from *T. lanuginosus* and GRN 000075 for the lipase from *F. oxysporum*).

Novozymes describes the catalytic activity of the lipase as hydrolyzing ester bonds in triglycerides, resulting in the formation of free fatty acids, diglycerides, monoglycerides, and glycerol. The enzyme also catalyzes the hydrolysis of the sn-1 ester bond of diacylphospholipids to form 2-acyl-1-lysophospholipid and a free fatty acid. Compared to other lipases, the subject lipase has a higher preference for long chain fatty acids in position 1 in the substrate, has increased activity towards phospholipids, and has a higher efficacy when used for the modification of egg, hydrolysis of lecithin, and vegetable oil degumming. The lipase enzyme is identified by the following classification numbers: Chemical Abstracts Service Registry No. 9001-62-1, Enzyme Commission No. 3.1.1.3, European Inventory of Existing Commercial Chemical Substances No. 232-619-9.

In assessing the safety of the host microorganism, *A. oryzae* strain BECh2, Novozymes describes the host as a derivative of a well-known industrial production strain of *A. oryzae* (Ahlburg) Cohn. Novozymes obtained the strain, designated IFO 4177 or A 1560, from the Institute for Fermentation, Osaka, Japan. Novozymes considers *A. oryzae* to be nontoxigenic and nonpathogenic based on published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients. Novozymes describes the donor microorganisms, *T. lanuginosus* and *F. oxysporum*, as nonpathogenic, nontoxigenic fungi.

Novozymes provides information about the components of the lipase expression plasmid pCaHj559 that was introduced into the host strain BECh2 by transformation. Novozymes cites published scientific articles to support its view that all of the DNA sequences that were used in the construction of the production strain are well-known, well-characterized, and commonly used. Novozymes assessed the identity and stability of the introduced DNA using the technique of Southern hybridization and concluded that the DNA is integrated into the *A. oryzae* chromosome as expected and is not prone to genetic transfer to other organisms. The resulting production strain meets the criteria for Good Industrial Large-Scale Practice published in the Organization for Economic Co-operation and Development's 1992 report entitled "Safety Considerations for Biotechnology."

Novozymes describes the manufacturing process for lipase enzyme preparation, which is produced by submerged, fed-batch pure culture fermentation of the *A. oryzae* production strain. The enzyme is secreted into the fermentation broth and separated from the cells using filtration. The enzyme preparation is concentrated

by ultrafiltration and evaporation. The enzyme preparation is then preserved and stabilized with the addition of sodium chloride. Novozymes follows standard industry practices and uses a quality management system that complies with the requirements of ISO 9001. Novozymes cites several published sources to support the conclusion that the production and control methods used are generally accepted methods that are commonly used for the production of microbial enzyme preparations.

Novozymes describes unpublished toxicity studies performed on its lipase preparation. The test article for these studies was prepared according to the standard production method, except that stabilization and standardization were omitted. These studies include two-week and 13-week oral gavage studies in rats and tests for genetic toxicity, including an Ames test and a chromosome aberration test with human lymphocytes. Novozymes concludes that these toxicity studies showed no treatment related toxicity and no induction of gene mutation in bacteria or chromosomal aberrations in cultured human blood lymphocytes.

Based on the information provided by Novozymes, as well as other information available to FDA, the agency has no questions at this time regarding Novozymes' conclusion that lipase enzyme preparation from *Aspergillus oryzae* carrying a gene constructed from a modified *Thermomyces lanuginosus* lipase gene and a portion of the *Fusarium oxysporum* lipase gene is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of this lipase enzyme preparation. As always, it is the continuing responsibility of Novozymes to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in the notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,
Alan M. Rulis, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

Links on this page:

Food

Agency Response Letter GRAS Notice No. GRN 000106

CFSAN/Office of Food Additive Safety
October 3, 2002

Lori Gregg
Novozymes North America, Inc.
77 Perry Chapel Church Road
Box 576
Franklinton, NC 27525

Re: GRAS Notice No. GRN 000106

Dear Ms. Gregg:

The Food and Drug Administration (FDA) is responding to the notice, dated April 29, 2002, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on April 29, 2002, filed it on April 29, 2002, and designated it as GRAS Notice No. GRN 000106.

The subject of the notice is a glucose oxidase enzyme preparation from *Aspergillus oryzae* carrying the gene encoding glucose oxidase from *A. niger*. The notice informs FDA of the view of Novozymes North America, Inc. (Novozymes) that the glucose oxidase enzyme preparation is GRAS, through scientific procedures, for use in baking applications as an enzyme, at minimum levels necessary in accordance with good manufacturing practice. Novozymes estimates that the glucose oxidase enzyme preparation would be used at the following levels: 0.25-5 g per 100 kilogram (kg) flour (corresponding to 25-500 glucose oxidase units (GODU)/kg flour).

In an amendment dated July 19, 2002, Novozymes notes that there are additional applications for the glucose oxidase enzyme preparation in cheese, beer, carbonated beverages, and fruit juice. These additional uses would require the use of a catalase enzyme preparation in combination with the glucose oxidase enzyme preparation. In an amendment dated August 28, 2002, Novozymes further notes that the estimated use levels for the glucose oxidase enzyme preparation in these other applications would fall within the range estimated for the baking applications.

Commercial enzyme preparations that are used in food processing typically contain an enzyme component, which catalyzes the chemical reaction that is responsible for its technical effect, as well as substances used as stabilizers, preservatives or diluents. Enzyme preparations may also contain constituents derived from the production organism and constituents derived from the manufacturing process, e.g., components of the fermentation media or the residues of processing aids. Novozymes' notice provides information about each of these components of the glucose oxidase enzyme preparation from *A. oryzae*.

Novozymes describes the glucose oxidase enzyme that is the subject of its notice as a protein transcribed and translated from the *A. niger* gene coding for glucose oxidase. The glucose oxidase encoding sequence from *A. niger* was incorporated into the DNA of the *A. oryzae* production strain without modification. Novozymes states that the subject glucose oxidase is catalytically and functionally equivalent to the glucose oxidase from *A. niger*. Novozymes concludes that the *A. niger* glucose oxidase expressed in *A. oryzae*v is the same enzyme as the glucose oxidase from *A. niger* that has been in use in food production since 1957.

Novozymes identifies the glucose oxidase by the following classification numbers: EC 1.1.3.4, CAS Registry No. 9001-37-0. Novozymes describes the enzymatic activity of the glucose oxidase as catalyzing the oxidation of beta-D-glucose to hydrogen peroxide and D-glucono-1,5-lactone, which spontaneously hydrolyzes to gluconic acid. In an amendment dated June 21, 2002, Novozymes notes that the primary intended use of this glucose oxidase is in baking applications to modify gluten. The hydrogen peroxide produced from the enzyme-catalyzed reaction oxidizes free sulfhydryl groups of gluten protein. In an amendment dated July 19, 2002, Novozymes further notes that glucose oxidase may be used in other non-baking applications, such as in the manufacture of cheese, beer, carbonated beverages, and fruit juice. For these applications, glucose oxidase would be used in combination with a catalase enzyme preparation to remove oxygen from the food product. Glucose oxidase would generate hydrogen peroxide, which would be removed by the action of catalase to yield oxygen and water.

In assessing the safety of the enzyme itself, Novozymes discusses the history of safe use of glucose oxidases in food processing. Novozymes cites published articles reporting the use of fungal glucose oxidases in food production since 1957. Novozymes describes specific glucose oxidase enzyme preparations that have been used in food, including glucose oxidase from *A. niger*. This preparation is one of several enzymes in a GRAS affirmation petition (GRP 3G0016), which was filed in 1973. In GRP 3G0016, the petitioner requested that FDA affirm GRAS status through experience based on common use in food, as evidenced by published articles that discussed the pre-1958 uses of the enzymes in the petition. In 2001, the petitioner requested that FDA partially convert GRP 3G0016 to a GRAS notice. As a result, the glucose oxidase from *A. niger* was one of several enzymes that were the subject of GRAS Notice No. GRN 000089. The uses of glucose oxidase enzyme preparation described in the articles originally provided in GRP 3G0016 focused on uses of glucose oxidase enzyme preparation in combination with catalase to remove glucose or oxygen from food.

In assessing the safety of the host microorganism, *A. oryzae* strain BECh2, Novozymes describes the host as a derivative of a well-known industrial production strain of *A. oryzae* (Ahlburg) Cohn. Novozymes obtained the strain, designated IFO 4177 or A 1560, from the Institute for Fermentation, Osaka, Japan. Novozymes considers *A. oryzae* to be nontoxigenic and nonpathogenic based on published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients.

Novozymes provides information about the components of the glucose oxidase expression plasmid pHUda107 that was introduced into the host strain BECh2 by transformation. Novozymes cites published scientific articles to support its view that all of the DNA sequences that were used in the construction of the production strain are well-known, well-characterized, and commonly used. Novozymes assessed the identity and stability of the introduced DNA using the technique of Southern hybridization and concluded that the DNA is integrated into the *A. oryzae* chromosome as expected and is not prone to genetic transfer to other organisms. The resulting production strain meets the criteria for Good Industrial Large-Scale Practice published in the Organization for Economic Co-operation and Development's 1992 report entitled "Safety Considerations for Biotechnology."

Novozymes describes the manufacturing process for glucose oxidase preparation, which is produced by submerged, fed-batch pure culture fermentation of the *A. oryzae* production strain. The enzyme is secreted into the fermentation broth and separated from the cells using filtration. The enzyme preparation is concentrated by ultrafiltration and evaporation. The enzyme preparation is then preserved and stabilized with the addition of sodium chloride. Novozymes follows standard industry practices and uses a quality management system that complies with the requirements of ISO 9001. Novozymes cites several published sources to support the conclusion that the production and control methods used are generally accepted methods that are commonly used for the production of microbial enzyme preparations.

Novozymes describes unpublished toxicity studies performed on its glucose oxidase preparation. The test article for these studies was a glucose oxidase preparation known as Gluzyme™. Novozymes describes Gluzyme™ as a liquid enzyme concentrate, predominately with glucose oxidase activity, but also a minor catalase side activity. Gluzyme™ is produced by submerged fermentation of a strain of *A. oryzae* expressing the glucose oxidase gene from *A. niger*. These studies include a 13-week oral gavage study in rats and tests for genetic toxicity, including an Ames test and a chromosome aberration test with human lymphocytes. Novozymes concludes that these toxicity studies showed no treatment related toxicity and no induction of gene mutation in bacteria or chromosomal aberrations in cultured human blood lymphocytes.

Based on the information provided by Novozymes, as well as other information available to FDA, the agency has no questions at this time regarding Novozymes' conclusion that glucose oxidase enzyme preparation from *Aspergillus oryzae* carrying the gene encoding glucose oxidase from *Aspergillus niger* is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of this glucose oxidase preparation. As always, it is the continuing responsibility of Novozymes to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in the notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,
Alan M. Rulis, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

The location of this letter on FDA's website as described in the text is out of date. To view or obtain an electronic copy of the text of the letter, follow the hyperlinks from the "Food" topic on the FDA home page at http://www.fda.gov to the "Food Ingredients and Packaging" section to the "Generally Recognized as Safe (GRAS)" page where the GRAS Inventory is listed.

Links on this page:

1425

Food

Agency Response Letter GRAS Notice No. GRN 000113

CFSAN/Office of Food Additive Safety
January 27, 2003

Jack Harris
Enzyme Technical Association
c/o Gary L. Yingling
1800 Massachusetts Avenue, NW, Second Floor
Washington, DC 20036-1800

Re: GRAS Notice No. GRN 000113

Dear Dr. Harris:

The Food and Drug Administration (FDA) is responding to your notice, dated August 5, 2002, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on August 15, 2002, filed it on August 21, 2002, and designated it as GRAS Notice No. GRN 000113.

The subject of the notice is lipase enzyme preparation from a nontoxigenic and nonpathogenic strain of *Aspergillus oryzae*. The notice informs FDA of the view of the Enzyme Technical Association (ETA) that the lipase enzyme preparation is GRAS, through scientific procedures, for use as an enzyme in dairy-based flavoring preparations, cheeses, liquid and dried egg white, bread, flour, bakery products not subject to a standard of identity, modified triglycerides, hydrolyzed lecithin, edible fats and oils, and modified egg yolk. The lipase enzyme preparation is used in food at minimum levels necessary to achieve the intended technical effect. This enzyme preparation is also the subject of a GRAS affirmation petition (GRP 3G0016) submitted to FDA in 1973 by the Ad Hoc Enzyme Technical Committee (now known as ETA) and amended a few times thereafter. In its notice, the ETA requested that FDA convert the filed GRAS affirmation petition GRP 3G0016 for this enzyme preparation to a GRAS notice in accordance with the agency's GRAS proposal.

Commercial enzyme preparations that are used in food processing typically contain an enzyme component, which catalyzes the chemical reaction that is responsible for its technical effect, as well as substances used as stabilizers, preservatives or diluents. Enzyme preparations may also contain constituents that derive from the source organism and constituents that derive from the manufacturing process, e.g., components of the fermentation media or the residues of processing aids. ETA's notice provides information about each of these components of the lipase enzyme preparation from *A. oryzae*.

In assessing the safety of the enzyme itself, ETA discusses the history of safe use of lipases in food processing. ETA cites published articles reporting the use of microbial lipases in food production since 1952. ETA describes specific lipase enzyme preparations that have been used in food, including the following:

- Lipase from *A. niger*, which FDA has included in its generally available document entitled "Partial List of Enzyme Preparations That Are Used in Foods," (available on the Internet at http://www.cfsan.fda.gov/~dms/opa-enzy.html)
- Animal lipase, which FDA affirmed as GRAS for use as an enzyme to hydrolyze fatty acid glycerides (21 CFR 184.1415)
- Lipase enzyme preparation derived from *Rhizopus niveus*, which FDA affirmed as GRAS for use as an enzyme for the interesterification of fats and oils (21 CFR 184.1420)
- Esterase-lipase derived from *Mucor miehei* (now known as *Rhizomucor miehei*), which FDA approved for use as a flavor enhancer (21 CFR 173.140)
- Immobilized esterase-lipase enzyme preparation from *M. miehei*, which FDA filed as GRAS affirmation petition GRP 7G0323 in 1989 (54 FR 9565)
- Lipase enzyme preparation from *A. oryzae* carrying a gene encoding lipase from *Thermomyces lanuginosus*, which Novozymes North America, Inc. (Novozymes) determined to be GRAS for use in dough, baked goods, and the fats and oil industry (GRAS Notice No. GRN 000043)
- Lipase enzyme preparation from *A. oryzae* carrying a recombinant gene encoding lipase from *Fusarium oxysporum*, which Novozymes determined to be GRAS for use as a processing aid in the modification of fats and oils, and in baking applications (GRAS Notice No. GRN 000075)
- Lipase enzyme preparation from *A. oryzae* carrying a recombinant gene encoding a modified lipase gene from *T. lanuginosus* and a portion of the *F. oxysporum* lipase gene, which Novozymes determined to be GRAS for use as a processing aid in the modification of fats and oils (GRAS Notice No. GRN 000103).

ETA describes the catalytic activity of the lipase as hydrolyzing ester bonds in triglycerides, resulting in the formation of free fatty acids, diglycerides, and monoglycerides. The hydrolysis reaction is reversible. Therefore, the enzyme can also catalyze the synthesis of ester bonds under appropriate conditions. The systematic name of lipase is triacylglycerol acylhydrolase and the Enzyme Commission number is 3.1.1.3.

In assessing the safety of the production organism, *A. oryzae*, ETA cites scientific review articles in support of its view that the safety of the production organism is the prime consideration in assessing the safety of an enzyme preparation intended for use in food. ETA also cites a publication of the International Food Biotechnology Council which concludes that if the production microorganism is nontoxigenic and nonpathogenic and the manufacturing process is conducted using current Good Manufacturing Practices, the food or food ingredient produced from that microorganism is safe to consume. ETA considers *A. oryzae* to be nontoxigenic and nonpathogenic based on published criteria for safety assessment and a long history of safe use documented in numerous scientific publications.

ETA describes the manufacturing process for the lipase enzyme preparation, which is produced from a nontoxigenic and nonpathogenic strain of *A. oryzae* by pure culture fermentation. The fermentation procedure is carried out by submerged culture, solid culture, or a semi-solid culture method. The controlled process is monitored for contamination with other microorganisms and if contamination is detected, the fermentation is terminated and the batch is rejected. After fermentation, the lipase is recovered, purified and concentrated. The enzyme is formulated as either a liquid or dry product with stabilizers, diluents, and/or preservatives added to the formulated enzyme preparation. ETA states that the fermentation media and all substances added to the enzyme preparation are suitable for general use in food. The enzyme preparation meets the general and additional requirements for enzyme preparations in the monograph on enzyme preparations in the Food Chemicals Codex, 4th edition (1996).

Based on the information provided by ETA, as well as the information in GRP 3G0016 and other information available to FDA, the agency has no questions at this time regarding ETA's conclusion that the lipase enzyme preparation from *A. oryzae* is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of this lipase enzyme preparation. As always, it is the continuing responsibility of each manufacturer to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with the interim policy discussed in the GRAS proposal (62 FR 18938 at 18954), FDA has not committed any resources to review of GRP 3G0016 since the date that we received ETA's conversion request.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in the notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,
/s/
Alan M. Rulis, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

The location of this letter on FDA's website as described in the text is out of date. To view or obtain an electronic copy of the text of the letter, follow the hyperlinks from the "Food" topic on the FDA home page at http://www.fda.gov to the "Food Ingredients and Packaging" section to the "Generally Recognized as Safe (GRAS)" page where the GRAS Inventory is listed.

Links on this page:

Food

Agency Response Letter GRAS Notice No. GRN 000122

CFSAN/Office of Food Additive Safety
July 18, 2003

Denise Bernstein
Novozymes North America, Inc.
77 Perry Chapel Church Road
P.O. Box 576
Franklinton, North Carolina 27525

Re: GRAS Notice No. GRN 000122

Dear Ms. Bernstein:

The Food and Drug Administration (FDA) is responding to the notice, dated January 9, 2003, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on January 17, 2003, filed it on January 22, 2003, and designated it as GRAS Notice No. GRN 000122.

The subject of the notice is laccase enzyme preparation from *Aspergillus oryzae* expressing the gene encoding a laccase from *Myceliophthora thermophila*. The notice informs FDA of the view of Novozymes North America, Inc. (Novozymes) that the laccase enzyme preparation is GRAS, through scientific procedures, for use as an enzyme in breath-freshening products (such as breath mints and chewing gum) and other food products at minimum levels necessary in accordance with good manufacturing practice. Novozymes estimates that this use of laccase enzyme preparation as a direct food ingredient would result in the consumption of up to approximately 14 milligrams per person per day of the total organic solids present in the laccase enzyme preparation.

Commercial enzyme preparations that are used in food typically contain an enzyme component, which catalyzes the chemical reaction that is responsible for its technical effect, as well as substances used as stabilizers, preservatives or diluents. Enzyme preparations may also contain constituents derived from the production organism and constituents derived from the manufacturing process, e.g., components of the fermentation media or the residues of processing aids. Novozymes' notice provides information about each of these components of the laccase enzyme preparation from *A. oryzae*.

Novozymes describes generally available information about the identity and technical effect of laccase. The subject laccase is an 85 kDa protein, with three internal disulfide bonds and four copper atoms. Novozymes identifies the laccase enzyme by the following classification numbers: EC 1.10.3.2 and CAS Registry No. 80498-15-3. Laccase catalyzes the oxygen-dependent oxidation of phenolic substrates such as o-diphenols and p-diphenols to form their corresponding quinones and water. In the applications described in the notice, these quinone reaction products react non-enzymatically with odor causing compounds such as sulfides, thiols, and amines to eliminate odors in the oral cavity. Novozymes describes a published article that discusses the characterization of the gene encoding the laccase of *M. thermophila* and the subsequent analysis of the recombinant laccase as expressed in *A. oryzae*.

Novozymes cites published articles reporting the presence of laccase enzymes and phenolic compounds as naturally occurring components commonly found in human food sources such as fruits, vegetables, fungi, herbs, spices, teas, and coffee. Novozymes also notes that interactions involving laccase and phenolic substrates are responsible for the browning of freshly peeled or bruised fruit and vegetables. The browning reaction plays a role in the curing of tea and coffee. Novozymes describes the use of laccases in beverage production. In Denmark, the laccase enzyme is approved for use in brewing beer, where it is applied during the mashing process to prevent the formation of an off-flavor compound, trans-2-nonenal. Laccase has also been immobilized on a copper-chelate carrier and used to remove phenols from white grape must during clarification of wine.

Novozymes describes the host microorganism, *A. oryzae* strain How B711, as a derivative of a well-known industrial production strain of *A. oryzae* (Ahlburg) Cohn, which Novozymes obtained from the Institute for Fermentation, Osaka, Japan.[(1)] Novozymes considers *A. oryzae* to be nontoxigenic and nonpathogenic based on published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients.

Novozymes describes the development of its bioengineered production strain as a three-phase process: construction of a host strain with the necessary genetic background for subsequent genetic manipulations, transformation of the host strain with the laccase gene, and mutagenesis of the transformed host strain to obtain a high-yielding laccase production strain (designated Mt-3). Novozymes cites published scientific articles to support its view that all of the DNA sequences that were used in the construction of the production strain are well-known, well-characterized, and commonly used. Novozymes assessed the identity and stability of the introduced DNA using the technique of genomic DNA hybridization and concluded that the DNA is integrated into the *A. oryzae* chromosome as expected and is not prone to genetic transfer to other organisms. The resulting production strain meets the criteria for Good Industrial Large-Scale Practice published in the Organization for Economic Co-operation and Development's 1992 report entitled "Safety Considerations for Biotechnology."

Novozymes describes the manufacturing process for the laccase enzyme preparation, which is produced by submerged, fed-batch pure culture fermentation of the *A. oryzae* production strain. The enzyme is secreted into the fermentation broth and separated from the cells using filtration. The enzyme preparation is concentrated by ultrafiltration and evaporation. The enzyme preparation is then preserved and stabilized with the addition of sodium chloride. Novozymes follows standard industry practices and uses a quality management system that complies with the requirements of ISO 9001. Novozymes cites several published sources to support the conclusion that the production and control methods used are generally accepted methods that are commonly used for the production of microbial enzyme preparations. Novozymes states that the three mycotoxins reported to be made by some strains of *A. oryzae* (i.e., kojic acid, cyclopiazonic acid, and beta-nitropropionic acid) were not detected in four different batches of laccase preparation. Novozymes states that the enzyme preparation complies with the general and additional requirements for enzyme preparations set forth in the Food Chemicals Codex (4th ed., 1996) and the specifications established by the Joint Food and Agriculture Organization/World Health Organization's (FAO/WHO) Expert Committee on Food Additives (Compendium of Food Additive Specifications, Volume 2, Annex 1, Food and Agriculture Organization of the United Nations, 1992 as supplemented in Appendix B to Annex 1, FAO Food and Nutrition Paper 52, Addendum 6, 1998).

Novozymes includes in the notice a published summary of toxicological studies performed on the laccase preparation produced as described in the notice, except that stabilization and standardization were omitted. Novozymes also includes in the notice an unpublished summary of the same studies. These studies include a two-week and 13-week oral gavage study in rats, acute inhalation and acute dermal toxicity studies in rats, acute skin and acute eye irritation studies in rats, human skin sensitization test, and tests for genetic toxicity, including an Ames test and a chromosome aberration test with human lymphocytes. Novozymes concludes that these toxicity studies showed no treatment related toxicity and no induction of gene mutation in bacteria or chromosomal aberrations in cultured human blood lymphocytes.

Non-food uses of laccase enzyme preparation

In its notice, Novozymes provides use levels for non-food items such as toothpaste and mouthwash. Under proposed 21 CFR 170.36, FDA evaluates the view of a notifier that a particular use or uses of a substance in food is safe and that this safety is generally recognized by experts qualified by scientific training and experience to evaluate the safety of substances added to food. In its evaluation of GRN 000122, FDA did not evaluate the use of laccase enzyme in non-food items.

Conclusions

Based on the information provided by Novozymes, as well as other information available to FDA, the agency has no questions at this time regarding Novozymes' conclusion that laccase enzyme preparation produced by *A. oryzae* expressing the gene encoding laccase from *M. thermophila* is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of this laccase preparation. As always, it is the continuing responsibility of Novozymes to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in the notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Laura M. Tarantino, Ph.D.
Acting Director
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

---

[1]The Institute for Fermentation, Osaka designates *A. oryzae* (Ahlburg) Cohn as IFO 4177 or A1560.

Links on this page:

1429

Food

Agency Response Letter GRAS Notice No. GRN 000142

CFSAN/Office of Food Additive Safety

June 23, 2004

Lori Gregg
Novozymes North America, Inc.
77 Perry Chapel Church Road
P.O. Box 576
Franklinton, NC 27525

Re: GRAS Notice No. GRN 000142

Dear Ms. Gregg:

The Food and Drug Administration (FDA) is responding to the notice, dated December 18, 2003, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on December 24, 2003, filed it on December 29, 2003, and designated it as GRAS Notice No. GRN 000142. We received additional information regarding this notice on May 7, 2004.

The subject of the notice is phospholipase A1 (PLA1) enzyme preparation from *Aspergillus oryzae* expressing a gene encoding a PLA1 from *Fusarium venenatum*. For the purposes of this letter, this notified substance will be referred to as PLA1 enzyme preparation. The notice informs FDA of the view of Novozymes North America Inc. (Novozymes) that PLA1 enzyme preparation is GRAS, through scientific procedures, for use as an enzyme in cheese manufacturing at levels up to 5 grams of enzyme preparation per kilogram of milkfat. Novozymes estimates that this use of PLA1 enzyme preparation as a direct food ingredient would result in an estimated daily intake (EDI) of 0.7 milligrams per person per day of the total organic solids present in the PLA1 enzyme preparation.

Commercial enzyme preparations that are used in food processing typically contain an enzyme component, which catalyzes the chemical reaction that is responsible for its technical effect, as well as substances used as stabilizers, preservatives or diluents. Enzyme preparations may also contain constituents derived from the source organism and constituents derived from the manufacturing process, e.g., components of the fermentation media or the residues of processing aids. Novozymes' notice provides information about each of these components of the PLA1 enzyme preparation.

Novozymes describes generally available information about the identity and technical effect of PLA1, as well as specific information about the identity and activity of the PLA1 enzyme preparation from *A. oryzae* that is the subject of GRN 000142. Phospholipases are classified as hydrolases and PLA1 specifically hydrolyzes the *sn*-1 ester bond of diacylphospholipids to form 2-acyl-1-lysophospholipid and a free fatty acid. Phospholipase A1 is also known as phosphatidylcholine 1-acylhydrolase and is identified by the following classification numbers: Enzyme Commission number 3.1.1.32 and Chemical Abstracts Service Registry Number 9043-29-2. Phospholipase A1 enzyme preparation is intended for use as an enzyme in cheesemaking to produce modified phospholipids in cheesemilk with improved emulsification properties.

Novozymes describes published information about the presence of PLA1 as a naturally-occurring component of animal and plant tissues. Novozymes notes that PLA1 has been found in cells and tissues of various organisms, including animal pancreas and small intestines. Novozymes notes that animal-derived pancreatic lipases have been affirmed as GRAS substances (21 CFR 184.1415). Novozymes concludes that PLA1, because it is found in cells and tissues that are consumed by man, should be digested like any other protein in food.

Novozymes describes the host microorganism, *A. oryzae* strain BECh2, that is used in the construction of the PLA1 production strain PFJo142 as a derivative of a well-known industrial production strain of *A. oryzae* (Ahlburg) Cohn. Novozymes obtained the strain, which is designated as IFO 4177(A 1560), from the Institute for Fermentation, Osaka, Japan. Novozymes considers *A. oryzae* to be nontoxigenic and nonpathogenic based on published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients.

Novozymes notes that the donor organism for the PLA1 gene, *F. venenatum*, is a saprophytic fungus found in soil, and is known to occur on several food crops (hops, potato, spinach, and corn). Novozymes also notes that *F. venenatum* is not considered to be a human pathogen. Novozymes acknowledges that *F. venenatum* is known to produce secondary metabolites such as trichothecences, culmorins, enniatins and fusarins. Batch analyses of the enzyme for secondary metabolites were provided and none were detected at specified detection limits. Novozymes also confirmed that no unintended *F. venenatum* coding sequences, including those related to secondary metabolite production, were introduced into the *A. oryzae* production strain.

Novozymes provides information about the components of the phospholipase A1 expression plasmid pPFJo142 that was introduced into the host *A. oryzae* strain BECh2 by plasmid transformation. Novozymes cites published scientific articles to support its view that all of the DNA sequences that were used in the construction of the production strain are well-known, well-characterized, and commonly used.

Novozymes assessed the identity and stability of the introduced DNA using Southern hybridization and concluded that the DNA is integrated into the *A. oryzae* chromosome as expected and is not prone to genetic transfer to other organisms. The resulting pPFJo142/BECh2 *A. oryzae* production strain meets the criteria for Good Industrial Large-Scale Practice published in the Organization for Economic Co-operation and Development's 1992 report entitled "Safety Considerations for Biotechnology."

Novozymes describes the manufacturing process for PLA1 enzyme preparation, which is produced by a submerged, fed-batch pure culture fermentation of the *A. oryzae* production strain. Each fermentation is initiated with a lyophilized stock culture of the production organism. The enzyme is secreted into the fermentation broth. When the fermentation is completed, the broth is separated from the cells using vacuum drum filtration. The enzyme is concentrated by ultrafiltration and/or evaporation, followed by filtration. The resulting enzyme is preserved and stabilized. The stabilized concentrate is blended with water, glycerol and sucrose, and then preserved with sodium benzoate and potassium sorbate. Novozymes follows standard industry practices and uses a quality management system that complies with the requirements of ISO 9001. Novozymes cites several published sources to support the conclusion that the production and control methods used are generally accepted methods that are commonly used for the production of microbial enzyme preparations.

Novozymes states that the enzyme preparation complies with the general and additional requirements for enzyme preparations set forth in the Food Chemicals Codex (Fourth edition, Third supplement, 2001) and the specifications established by the Joint Food and Agricultural Organization/World Health Organization's (FAO/WHO) Expert Committee on Food Additives (General Specifications and Considerations for Enzyme Preparations Used in Food Processing, Compendium of Food Additive Specifications, FAO Food and Nutrition Paper 52, Addendum 9, 2001).

Novozymes cites publications in the toxicological and regulatory literature that support the conclusion that a wide variety of enzymes are used in food processing and that enzyme proteins do not generally raise safety concerns. Novozymes also notes that very few toxic agents have enzymatic properties. Novozymes states that phospholipases, in general, break down substrates into smaller units that do not have toxic properties and that are readily metabolized by the human body.

In the notice, Novozymes summarizes unpublished toxicological studies performed on the PLA1 enzyme preparation produced without the stabilization and standardization steps. These studies include two *in vitro* studies consisting of an Ames mutagenicity assay and a neutral red uptake in L929 monolayer culture cytotoxicity assay. Novozymes concludes that these studies showed no treatment related induction of gene mutation in bacteria or cytoxicity.

Novozymes provided information regarding the potential allergenicity of PLA1. Novozymes relates that there are no known cases of allergic responses to phospholipases in foods, reactions to food enzymes in general are very rare, and the level of exposure to this enzyme is very low. Based on these statements, Novozymes concludes that the risk of allergy due to ingestion of their PLA1 is negligible.

Based on the information provided by Novozymes, as well as other information available to FDA, the agency has no questions at this time regarding Novozymes' conclusion that PLA1 enzyme preparation from *A. oryzae* expressing the gene encoding a phospholipase A1 from *F. venenatum* is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of PLA1 enzyme preparation. As always, it is the continuing responsibility of Novozymes to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,

Laura M. Tarantino, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

Links on this page:

1431

Food

Agency Response Letter GRAS Notice No. GRN 000201

CFSAN/Office of Food Additive Safety
November 24, 2006

Ms. Lori Gregg
Novozymes North America, Inc.
77 Perry Chapel Church Road
P.O. Box 576 Franklinton, NC 27525

Re: GRAS Notice No. GRN 000201

Dear Ms. Gregg:

The Food and Drug Administration (FDA) is responding to the notice, dated May 30, 2006, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on June 1, 2006, filed it on June 5, 2006, and designated it as GRAS Notice No. GRN 000201. Novozymes responded to questions from FDA in amendments dated July 27, 2006, and August 5, 2006.

The subject of the notice is asparaginase enzyme preparation from *Aspergillus oryzae* expressing a gene encoding an asparaginase from *A. oryzae* (*A. oryzae* asparaginase enzyme preparation). The notice informs FDA of the view of Novozymes North America, Inc (Novozymes) that *A. oryzae* asparaginase enzyme preparation is GRAS, through scientific procedures, for use in reducing asparagine levels in wheat dough-based products such as cookies and crackers, fabricated potato chips, and cut or sliced potato products.[1]

Novozymes provides general information about the identity and technical effect of asparaginases as well as specific information about the identity and activity of the asparaginase enzyme preparation that is the subject of GRN 000201. It is identified by IUB name L-asparagine amidohydrolase, EC No. 3.5.1.1, and Chemical Abstracts Services (CAS) Registration No. 9015-68-3. Asparaginase hydrolyzes the amide of asparagine to form aspartic acid and ammonia. The amino acid sequence of this asparaginase and the nucleotide sequence of the gene encoding it have been determined. Novozymes notes that asparaginase also hydrolyzes glutamine, but not other free amino acids or asparagine residues within peptides or proteins. Novozymes characterizes the asparaginase in its *A. oryzae* asparaginase enzyme preparation by its molecular weight (approximately 36,000 Daltons (Da)), temperature and pH optima (60 degrees Celsius and 7.0, respectively), and activity range (pH 5.0 to 9.0).

Novozymes notes that a variety of bacteria and fungi produce asparaginases in cytoplasmic, periplasmic, or extracellular forms. Novozymes notes that extracellular asparaginases, such as the *A. oryzae* asparaginase, have higher affinity for asparagine than cytoplasmic forms.

The production strain was bioengineered to produce asparaginase under the control of the neutral amylase promoter of *A. niger*. Novozymes considers that an enzyme preparation derived from a recombinant microorganism will be safe if the host microorganism is nontoxigenic and nonpathogenic, the genetic information that is introduced into the host microorganism is well characterized, and the added DNA does not encode and express any known harmful or toxic substances. The framework for this conclusion is based on published comprehensive reviews and reports.

Novozymes discusses that the recipient strain *A. oryzae* BECh2 is derived from a safe lineage including strains that Novozymes has used for enzyme production for over 30 years. Novozymes published safety studies on two products from *A. oryzae* strains developed from *A. oryzae* A1560, the parent of BECh2. The recipient strain was used to construct production strains for modified lipase, glucose oxidase, and phospholipase enzyme preparations (the subjects of GRN Nos. 000103, 000106, and 000142, respectively). Novozymes notes that the changes made in BECh2 are well characterized, specific, and do not encode for elements that might adversely affect safety.

Novozymes describes the genes on its asparaginase expression plasmid, similar in construction to expression plasmids used to construct production strains for lipase (GRN 000103) and phospholipase A1 (GRN 000142), and describes the differences among these expression plasmids. Novozymes states that the DNA is randomly integrated into the chromosome, and that the production strain is well characterized by qualified scientists and technicians with sufficient expertise in identifying and characterizing strains to prevent contamination and ensure acceptable yields of a functional enzyme product. Novozymes considers its monitoring sufficient to detect unexpected secondary effects from genetic modifications.

Novozymes states that the method of manufacture for its asparaginase enzyme follows standard industry practices, complies with requirements of ISO 9001, and is in accordance with current good manufacturing practices. Novozymes describes raw materials used in fermentation and recovery for its asparaginase enzyme preparation as standard ingredients in the enzyme industry, conforming either to the Food Chemicals Codex (FCC) specifications or to internal specifications consistent with FCC requirements. Novozymes' quality control department ensures that raw materials meet specifications.

Novozymes describes asparaginase enzyme preparation as produced under submerged fed-batch pure culture of the production strain with equipment designed, operated, and cleaned to prevent microbial contamination. Novozymes recovers their asparaginase from culture broth by pH adjustment, primary filtration, concentration by ultrafiltration or evaporation, followed by prefiltration and germ filtration to remove residual microorganisms. The fluid concentrate is then preserved, stabilized, and then further evaporated or ultrafiltered as needed to achieve the appropriate activity. Stabilized concentrate is blended with glycerol and water and preserved with sodium benzoate and potassium sorbate.

Novozymes provides specifications for the asparaginase enzyme preparation, noting total organic solids (TOS) at approximately four percent, with the remainder as glycerol, water, sodium benzoate, and potassium sorbate at 50, 46, 0.3 and 0.1 percent, respectively. Novozymes states that the specifications for its asparaginase enzyme preparation comply with purity criteria for enzyme preparations in the FCC and with General Specifications for Enzyme Preparations used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in the Compendium of Food Additive Specifications.

Novozymes states that dough-based applications would use from 200-2500 ASNU[2] per kg processed food, corresponding to 6-70 g of asparaginase preparation per 100 kg processed food. Cut vegetables would use approximately 200 ASNU per kg final product, reflecting treatment in enzyme baths designed to pick up approximately 600 ASNU per kg treated potatoes.

Although Novozymes acknowledges that asparaginase would be heat inactivated by food processing, Novozymes calculates exposure (as estimated daily intake, EDI) assuming that added enzyme would be retained in the final products. Novozymes presumed that all processed food products (half of all food intake) would use the enzyme preparation at the highest recommended usage level and that all TOS would remain in the final product. Novozymes calculated an EDI of 0.35 mg TOS per kilogram body weight per day.

Novozymes includes results from unpublished studies; anin vitro bacterial reverse mutation assay with and without metabolic (S9) activation and anin vitro cytotoxicity test to conclude that the test preparation (a liquid asparaginase enzyme concentrate prepared like the commercial preparation, but without stabilization and standardization) is nonmutagenic and noncytoxic. Novozymes also discusses results from published and unpublished 13 week oral toxicity studies of enzyme preparations from *A. oryzae* strains, emphasizing studies using enzymes produced from strains derived from strain BECh2 or its parent A1560. These studies concluded that test preparations did not exhibit toxicity or mutagenicity under the conditions of the tests. Novozymes concludes that these studies support the safe use of enzyme preparations produced by strains derived from *A. oryzae* BECh2.

Based on the information provided by Novozymes, as well as other information available to FDA, the agency has no questions at this time regarding Novozymes' conclusion that *A. oryzae* asparaginase enzyme preparation is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of *A. oryzae* asparaginase enzyme preparation. As always, it is the continuing responsibility of Novozymes to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000201, as well as a copy of the information in this notice that conforms to the information in the proposed GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,
Laura M. Tarantino, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

---

[1]Novozymes describes the intended effect of the asparaginase as the conversion of asparagine to aspartic acid to reduce the formation of acrylamide in specified products. FDA neither evaluated the efficacy of such treatments nor determined whether acrylamide levels detected by Novozymes in untreated foods represent a significant health concern.

[2]One ASNU (asparaginase unit) produces one micromole ammonia per minute under specific reaction conditions.

Links on this page:

Biotechnology Program under the Toxic Substances Control Act (TSCA)

Aspergillus oryzae Final Risk Assessment

ATTACHMENT I--FINAL RISK ASSESSMENT OF

Aspergillus oryzae

(February 1997)

I. INTRODUCTION

Aspergillus oryzae is an asexual, ascomycetous fungus used for hundreds of years in the production of soy sauce, miso and sake without recorded incidents. There are conflicting opinions about whether *A. oryzae* can be isolated in nature. Although the details of the genetic relationship between *A. oryzae* and *A. flavus* remain unclear, the two species are so closely related that all strains of *A. oryzae* are regarded by some as natural variants of *A. flavus* modified through years of selection for fermenting of foods. *A. oryzae* is regarded as not being pathogenic for plants or animals, though there are a handful of reports of isolation of *A. oryzae* from patients. There are also several reports of products of *A. oryzae* fermentations, e.g. a-amylase, that seem to be associated with allergic responses in certain occupations with high exposure to those materials. *A. oryzae* can produce a variety of mycotoxins when fermentation is extended beyond the usual time needed for production of these foods. While wild *A. flavus* isolates readily produce aflatoxins and other mycotoxins, *A. oryzae* has not been shown to be capable of aflatoxin production.

History of Commercial Use and Products Subject to TSCA Jurisdiction

Aspergillus oryzae has apparently been an essential part of oriental food production for centuries and is now used in the production of many different oriental foods such as soy sauce, sake and miso. Potential uses under TSCA include fermentations of numerous enzymes, e.g., amylase, protease, B-galactosidase, lipase, and cellulase, and organic compounds such as glutamic acid. While these products have a variety of potential commercial uses, some of them are mostly frequently used in food processing.

The experience of safe commercial use of *A. oryzae* is extraordinarily well established. As a "koji" mold it has been used safely in the food industry for several hundred years. *A. oryzae* is also used to produce livestock probiotic feed supplements. Even the commercialization of byproducts of the fermentation was established nearly a century ago. The "koji" mold enzymes were among the first to be isolated and commercialized. In 1894, Dr. J. Takamine isolated and soldTakadiastase from a commercial firm he started in Clifton, New Jersey (Bennett, 1985a).

EPA has reviewed, under TSCA, two genetically modified strains of *A. oryzae* used for the production of enzymes (Premanufacture Notice (PMN) numbers P89-134 and P94-1475).

II. IDENTIFICATION AND TAXONOMY

A. Overview

The candidate species is a member of the genus *Aspergillus* and belongs to the group of fungi that are generally considered to reproduce asexually (Fungi Imperfecti or Deuteromycetes), although perfect forms (forms that reproduce sexually) of some aspergilli have been found. The form genus *Aspergillus* represents a taxonomic grouping of a very large number of asexual fungi which are characterized by the production of spores on large black or brown conidia in phialides arranged on a characteristic spherical conidiophore

termed the vesicle. This definition leads to inclusion of a complex assortment of organisms within the taxon. To simplify the taxonomy of such a large number of organisms, the genus *Aspergillus* has been divided into sections or groups based on color, size and roughness of the spore, conidiophore and vesicle as well as the arrangement of phialides and the presence of sclerotia. The separation of individual species into groups is somewhat tenuous and based on distinguishing measured characters with overlapping means. This resulted in the 132 species arranged in 18 groups by Raper and Fennell (1965) due to overlapping morphological or physiological characteristics. However, it is important to remember that taxonomy is "dealing with living variable organisms and that species and group concepts must be reasonably elastic" (Raper & Fennell, 1965).

As is the case of many fungi, the taxonomy of *Aspergillus* is primarily based on morphological features, rather than physiological, biochemical features and genetic characteristics often used to classify bacteria. Nomenclature problems of the genus *Aspergillus* arise from their pleomorphic life cycle. The newer findings show that this group of fungi has both a perfect (teleomorphic) and an imperfect (anamorphic) state.

The morphological approach to taxonomy has led to the existence of several synonyms for the genus *Aspergillus.* They are: *Alliospora* Pim; *Aspergillonsis* Spegazzini; *Cladaspergillus* Ritg; *Cladosparum* Yuill and Yuill; *Euaspergilus* Ludwig; *Gutturomyces* Rivolta; *Raperia* Subramaniam and Grove; *Sceptromyces* Corda; *Spermatoloncha* Spegazzini; *Sphaeromyces* Montagne; *Sterigmatocystis* Cramer; and *Stilbothamnium* Hennings (Bennett, 1985b).

Aspergilli are ubiquitous in nature. They are geographically widely distributed and have been observed in a broad range of habitats, because they can colonize a wide variety of substrates.

B. The *Aspergillus flavus* Group

Aspergillus oryzae is a member of the *A. flavus* group of *Aspergillus* species. The *A. flavus* group, which also now includes *A. sojae, A. nomius* and *A. parasiticus* (see below) is defined by the production of spore chains in radiating heads which range in color from yellow-green to olive brown. The conidiophores are roughened and colorless. The spores themselves have conspicuous ridges and echinulations (spines). Sclerotia are occasionally produced (Raper & Fennell, 1965). *A. oryzae/flavus* species have never been connected to a sexual or teleomorphic stage. However, the teleomorphic stages of other *Aspergillus* species have been demonstrated by the formation of cleistothecia. These species belong to the genera *Emericella, Neosartorya* and *Eurotium,* all belonging to the ascomycetous family *Eurotiaceae* (Fennel, 1973). Either the sexual stages of the *A. flavus* group have not been recognized as such, being identified as completely different species based on morphology, or this group of fungi are "degenerate", having lost the ability to form sexual spores and mycelia.

A. oryzae is considered by some experts to be a domesticated variant of *A. flavus* (Kurtzman et al. 1986). Through long-time use, *A. oryzae* strains seem to have been selected to exhibit reduced sporulation, have more aerial mycelia and exhibit no environmental survival structures like sclerotia or the presence of aflatoxins that might function to inhibit grazing by insects. These morphological features that differentiate *A. oryzae* from *A. flavus* may represent adaptations to the artificial culture conditions of the koji fermentation. Misidentification of new isolates not obtained from well established cultures is always a possibility, since the key morphological differences between the two species seem related to culture adaptation. However, the source of *A. oryzae* strains for industrial fermentations today is likely to be standard culture collections. Environmental isolates of aspergilli would likely be identified as *A. flavus*

rather than the laboratory-adapted *A. oryzae*.

C. Related Species of Concern

The taxonomy of *Aspergillus* has public health implications due to the production of potent mycotoxins by members of this genus. Most notable is the association of aflatoxins with members of the *A. flavus* group (Bennett, 1985b; Semeniuk et al., 1971). *A. oryzae* is a member of that group and in spite of the above mentioned morphological distinctions, *A. oryzae* appears to be very closely related to *A. flavus*. Numerous

studies have beendone to distinguish the koji molds from their toxicogenic relatives. The results are unambiguous in their confirmation of the conspecificity of *A. oryzae* and *A. flavus.* (see Section IV. below).

In a similar way, A. *sojae* is considered to be a domesticated form of *A. parasiticus* and shares a 92% DNA homology with its wild progenitor. *A. sojae* also has a history of safe use in the food industry. *A. parasiticus* in nature is an active colonizer of cereal grains and seeds with concurrent mycotoxin production. While these species can be distinguished from *A. flavus/oryzae* using morphological criteria, all four species intergrade. The hazard concerns for these species, thus, are equivalent to those associated with *A. flavus/oryzae*.

A. nomius is a newly classified species of toxigenic strains originally described in the *A. flavus* group, but not having the same level of DNA homology as shown among the four varieties mentioned above (Kurtzman et al., 1987). *A. nomius* produces aflatoxin and includes strains isolated from diseased bees. *A. oryzae* is distinguishable both morphologically and genetically from *A. nomius*.

III. HAZARD ASSESSMENT

A. Human Health Hazards

1. Toxin Production by *A. oryzae*

The close relationship between *A. oryzae* and *A. flavus* and the production of highly toxic mycotoxins by the latter has resulted in careful examination of the toxigenic potential of *A. oryzae*. However, *A. oryzae*, as a koji mold, has toxigenic potential in its own right. Those aspergilli used for manufacture of Japanese fermented foods have long been called koji molds. Prominent among the 25 koji molds listed is *A. oryzae* (Manabe et al., 1984). This fungus is used for sake, an alcoholic beverage, miso a soy bean paste, shoyu, soy sauce, amasake, a sweet beverage, and shouchu, a distilled liquor.

A. flavus commonly colonizes damaged cereal grains, soybeans and peanuts, actively producing mycotoxins (Stoloff, 1982). Certain strains of *A. oryzae* have themselves been shown to produce the mycotoxins aspergillic, kojic, cyclopiazonic and B-nitropropionic acids and maltoryzine (Ciegler & Vesonder, 1987).

Even with the food industry strains, a caveat of safety is that the fungal incubation not exceed the normal three day period. *A. oryzae* has been shown to produce toxic compounds under incubations longer than the typical koji fermentation(Semeniuk et al, 1971; Yokotsuka & Sasaki, 1986). The following are toxins produced by some strains of *A. oryzae*.

a. Kojic acid

Kojic acid (discovered by Saito, 1907) is produced by koji, a solid culture of the koji mold. It is a commonly produced metabolite that possesses antibacterial and antifungal activity. Few oral studies exist for this byproduct. Giroir reported toxic effects on chickens at four to eight mg/kg feed. Older studies (Friedemann, 1934, Werch et al. 1957, Morton et al., 1945) using intravenous or intraperitoneal challenges show moderate toxicity for kojic acid. Later work had similar results (Ueno and Ueno, 1978). Kojic acid also is reported to have moderate cardiotoxic and cardiotonic activity (Manabe et al., 1984., Bajpai et al. 1982). Nineteen of 47 *A. oryzae* strains tested produced kojic acid (Manabe et al., 1984). Even though it is apparent that the koji molds, including *A. oryzae* can produce the toxin kojic acid, this toxin may not be present in the fermented foods. The incubation period for sake, shoyu and miso is about two days and no kojic acid is found at that time (Manabe et al., 1984). However, these authors concluded that they were unable to prove kojic acid was not present in any fermented food in Japan, because conditions of production and materials were different for each industry, and were often uncontrolled. Semeniuk et al. (1971) warned that even with food industry strains, fungal incubation must not exceed three days. Thus, as the culture adjusts to changing conditions, *A. oryzae* may produce toxic compounds when incubation time exceeds typical koji fermentation time.

b. Maltoryzine

Maltoryzine, another toxic metabolite isolated and characterized by Iisuka and Iida (1962), was produced by *A. oryzae* var. *microsporus*. This metabolite was determined to be the cause of poisoning among dairy cows. While highly toxic (LD_{50} 3 mg/kg; Iizuka, 1974; Ciegler and Vesonder, 1987), the substance may only be found in one or a very few strains of *A. oryzae*. The single isolate, IAM 2950, produced enough of the toxin when grown on malt rootlets to poison some milk cows, prompting the determination of its LD_{50}. The production of these toxins is related to the composition of the growth substrate and usually occurs in stationary phase cultures. Commercial strains of *A. oryzae* and *A. sojae* apparently do not produce maltoryzine.

c. Cyclopiazonic acid

Pitt and Cruickshank (1990), note that many isolates of *Aspergillus oryzae* are found to produce cyclopiazonic acid. Orth (1977), reporting on food industry strains of *A. oryzae*, indicated that eight of 16 strains produced cyclopiazonic acid. This acid is a natural contaminant of foods and feeds and is produced by several molds including those used in fermented food production. These included *A. flavus, A. versicolor, A. tamarii*, several *Penicillium* species, including *P. camemberti*, and *A. oryzae.* This mycotoxin has been shown to occur naturally in corn, cheese, peanuts and in Kodo millet that was implicated in natural human intoxication in India (CAST Task Force Report No. 116, 1989a). Benkhemmar et al. (1985) showed that when cyclopiazonic acid producing (CPA+) strains are mated with CPA-strains, the CPA+ phenotype is dominant in the heterokaryon. Oral administration produced effects at levels ranging from 0.25 to >50 mg/kg with dogs among the most sensitive species and rats among the least (Purchase, 1971; Nuehring et al., 1985). LO(A)ELs for sensitive species were at or under 1mg/kg. Nishie et al. (1985) noted that Rao and Husain (1985) identified cyclopiazonic acid as the cause of debilitating illnesses in cattle and man in India.

d. b-nitropropionic acid

A. oryzae can produce b-nitropropionic acid, along with other food-borne molds (Gilbert et al., 1977). Its mode of action is apparently irreversible succinate dehydrogenase inhibition which can cause a variety of symptoms often neurological in nature. These symptoms have been studied in mice (Gould and Gustine, 1982; Umezawa, 1967) and rats (Hamilton and Gould, 1987) where intravenous or subcutaneous LD_{50}s of 20-50 mg/kg were determined. Reports of livestock poisoning via ingestion in feed (James et al., 1980; James, 1983) showed that ingestion of b-nitropropionic acid could produce significant toxic effects up to and including death. When *A. oryzae* (ATCC 12892) was studied for its ability to produce b-nitropropionic acid on various high protein and carbohydrate-rich foods, it flourished and produced this toxin in cooked sweet potato, potato and ripe banana (Penel and Kosikowski, 1990). Ames type assays for mutagenicity (Dunkel, 1985) showed positive responses with and without activation for two *Salmonella* strains, but not for three others. This assay uses multiple indicator strains in order to ensure that each potential mutation mode is detectable; the failure in three strains merely implies that the mutation modes to which each is sensitive are not the ones associated with the test substance.

2. Taxonomic and Genetic Relationship to Other Aspergilli

The closest taxon to *A. oryzae* is *A. flavus* which Kurtzman et al. (1986) regard as conspecific. Many strains of *A. flavus* produce aflatoxins which are acutely toxic to mammals (oral LD_{50}s ranging from 1 to 15 mg/kg depending on test species (Ceigler, 1975). Aflatoxins are animal carcinogens (Barnes and Butler, 1964; Dickens and Jones, 1964; Sinhuber, 1968) and also probablehuman carcinogens (Council for Agricultural Science and Technology, 1989). Developmental effects have also been found (Elis and DiPaolo, 1967, Le Breton et al., 1964).

While the koji molds like *A. oryzae* are distinguishable from, they are nevertheless very closely related to, *A. flavus*. Distinguishing between *A. oryzae* and *A. flavus* by physical traits is elusive. The toxigenic subspecies/variety *A. flavus* has numerous spores chains that remain yellow-green; sterigmata that are always biseriate; spiny (echinulate) individual spores; roughened conidiophores up to 600æm in length and sclerotia often present. The variety called *A. oryzae* specifically has fewer spore chains, fading to brown with age; longer average conidiophores (about two to three mm); smoother individual spores; sterigmata usually in 1 series and sclerotia rarely produced (Raper & Fennel, 1965).

3. Lack of Aflatoxin Production in *A. oryzae*

Despite this strong similarity between the two species, production of aflatoxins has not been demonstrated by *A. oryzae.* Many studies affirm that the currently available strains confirmed to be *A. oryzae* are not capable of producing aflatoxins (Wei and Jong, 1986; Yokotsuka and Sasaki, 1986). In one test, no strains of *A. oryzae* or *A. sojae* (another koji mold) produced detectable levels of aflatoxins, while 33% and 85% of the strains of *A. flavus* and *A. parasiticus*, respectively, were toxigenic. As mentioned above, Kurtzman, et al. (1986) regard *A. oryzae* and *A. sojae* as domesticated varieties of their respective subspecies. Only one study (El-Hag and Morse, 1976) describes aflatoxin production by a strain reported to be *Aspergillus oryzae* (NRRL strain 1988). This observation is notable as an exception to the rule of no aflatoxin production by *A. oryzae*.

It has been noted that *A. flavus* strains upon extended laboratory cultivation lose morphologically distinguishing characteristics, making them appear much like *A. oryzae* (Kurtzman, et al., 1986). Wicklow (1984) details the competitive disadvantages of *A. oryzae* and implies that *A. flavus* is the "wild" form. Kurtzman et al. (1986) ask whether the separation between toxigenic and non-toxigenic *A. flavus* group species occurs through ecological adaptation or chromosomal changes such as translocations or inversions.

The elucidation of metabolic pathways responsible for the production of aflatoxins by *A. flavus* group fungi has progressed rapidly. Recently Payne (Bhatnagar, et al. 1992 and Payne, 1994) reported on the conversion of an aflatoxin non-producing strain of *A. flavus* to aflatoxin B_1 positive using a cosmid library developed from a toxigenic *A. flavus*. While added metabolic precursors could not stimulate toxin production in the mutant, the addition of an appropriate cosmid carrying a <5 kb fragmentof the genome of the toxin producer converted the non-toxigenic strain to significant levels of aflatoxin production. Further work has resulted in isolation of a small segment specifying a regulatory, rather than structural, gene that affects early parts of the pathway. Probes for this regulatory gene, designated *afl* R, have been positive in both *A. oryzae* and *A. sojae*, even though those strains do not produce aflatoxin. In addition, Payne stated that probes for structural genes for aflatoxin production were also positive in some, but not all, *A. oryzae* strains examined.

It appears that evidence is mounting towards multiple reasons for failure to produce aflatoxins in *A. oryzae* cultures. One explanation is a lack of functional regulators, specifically *afl* R, that activate aflatoxin production. Another is that some or all of the structural genes in the aflatoxin pathway may be non-functional. For both types of genes, those sequences could be absent or present in the wrong orientation or split by insertions or modified slightly so as to be non-functional. Except for substantial deletion or absence of the necessary sequences, all of these alternatives are potentially reversible. However, Payne indicated that he doubted that industrial strains of *A. oryzae* were likely to revert to aflatoxin production. He indicated that, even though probes found the presence of appropriate gene sequences, the genes so detected could easily be incomplete enough so as to be completely non-functional.

Thus, complete absence of genetic potential is not the only plausible explanation for the non-expression of characters such as aflatoxin production in *A. oryzae*. In a related study, researchers attempting to improve strains of a mold identified as *A. oryzae* used for food fermentation in Thailand acquired a toxin producing strain by simple UV mutagenesis of a known "safe" strain (Kalayanamitr, et al. 1987). The toxins produced by this strain and other toxigenic *A. oryzae* strains are not aflatoxins but rather other types of mycotoxins. The exact composition of the toxins involved in *A. oryzae* toxicosis in these studies, as in other anecdotal studies, was not determined (Semeniuk, et al., 1971; Wicklow and Dowd, 1989, and Kalayanamitr, et al., 1987). The mechanism for this conversion to toxigenicity was not investigated, but the mutations required could have affected either structural or regulatory genes and produced the new observed toxigenic phenotype.

4. Colonization and Pathogenicity

Aspergillus oryzae does not appear to be a human pathogen. Available information documents infections in humans possibly caused by *A. oryzae* in only three instances. The first was a case of meningitis (Gordon, et al., 1976). In the second case, *A. oryzae* invaded the paranasal sinuses, causing fever and right periorbital swelling (Byard, et al., 1986). The third case was apulmonary aspergilloma caused by *A. oryzae* (Liao,

1988). Care must be exercised in evaluating these three cases as having been caused by this organism due to its close taxonomical relationship to *A. flavus* and the possibility of incorrect identification. The relative rarity of such cases in light of the commonplace use of *A. oryzae* suggests this species has a low potential for expressing pathogenic traits.

5. Allergic Reactions to *Aspergillus oryzae*

Allergic reactions are not uncommon for aspergilli in general. There is one reported case of an allergic bronchopulmonary aspergillosis due to *A. oryzae* in a 19-year old female (Akiyama et al., 1987). However, the a-amylase produced by *A. oryzae*, that is used by bakers in bread making, was reported by Birnbaum, et al. (1988) to have caused asthma in a baker. Based on an observation of a case of baker's asthma due to monovalent sensitization to a-amylase used as an additive to flour, investigators tested 31 bakers who had occupational asthma and/or rhinitis by skin tests and serologic RAST examinations. Thirty-two percent of the bakers had RAST specific IgE to a-amylase from *A. oryzae.* Baker's asthma is reported to be the most frequent occupational lung disease in Switzerland and West Germany (Wuthrich and Baur, 1990). However, allergic reactions in bread bakers are quite common, both to the flours of various grains, as well as to the flour additives such as fungal amylases. Allergic reactions in bakers are not specific to *A. oryzae*, nor the enzymes produced by *A. oryzae* (O'Neil and Lehrer, 1991). In addition, the exposure scenario of a bread baker to flour and the additives contained therein is quite different from that of workers in a fermentation facility using general worker hygiene and protection practices.

6. Conclusions

There are two possible concerns for human health hazards associated with *A. oryzae*. The first, which is directly tied to *A. oryzae*, is the potential for mycotoxin production with extended fermentation. A variety of toxins can be produced, with the most common being the moderately toxic kojic acid. Other more potent toxins may only be produced by a few strains or in lesser quantities. These mycotoxins seem to be produced only under conditions of extended fermentation, and therefore, their production could be averted under proper fermentation conditions i.e., short fermentation times.

The second issue is the possibility for the production of aflatoxins because of the nearly indistinguishable identity of *A. oryzae* and *A. flavus*. Kurtzman, et al. (1986) have shown that *A. flavus* and *A. oryzae* are essentially the same based on DNA comparisons. *A. oryzae* appears so closely related to its aflatoxin-producing counterpart as to be viewed as consisting ofculture-attenuated strains of *A. flavus* (Kurtzman, 1994; Wicklow, 1984). It has been hypothesized that *A. oryzae* evolves under culture from *A. flavus* strains due to selection for features that would be ecologically detrimental in the wild.

Hypothetically, then, if *A. oryzae* has evolved to non-aflatoxigenic status after centuries in culture, the question remains whether it can revert to the "wild" type. The experience of oriental food production would seem to suggest not, or at least not frequently enough as to be detectable. Recent studies (Payne, 1994; Klich, 1994) suggest homology between parts of the *A. oryzae* genome and structural genes for aflatoxin production. It is conceivable that reintroduction of regulatory genes or their gene products could activate a dormant aflatoxin synthetic potential. There is no evidence to show that the required gene transfer or gene rearrangement that might provide the needed functional sequences for an aflatoxin producing *A. oryzae* strain occurs naturally. The question is, therefore, whether this type of genetic modification is possible in culture. Gene transfer from a toxigenic strain during fermentation is highly unlikely due to the need for maintaining axenic conditions during fermentation. The theoretical possibility of genetic rearrangement occurring in culture resulting in reversion back to the "wild-type" seems unlikely. Anecdotal evidence gathered over centuries suggests that *A. oryzae* commercial food strains do not produce aflatoxins, nor have there been reports of any adverse human health effects from aflatoxin.

B. Environmental Hazards

1. Hazards to Animals

The potential for toxin production is the main environmental hazard issue of concern for *A. oryzae.* If there were a method to distinguish between toxicogenic and non-toxicogenic strains, there would be no

environmental concern for *A. oryzae*. Two recent studies that addressed the question of differentiating between toxin producing and non-toxicogenic strains of the related species *A. flavus*, *A. parasiticus* and *A. nomius* were unable to correlate either mitochondrial or chromosomal DNA RFLPs with mycotoxin production (Moody & Tyler, 1990a, 1990b). This again points to differences that may only involve small regulatory regions or that involve differences in structural gene complements that are beyond the detection limit of current DNA typing technologies.

Compounding this is the observation that *A. oryzae* and *A. flavus* are essentially indistinguishable by most molecular techniques. *A. flavus* is believed to be second in frequency only to the frank fungal pathogen, *A. fumigatus*, as a cause of aspergillosis in many species. *A. flavus* is associated specifically with invasive diseases of insects as well as toxicosis (Austwick, 1965). Recently, some insect pathogenic *A.flavus* strains were reclassified into *A. nomius* (Kurtzman et al., 1987). Whether *A. oryzae* is involved depends on how one defines the species of the *A. flavus* group. The effects on livestock of the various toxins that occur after extended koji fermentations, or in contaminated feed, show that the "minor" mycotoxins can still cause economic loss. No anecdotal accounts have been found that demonstrate that these potential effects occur in wildlife outside the agricultural environment.

2. Hazards to Plants

No reports of *A. oryzae* effects on living plants have been found. This species does not appear to be pose a hazard to plants.

3. Conclusions

The issues for environmental hazards are similar to those for human health hazards. The primary hazard concerns are for toxin production by *A. oryzae* strains. Under usual conditions of culture, well established commercial strains of this species do not seem to produce significant levels of mycotoxins, although certain moderately potent toxins can be produced after extended culture. Aflatoxins appear not to be produced by such cultures. The potential for environmental hazard is dependent on the likelihood that commercial strains could escape and establish themselves in the wild and grow under conditions analogous to those resulting in toxin production in extended culture. The few examples of livestock poisoning associated with the "minor" toxins, b-nitropropionic acid, maltoryzine and cyclopiazonic acid cited above imply that, for a short time at least, strains of *A. oryzae* may be able to survive in the wild.

IV. EXPOSURE ASSESSMENT

A. Worker Exposure

Aspergillus oryzae is considered a Class 1 Containment Agent under the National Institute of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules (U.S. Department of Health and Human Services, 1986). In Europe, *Aspergillus* spp. are treated as low-risk-class microorganisms, i.e., category 2 of the European Federation of Biotechnology (Frommer et al., 1989) or category 1 on the OECD containment scale. Category 1 of the European Federation of Biotechnology scale includes organisms deemed harmless, which can be grown under good industrial large scale practices (GILSP), while category 2 organisms like *Aspergillus* require more stringent containment.

No data were available for assessing the release and survival specifically for fermentation facilities using *A.oryzae*. Therefore, the potential worker exposures and routine releases to the environment from large-scale, conventional fermentation processes were estimated on information available from eight premanufacture notices submitted to EPA under TSCA Section 5 and from published information collected from non-engineered microorganisms (Reilly, 1991). These values are based on reasonable worst-case scenarios and typical ranges or values are given for comparison.

During fermentation processes, worker exposure is possible during laboratory pipetting, inoculation, sampling, harvesting, extraction, processing and decontamination procedures. A typical site employs less than 10 workers/shift and operates 24 hours/day throughout the year. NIOSH has conducted walk-through surveys of several fermentation facilities in the enzyme industry and monitored for microbial air

contamination. These particular facilities were not using recombinant microorganisms, but the processes were considered typical of fermentation process technology. Area samples were taken in locations where the potential for worker exposure was considered to be potentially greatest, i.e., near the fermentor, the seed fermentor, sampling ports, and separation processes (either filter press or rotary drum filter). The workers with the highest potential average exposures at the three facilities visited were those involved in air sampling. Area samples near the sampling port revealed average airborne concentrations ranging from 350 to 648 cfu/m^3. Typically, the Chemical Engineering Branch would not use area monitoring data to estimate occupational exposure levels since the correlation between area concentrations and worker exposure is highly uncertain. Personal sampling data are not available at the present time. Thus, area sampling data have been the only means of assessing exposures for previous PMN biotechnology submissions. Assuming that 20 samples per day are drawn and that each sample takes up to 5 minutes to collect, the duration of exposure for a single worker will be about 1.5 hours/day. Assuming that the concentration of microorganisms in the worker's breathing zone is equivalent to the levels found in the area sampling, the worst-case daily inhalation exposure is estimated to range up to 650 to 1200 cfu/day. The uncertainty associated with this estimated exposure value is not known (Reilly, 1991).

B. Environmental and General Exposure

1. Fate of Organism

Controversy exists over the ability to isolate *A. oryzae* from the natural environment. Some researchers believe that *A. oryzae* is widely distributed in nature while other maintain that all strains of *A. oryzae* are variants of *A. flavus* which have been modified through years of selection in an artificial environment. Specific data which indicates the survivability of industrial "domesticated" strains of *A. oryzae* in the environmentare not available. The process of domestication may have resulted in lessened survivability in the environment. However, its ability to produce spores suggests that it may survive in the environment (Versar, 1991).

2. Releases

Estimates of the number of *A. oryzae* organisms released during production are tabulated in Table 1 (Reilly, 1991). The uncontrolled/untreated scenario assumes no control features for the fermentor offgases, and no inactivation of the fermentation broth for the liquid and solid waste releases. The containment criteria required for the full exemption scenario assume the use of features or equipment that minimize the number of viable cells in the fermentor off-gases. They also assume inactivation procedures resulting in a validated 6log reduction of the number of viable microorganisms in the liquid and solid wastes relative to the maximum cell density of the fermentation broth.

TABLE 1. Estimated Number of Viable *A. oryzae*

Organisms Released During Production

Uncontrolled/ Full

Release Media Untreated Exemption Release

(cfu/day) (cfu/day) (days/year)

Air Vents $2x10^8$ - 1x1011 $<2x10^8$ - 1x1011 350

Rotary Drum Filter 250 250 350

Surface Water $7x10^{12}$ $7x10^6$ 90

Soil/Landfill $7x10^{14}$ $7x10^{8}$ 90

Source: Reilly, 1991

These are "worstcase" estimates which assume that the maximum cell density in the fermentation broth for fungi is 10^7 cfu/ml, with a fermentor size of 70,000 liters, and the separation efficiency for the rotary drum filter is 99 percent.

3. Air

Specific data which indicate the survivability of *A. oryzae* in the atmosphere after release are currently unavailable. Survival of vegetative cells during aerosolization is typically limited due to stresses such as shear forces, desiccation, temperature, and UV light exposure. As with naturally-occurring strains, human exposure may occur via inhalation as the organisms are dispersed in the atmosphere attached to dust particles, or lofted through mechanical or air disturbance.

Air releases from fermentor offgas could potentially result in nonoccupational inhalation exposures due to point source releases. To estimate exposures from this source, the sector averaging form of the Gaussian algorithm described in Turner(1970) was used. For purposes of this assessment, a release height of 3 meters and downward contact at a distance of 100 meters were assumed. Assuming that there is no removal of organisms by controls/equipment for offgases, potential human inhalation dose rates are estimated to range from 3.0×10^3 to 1.5×10^6 cfu/year for the uncontrolled/untreated scenario and less than that for systems with full exemptions. It should be noted that these estimates represent hypothetical exposures under reasonable worst case conditions (Versar, 1991).

4. Water

The concentrations of *A. oryzae* in surface water were estimated using stream flow values for water bodies receiving process wastewater discharges from facilities within SIC Code 283 (drugs, medicinal chemicals, and pharmaceuticals). The surface water release data (cfu/day) tabulated in Table 1 were divided by the stream flow values to yield a surface water concentration of the organism (cfu/l). The stream flow values for SIC Code 283 were based on discharger location data retrieved from the Industrial Facilities Dischargers (IFD) database on December 5, 1991, and surface water flow data retrieved from the RXGAGE database. Flow values were obtained for water bodies receiving wastewater discharges from 154 indirect (facilities that send their waste to a POTW) and direct dischargers facilities that have a NPDES permit to discharge to surface water). Tenth percentile values indicate flows for smaller rivers within this distribution of 154 receiving water flows and 50th percentile values indicate flows for more average rivers. The flow value expressed as 7Q10 is the lowest flow observed over seven consecutive days during a 10year period. The use of this methodology to estimate concentrations of *A. oryzae* in surface water assumes that all of the discharged organisms survive wastewater treatment and that growth is not enhanced by any component of the treatment process. Estimated concentrations of *A. oryzae* in surface water for the uncontrolled/untreated and the full exemption scenarios are tabulated in Table 2 (Versar, 1991).

TABLE 2. *A. oryzae* Concentrations in Surface Water

Receiving Flow Stream Flow (MLD*)	Organisms (cfu/l)	
	Mean 7Q10	Mean 7Q10

Uncontrolled/Untreated

10th Percentile 156 5.60 $4.5x10^{4}$ $1.25x10^{6}$

50th Percentile 768 68.13 $9.11x10^{3}$ $1.03x10^{5}$

Full Exemption

10th Percentile 156 5.60 $4.5x10^{-2}$ $1.25x100^{0}$

50th Percentile 768 68.13 $9.11x10^{-3}$ $1.03x10^{-1}$

---

*MLD = million liters per day

Source: Versar, 1991

5. Soil

Since soil is a possible natural habitat for *A. oryzae*, long-term survival in the environment, particularly as spores, may occur. Human exposures via dermal contact and ingestion routes, and environmental exposures [i.e., to terrestrial, avian, and aquatic organisms (via runoff)] may occur at the discharge site if there is establishment of *A. oryzae* within the soil (Versar, 1991).

6. Summary

Although direct monitoring data are unavailable, worst case estimates do not suggest high levels of exposure of *A. oryzae* to either workers or the public resulting from normal fermentation operations.

V. INTEGRATION OF RISK

In the previous sections, information regarding the potential exposures and hazards to workers, the general public, animals, plants and the environment was reviewed. This section serves to integrate this information to evaluate the potential risks associated with the industrial use of *Aspergillus oryzae*.

A. Discussion

The only major concerns identified are associated with human and animal toxicity due to mycotoxin production. *A. oryzae* and *A. flavus* are designations of taxa that represent the extremes of a spectrum of traits associated with a common fungus. Current evidence points to *A. oryzae* as a domesticated derivative of *A. flavus*. The evidence is not complete enough to indicate whether *A. oryzae* represents a unique genotype as well as a stable phenotype. It appears that under prolonged cultivation the phenotype of *A. oryzae* will be exhibited and that aflatoxins will not be produced from such strains. Other toxins such as cyclopiazonic acid and kojic acid may, however, be expressed.

1. Aflatoxin Production

Although it is likely that *A. oryzae* held in cultivation for decades or even centuries are likely to represent strains having small, but key, deletions in an otherwise identical genome to *A. flavus*, it is remotely possible that the phenotypic differences between the two species may be due to differences in the arrangement and control of genes rather than the loss or gain of them. If *A. oryzae* strains have had reversible gene modifications that prevent the expression of aflatoxin genes, then environmental control of such rearrangements is possible and reversion can occur. It must be noted that there have been noreports of workers in the industrial setting suffering from aflatoxin effects.

There is a basic question as to the likelihood that *A. oryzae* exists in the wild. Some researchers (Klich, 1994) indicate that *A. oryzae* can be isolated in nature. Other researchers (Kutzman et al., 1986) contend that *A. oryzae* is a domesticated version of *A. flavus*, with decreased survival characteristics such as reduced sporulation and the lack of sclerotia. Wicklow (1984) has described the competitive disadvantages of *A. oryzae*. These observations suggest that this organism is highly adapted to conditions in the laboratory.

All this points to an incomplete knowledge base for *A. oryzae*. However, it appears that aflatoxin production is not a concern for established *A. oryzae* strains. Although there is a theoretical possibility for reversion to the aflatoxigenic phenotype of *A. flavus*, it has not been observed through hundreds of years of use in food production. In addition, controls on exposure mitigate concerns. While some workers might be exposed to the organism, much of that exposure would presumably be via an inhalation route rather than an ingestion. They would be exposed mostly to spores of *A. oryzae* during large-scale fermentation. Spores that escape the manufacturing site would be unlikely to persist in the environment because of less than optimal conditions for germination and growth. As pointed out in the hazard assessment, *A. oryzae,* lacks many survival features possessed by the related *A. flavus*. Therefore, from the information cited above and using the values in the assessment of exposure, environmental exposure relevant to aflatoxin production appears highly unlikely.

2. Other Toxins

There remains some concern for other mycotoxins produced by koji molds. These toxins are less potent than aflatoxins and their production is tied both to strain specificity and culture conditions. However, they can occur even with current domesticated strains, although there are no reports that their production in industrial fermentations have resulted in adverse effects on human health. The most toxic ones, such as cyclopiazonic acid, seem to be produced by a few strains under special conditions. The less toxic ones, such as kojic acid, may be limited by engineering controls on the fermentation process.

The exposure component of the risk for this concern is similar to that described for aflatoxin. Proper conditions of cultivation should limit production of these toxins and limit exposure to workers.

3. Other Issues

Allergenicity seems to be related more to the product of the fermentations than to *A. oryzae per se*. Sensitivity to a-amylasein particular, is a potential concern, but one that exists for all microorganisms producing this enzyme. There is thus no incremental risk specific to the use of this fungus.

4. Summary

Thus, the potential risks for *A. oryzae* include the theoretical possibility of genetic rearrangement resulting in the inadvertent production of aflatoxins in *A. oryzae*. Through centuries of use in food production, there are no reports of aflatoxin production. Mycotoxin production can most likely be avoided by properly controlling the fermentation conditions, and human health concerns are mitigated by controls on exposure and worker hygiene practices. *A. oryzae* is not a plant or animal pathogen, and survival in the environment is expected to be limited due to its decreased survival characteristic by years of domestication. The risk of the use of this organism under the specified conditions of this exemption is low.

B. Recommendation

Aspergillus oryzae is recommended for the tiered exemption.**VI. REFERENCES**

A. Primary Sources

Akiyama, K., H. Takizawa, M. Suzuki, S. Miyachi, M. Ichinohe and Y. Yanagihara. 1987. Allergic bronchopulmonary aspergillosis due to *Aspergillus oryzae*, Abstract. Chest 91:285-286.

Austwick, P.K.C. 1965. Pathogenicity of *Aspergillus* species, pp. 82126. In K.B. Raper and D.I. Fennell, (eds.), The genus *Aspergillus*. Williams and Wilkins, Baltimore, MD.

Bajpai, P., P.K. Agrawal, and L. Vishwanathan. 1982. Kojic acid: synthesis and properties. J. Scien. Indust. Research 41:185-194.

Barnes, J.M. and W.H. Butler. 1964. Carcinogenic activity of aflatoxin to rats. Nature 202:1016.

Beckwith, A., R. Vesonder, and A. Ciegler. 1976. Chemical methods investigated for detoxifying aflatoxin in foods and feeds, Chapter 4. In J. Roderick (ed.), Mycotoxins and other fungal related food problems, Advances in Chemistry Series #149. American Chemical Society, Washington, DC.

Benkhemmar, O., F. Gaudemer, and I. Bouvier-Fourcade. 1985. Heterokaryosis between *Aspergillus oryzae* cyclopiazonic acid-defective strains: Method for estimating the risk of inducing toxin production among cyclopiazonic acid-defective industrial strains. Appl. Environ. Microbiol. 50:1087-1093.

Bennett, J.W. 1985a. Molds, manufacturing and molecular genetics. In W.E. Timberlake, (ed.), Molecular genetics of filamentous fungi. Alan R. Liss, Inc., NY.

Bennett, J.W. 1985b. Taxonomy of fungi and biology of the Aspergilli. In A.L. Demain and N.A. Solomon, (eds.), Biology of industrial microorganisms. Benjamin Cummings Publishing Co. Menlo Park, CA.

Betina, V. 1989. Mycotoxins, Volume 9. In Bioactive molecules. Elsevier American Publishers, Oxford.

Bhatnagar, D., J.W. Cary, G.A. Payne, N.P. Keller, and T.E. Cleveland. 1992. Molecular regulation of aflatoxin biosynthetic pathway. Presented at 92nd General Meeting, American Society for Microbiology, New Orleans, LA.

Birnbaum, J., F. Latil, D. Vervoloet, M. Sneft, and J. Charpin. 1988. The role of alpha-amylase in baker's asthma, Abstract. Rev. Mal. Respir. 5:519-521.

Byard, R. W., R.A. Bonin, and A. Haq. 1986. Invasion of paranasal sinuses by *Aspergillus oryzae*. Mycopathologia 96:41-43.

Ciegler, A. 1975. Mycotoxins: Occurrence, chemistry, biological activity. *Lloydia* 38:21-35.

Ciegler, A. and R.F. Vesonder. 1987. Microbial food and feed toxicants: fungal toxins, pp. 19126. In The CRC handbook of microbiology, Volume VIII. CRC Press, Boca Raton, FL.

Cole, R.J. 1984. Screening for mycotoxins and toxinproducing fungi. In V. Betina, (ed.), Mycotoxins: Production, isolation, separation and purification. Elsevier Scientific Publishers, Amsterdam.

Council for Agricultural Science and Technology. Report No. 116, November 1989. Mycotoxins--Economic and health risks.

Cruickshank, R.H. and J.I. Pitt. 1990. Isoenzyme patterns in *Aspergillus flavus* and closely related species, pp. 250-265. In R.A. Samson and J.I. Pitt, (eds.), Modern concepts in *Penicillium* and *Aspergillus* classification, NATO Advanced Science Institute Series, Series A: Life Sciences, Volume 185. Plenum Press, NY.

Dickens, F. and H.E.H. Jones. 1964. The carcinogenic action of aflatoxin after its subcutaneous injection in the rat. Brit. J. Cancer. 17:691-698. (Cited by Ong, 1975)

Dunkel, V.C. and V.F. Simmon. 1980. Mutagenic activity of chemicals previously tested for carcinogenicity in the National Cancer Institute Bioassay Program, pp. 283-302. In R. Montesano, H. Bartsch, and L. Tomatis, (eds.), Molecular and cellular aspects of carcinogen screening tests. IARC Scientific Publication No. 27. International Agency for Research on Cancer, Lyon.

Edwards, J.H. and T.S. AlZubaidy. 1977. Medical aspects. In J.E. Smith and J.A. Pateman, (eds.), Genetics and physiology of *Aspergillus*. Academic Press, NY.

El-Hag, N. and R.E. Morse. 1976. Aflatoxin production by a variant of *Aspergillus oryzae* (NRRL strain 1988) on cowpeas (*Vigna inenisis*). Science 192:1345-1346.

Elis, J. and J.A. DiPaolo. 1967. Aflatoxin B_1 - Induction of malformations. Arch. Pathol. 83:53-57.

Fennell, D.I. 1973. Plectomycetes: Eurotiales, Chapter 4, pp. 45-68. In G.C. Ainsworth, F.K. Sparrow, and A.S. Sussman, (eds.), The fungi: An advanced treatise, Volume IVa. Academic Press, NY.

Friedemann, T.E. 1934. Science 30:34. (Cited by Bajpai, et al. 1982.)

Frisvad, J.C., D.L. Hawksworth, Z. Kozakiewicz, J.I. Pitt, R.A Samson, and A.C. Stolk. 1990. Proposals to conserve important species names in *Aspergillus* and *Penicillium*, pp. 83-89. In R.A. Samson and J.I. Pitt, (eds.), Modern concepts in *Penicillium* and *Aspergillus* classification. Plenum Press, NY.

Frommer, W., B. Ager, L. Archer, B. Brunius, C.H. Collins, R. Donakian, C. Frontali, S. Hamp, E.H. Houwink, M.T. Kuenzi, P. Kramer, H. Lagast, S. Lund, J.L. Mshler, F. Normand-Plessier, K. Sargeant, M.G. Tuijnenburg, S.P. Vranch and R.G. Werner. 1989. Safe biotechnology III. Safety precautions for handling microorganisms of different classes. Appl. Microbiol. Biotechnol. 30: 541-552

Gilbert, M., A. Penel, F.V. Kosikowski, J.D. Henion, G.A. Maylin and D.J. Lisk. 1977. Electron affinity gas chromatographic determination of b-nitropropionic acid as its pentafluorobenzyl derivative in cheeses and mold filtrates. J. Food Science 42:1650-1653.

Giroir, L.E., W.E. Huff, L.E. Kubena, R.B. Harvey, M.H. Elissalde, D.A. Witzel, A.G. Yersin, and G.W. Ivie. 1991. Toxic effects of kojic acid in the diet of male broilers. Poultry Science 70:499-503.

Gordon, M.A., R.S. Holzman, H. Senter, E.W. Lapa, and M.J. Kupersmith. 1976. *Aspergillus oryzae* meningitis. JAMA 235:2122-2123.

Gould, D.H. and D.L. Gustine. 1982. Basal ganglia degeneration, myelin alterations and enzyme inhibition induced in mice by the plant toxin 3-nitropropionic acid. Neurpathol. Appl. Neurobiol. 8:377-393.

Hamilton, B.F. and D.J. Gould. 1987. Correlation of morphologic brain lesions with physiologic alterations and blood-brain barrier impairment in 3-nitropropionic acid toxicity in rats. Acta Neuropathol. 74:67-74.

Hawksworth, D.L. 1990. Problems and prospects for improving the stability of names in *Aspergillus* and *Penicillium*. In R.A. Samson and J.I. Pitt, (eds.), Modern conceptsin *Penicillium* and *Aspergillus* classification. NATO Advanced Science Institute Series, Series A: Life Sciences, Volume 185. Plenum Press, NY.

Iizuka, H. 1974. Maltoryzine, pp. 405-418. In I.F.H. Purchase, (ed.), Mycotoxins. Elsevier, Amsterdam.

Iizuka, H. and M. Iida. 1962. Maltoryzine, a new toxic metabolite produced by a strain of *Aspergillus oryzae* var. *microsporus* isolated from the poisonous malt sprout. Nature 196:681-682.

James, L.F., J. Hartley, M.C. Williams, and K.R. Van Kampen. 1980. Field and experimental studies in cattle and sheep poisoned by nitro-bearing *Astragalus* or their toxins. J. Am. Vet. Res. 41:377-382.

James, L.F. 1983. Neurotoxins and other toxins from *Astragalus* and other related genera, pp. 445-462. In Keeler, R.F. and A.T. Tu, (eds.), Handbook of natural toxins, Vol. 1. Marcel Dekker, NY. (Cited by Ludolf et al., 1991.)

Jong,S.C. and M.J.Gantt, (eds.) 1987. Catalogue of fungi and yeasts, 17th edition. American Type Culture Collection, Rockville, MD.

Kalayanamitr, A., A. Bhumiratana, T.W. Flegel, T. Glinsukon, and N. Shinmyo. 1987. Occurrence of toxicity among protease, amylase and color mutants of a non-toxic soy sauce koji mold. Appl. Environ. Microbiol. 53:1980-1982.

Klich, M.A. and E.J. Mullaney. 1987. DNA restriction enzyme fragment polymorphism as a tool for rapid differentiation of *Aspergillus flavus* from *Aspergillus oryzae*. Experim. Mycology 11:170-175.

Klich, M.A. and J.I. Pitt. 1985. The theory and practice of distinguishing species of the *Aspergillus flavus* group, pp. 211-220. In R.A. Samson and J.I. Pitt, (eds.), Advances in *Penicillium* And *Aspergillus* systematics. Plenum Publishing, NY.

Klich, M.A. and J.I. Pitt. 1988. Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. Trans. Br. Mycol. Soc. 91:99-108.

Kozlowski, M. and P.P. Stepien. 1982. Restriction analysis of mitochondrial DNA of members of the genus *Aspergillus* as an aid in taxonomy. Jour. Gen. Microbiol. 128: 471476.

Kurtzman, C.P., M.J. Smiley, C.J. Robnett, and D.T. Wicklow. 1986. DNA relatedness among wild and domesticated species of the *Aspergillus flavus* group. Mycologia 78(6):955-959.

Kurtzman, C.P., B.W. Horn, and C.W. Hesseltine. 1987. *Aspergillus nomius*, a new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus tamarii*. Antonie van Leeuwenhoek 53: 147-158.

LeBreton, C., C. Frayssinet, C. LaFarge, and A.M. DeRecondo. 1964. Aflatoxin - mechanisms de l'action. Food Cosmet. Toxicol. 2:675-676. (Cited by Ong, 1975.)

Liao, W.Q., J.Z. Shao, S.Q. Li, G.T. Wan, L.W. Da, Y.C. Sun, Z.T. Qi, Q.T. Chen, and Z.M. Sun. Mycological identification of pulmonary aspegillioma caused by *Aspergillus oryzae* with proliferating heads, Abstract. Clin. Med. Jour. 101:601-604.

Ludolf, A.C., F. He, P.S. Spencer, J. Hammerstad, and M. Sabri. 1991. 3-nitropropionic acid-exogenous animal neurotoxin and possible human striatal toxin. Canad. J. Neurol. Sci. 18:492-498.

Manabe, M., K. Tanaka, T. Goto, and S. Matsuura. 1984. Producing capacity of kojic acid and aflatoxin by koji mold, pp. 4-14. In H. Kurata and Y. Ueno, (eds.), Toxigenic Fungi - their toxins and health hazard, Proceedings of the mycotoxin symposia held at the Third International Mycological Congress, Tokyo, 1983. Elsevier, Tokyo.

Moody, S.F. and B.M. Tyler. 1990a. Restriction enzyme analysis of mitochondrial DNA of the *Aspergillus flavus* group: *A. flavus*, *A. parasiticus*, and *A. nomius*. Appl. & Environ. Microbiol. 56:2441-2452.

Moody, S.F. and B.M. Tyler. 1990b. Use of nuclear DNA restriction fragment length polymorphisms to analyze the diversity of the *Aspergillus flavus* group: *A. flavus, A. parasiticus* and *A. nomius*. Appl. & Environ. Microbiol. 56:2453-2461.

Morton, H.E., W. Kocholaty, R. Junowicz-Kocholaty, and A. Kelner. 1945. Toxicity and antibiotic activity of kojic acid produced by *Aspergillus luteovirescens*. J. Bacteriol. 50:579-584.

Nishie, K., J.K. Porter, R.J. Cole, and J.W. Dorner. 1985. Neurochemical and pharmacological effects of cyclopiazonic acid, chlorpromazine and reserpine. Research Communications in Psychology, Psychiatry and Behavior 10:291-302.

Nuehring, L.P., G.N. Rowland, L.R. Harrison, R.J. Cole, and J.W. Dorner. 1983. Cyclopiazonic acid mycotoxicosis in the dog. Am. J. Vet. Res. 46:1670-1676.

O'Neil, C.E. and S.B. Lehrer. 1991. Occupation reactions to food allergens. In: D.D. Metcalfe, H.A. Sampson, and R.A. Simon (eds.). Food Allergy: Adverse Reactions to Foods and Food Additives, pp. 207-236. Blackwell Scientific Publications, Oxford.

Ong, T.M. 1975. Aflatoxin mutagenesis. Mutation Res. 32:35-53.

Orth, R. 1977. Mycotoxins of *Aspergillus oryzae* strains for use in the food industry as starters and enzyme producing molds. Ann. Nutr. Alim. 31:617-624.

Penel, A.J. and F.V. Kosikowsi. 1990. Beta-nitropropionic acid production by *A. oryzae* in selected high protein and carbohydrate-rich foods. J. Food Protect. 53:321-323.

Pitt, J.I. and R.H. Cruickshank. 1990. Speciation and synonymy in *Penicillium* subgenus *Penicillium* -- Towards a definitivetaxonomy. In R.A. Samson and J.I. Pitt, (eds.), Modern concepts in *Penicillium* and *Aspergillus* classification, NATO Advanced Science Institute Series, Series A: Life Sciences, Volume 185. Plenum Press, NY.

Purchase, I.F.H. 1971. The acute toxicity of the mycotoxin cyclopiazonic acid to rats. Toxicol. Appl. Pharmacol. 18:114-123.

Rao, B.L. and A. Husain. 1985. Presence of cyclopiazonic acid

in Kodo millet (*Paspalum serobiculatum*) causing Bodua poisoning in man and its production by associated fungi. Mycopathologia 89:177-180.

Raper, K.B. and D.I. Fennell. 1965. The genus *Aspergillus*, p. 686. Williams and Wilkins Company, Baltimore, MD.

Reilly, B. 1991. Analysis of environmental releases and occupational exposure in support of proposed TSCA 5(h)(4) exemption. Unpublished, U.S. Environmental Protection Agency, Washington, D.C.

Saito, K. 1907. Bot. Mag. (Tokyo) 21:240. (Cited by Bajpai et al. 1982.)

Semeniuk, G., G.S. Harshfield, C.W. Carlson, C.W. Hesseltine and W.F. Kwolek. 1971. Mycotoxins in Aspergillus. Mycopathologia 43:137152.

Sinhuber, R.O., D.J. Lee, J.H. Wales, and J.L. Ayers. 1968. Dietary factors and hepatomas in rainbow trout (*Salmo gaidneri.*) II. Carcinogenesis and the effects of grosypol and altered lipids on aflatoxin-induced liver cancer. J. Nat. Cancer Inst. 41:1293-1299. (Cited by Ong, 1975.)

Stoloff, L.. 1982. Mycotoxins as potential environmental carcinogens, Chapter, 9, pp. 97-120. In H.F. Stich, (ed.), Carcinogens and mutagens in the environment, Volume I: Food products. CRC Press, Boca Raton, FL.

Thom, C. and K.B. Raper. 1945. A Manual of the *Aspergilli*. Williams and Wilkins Company, Baltimore, MD.

Turner, B. 1970. Workbook of atmospheric dispersion estimates. U.S. Environmental Protection Agency, Research Triangle Park, NC.

Ueno, Y. and I. Ueno. 1978. Toxicology and biochemistry of mycotoxins. In K. Uraguchi and M. Yamazaki, (eds.), Toxicology, biochemistry, and pathology of mycotoxins. John Wiley and Sons, Halstead Press, NY.

Umezawa, H., (ed.) 1967. Index of antibiotics from actinomycetes. Univ. Tokyo Press, Tokyo.

U.S. Department of Health and Human Services. 1986. Guidelines for research involving recombinant DNA molecules; Notice. 51 FR 16958, May 7, 1986.

U.S. Department of Health and Human Services. Undated. Proposed biosafety guidelines for microbial and biomedical laboratories. Office of Biosafety, Center for Disease Control.

Voss, E.G., et al. 1983. International code of botanical nomenclature adopted at the Thirteenth International Botanical Congress, Sydney, August 1981. Regnum Vegetabile 111:1472. (1)

Wei, D.L. and S.C. Jong. 1986. Production of aflatoxins by strains of the *Aspergillus flavus* group maintained in ATCC. Mycopathologia 93:19-24.

Werch, S.C., Y.T. Oester, and T.E. Friedemann. 1957. Kojic acid-- A convulsant. *Science* 126:450-451.

Wicklow, D.T. 1983. Taxonomic features and ecological significance of sclerotia. In U.L. Diener, R.L. Asquith and J.W. Dickens, (eds.), Aflatoxin and *Aspergillus flavus* in corn. Proc. Symp. held in Atlanta, GA, Jan.26-27, 1982. *Southern Cooperative Extension Service Bulletin* 279:6-12.

Wicklow, D.T. 1984. Conidium germination rate in wild and domesticated yellow-green Aspergilli. Appl. & Environ. Microbiol. 47:299-300.

Wicklow, D.T. and O.L. Shotwell. 1983. Intrafungal distribution of aflatoxins among conidia and sclerotia of *Aspergillus flavus* and *A. parasiticus*. Can. J. Microbiol. 29:1-5.

Wicklow, D.T. and P.F. Dowd. 1989. Entomotoxigenic potential of wild and domesticated yellow-green Aspergilli: Toxicity to corn earworm and fall armyworm larvae. Mycologia 81:561-566.

Wuthrich, B. and X. Baur. 1990. Baking ingredients, especially alpha-amylase, as occupational inhalation allergens in the baking industry, Abstract. Schweiz-Med-Wochenschr. 120:446-450.

Yamatoya, K., J. Sugiyama and H. Kuraishi. 1990. Electrophoretic comparison of enzymes as a chemotaxonomic aid among *Aspergillus* taxa (2) *Aspergillus* section *flavi*, pp. 395-406. In R.A. Samson and J.I. Pitt, (eds.), Modern concepts in *Penicillium* and *Aspergillus* classification, NATO Advanced Science Institute Series, Series A: Life Sciences, Volume 185. Plenum Press, NY.

Yokotsuka, T. and M. Sasaki. 1986 Risks of mycotoxin in fermented foods, Chapter 15, pp. 259-287. In C.W. Hesseltine and H. Wang, (eds.), Indigenous fermented foods of non-western origin. Mycologia Memoirs No. 11. J. Cramer, Berlin.

B. Secondary Sources

(1)Versar. 1991. Screening level exposure assessment of *aspergillus* species for 5(h)(4) exemption under the proposed biotechnology rule. Unpublished, U.S. Environmental Protection Agency, Washington, D.C.

T-2

DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

DSM Nutritional Products
45 Waterview Boulevard
Parsippany
NJ 07054
United States of America

phone +1 800 526 0189
fax +1 973 257 8414

Dr. Andrea Krause
Center for Veterinary Medicine
Ingredient Safety Team (HFV 224)
7519 Standish Place
Rockville, Maryland 20855

January 2, 2013

Antifoam used in enzyme fermentation

Dear Dr. Krause

Enclosed are the paper copies you requested of the technical data sheet for the antifoam (b) (4) used in the fermentation process for the production of Ronozyme® HiPhos. Enclosed also is a general technical bulletin from (b) (4) on industrial antifoams.

Kind regards

James La Marta, Ph.D.
Senior Manager Regulatory Affairs

RECEIVED
JAN 08 2013
By

(b) (4)

Technical Data Sheet

(b) (4)

Antifoam agent

Chemical Composition

Polyalkylene glycols

Properties

Appearance	colourless liquid
Odour	neutral
Specific gravity	1020 kg/m^3 at 20 °C
Viscosity	465 mPa.s at 25 °C
Flash point	> 100 °C
Solidification Point	- 21°C
Toxicological Behaviour	inoffensive
Ecological Behaviour	inoffensive - see safety-data-sheet
Storage stability	at least 1 year at adequate storage

(b) (4) corresponds to FDA

The data given are typical values which are not intended for use in preparing specifications. For test methods refer to the corresponding supplement.

Application

The best possible addition point and necessary dosage quantity of (b) (4) depend on factory conditions and have to be found out by trials.

The suggestions for application and usage of our products as well as possible proposed formulations are meant to advise only to the best of our knowledge. This information is without obligation and does not release customers from their own testings to ensure suitability for intended processes and use. Liability is only accepted in case of intention or gross negligence. Liability for any defects caused by minor negligence are not accepted. Each producer is responsible and liable to observe legislation and patent rights of third parties.This new leaflet replaces all previously printed documentation.Alterations reserved. 07/01/2010

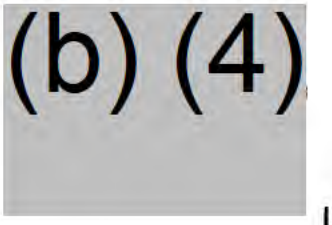
(b) (4)

(b) (4)

(b) (4)

Defoamers and Deaerators

FDA/CVM001784


(b) (4)

(b) (4)

Contents

Page

(b) (4)

Defoamers and Deaerators

1. Introduction

Apart from a few exceptions such as removing ink from used paper and in the flotation of ores and minerals, foams are generally undesirable. Foams disturb production sequences in which liquids, particularly water, have an important function as transporting media or solvents, and in the worst cases they can even bring such processes to a standstill. Furthermore, foams can lead to lower production yields and finished products of poorer quality.

Some examples to illustrate this are given below:

- During sugar production from sugar beet, considerable problems due to foam occur in the entire inside and outside working areas.

Sugar production from sugar beet

Fig. 1: Foam in a sugar factory

(b) (4)

- Many effluents from the chemico-technical industries as well as the paper and cellulose industry cause considerable environmental problems due to foam formation.

Effluents

- Entrapped air bubbles in the pulp used for paper manufacture can cause breaking down of the sheet. Air containing material necessitates increased pump work when conveying the material. In addition, defects are produced in the paper resulting from foam bubbles.

Pulp used for paper manufacture

- Entrapped air bubbles in dyes used in the paper industry lead to defects in the coating, and therefore to a reduction in quality. This leads to complaints by the user of such coated paper.

Dyes used in the paper industry

- Foam is undesirable in the production of dispersion paints. Foam causes a reduction in capacity of the mixing vessel and the formation of "craters" during application of the dispersion paint.

Production of dispersion paints

- In biotechnical processes, e. g. during the production of penicillin, enzymes, yeast, citric acid and glutamic acid, foam causes difficulties in controlling the reaction and results in a reduction in the yield.

Biotechnical processes

- In the wet attack of phosphate rock with sulphuric acid, foam production can cause a reduction in the capacity of the reactor, a decrease in the pump output and to difficulties in the filtration stage. Furthermore, considerable problems are likely in the evaporation stages.

Wet attack of phosphate rock with sulphuric acid

- Foam on lubricant emulsions in metal working leads to an insufficient cooling effect and thus to overheating of the cutting tools.

Lubricant emulsions

- Foam formed on drawing oil emulsions during the drawing of non-ferrous metal wires leads to an inadequate cooling effect. This results in hot running of the drawing dies and therefore premature wear and breaking of the wire in extreme cases.

Drawing oil emulsions

Fig. 2: Foam in a sewage plant

- In mining, flotation foam causes difficulties in the thickening of concentrates and in de-watering. Residual quantities of flotation reagents in the flotation tailings lead to problems with clarification.

Mining

- Effluents in industrial and local authority sewage plants contain a number of different foam-forming substances. In mechanical and biological sewage plants foam causes clarification problems and environmental pollution.

Mechanical and biological sewage plants

A number of terms used in connection with the subject of "foaming" are defined below:

- **Foaming**
  Foaming is a physical process which leads to the formation of foam.
- **Foam**
  Foam is defined as the entirety of all cells separated by liquid lamella, that are formed by an accumulation of bubbles. The liquid lamellae are very thin and often have a thickness of between only a few nanometres and 10 µm.
- **Gas bubble**
  A gas bubble is a cavity or cell filled with gas, that is surrounded by a thin liquid envelope.
- **Foaming capacity**
  Foaming capacity is the level of the ability to form a foam.
- **Foam stability**
  Foam stability is the degree of the ability of a foam to remain stable.
- **Foam dewatering**
  Foam dewatering is the return flow of excess liquid to the liquid phase. The excess liquid is entrained by the gas bubbles during foaming.
- **Defoaming agent (anti-foaming agent, defoamer, foam removing oil)**
  A defoaming agent is a product which prevents the formation of (surface) foam, or considerably decreases its stability and destroys it.
- **Deaerating agent**
  A deaerating agent is a product which causes rapid accumulation (enlargement) of the gas bubbles dispersed in a liquid. This results in a considerably improved ability of the air to escape from the liquid.

(b) (4)

2. Foam Formation

Pure water cannot form a stable foam, and even when a high mechanical force is applied, e. g. by pumping and stirring, the air introduced still escapes very quickly. The spontaneous escape of air bubbles from pure water is represented in figure 3.

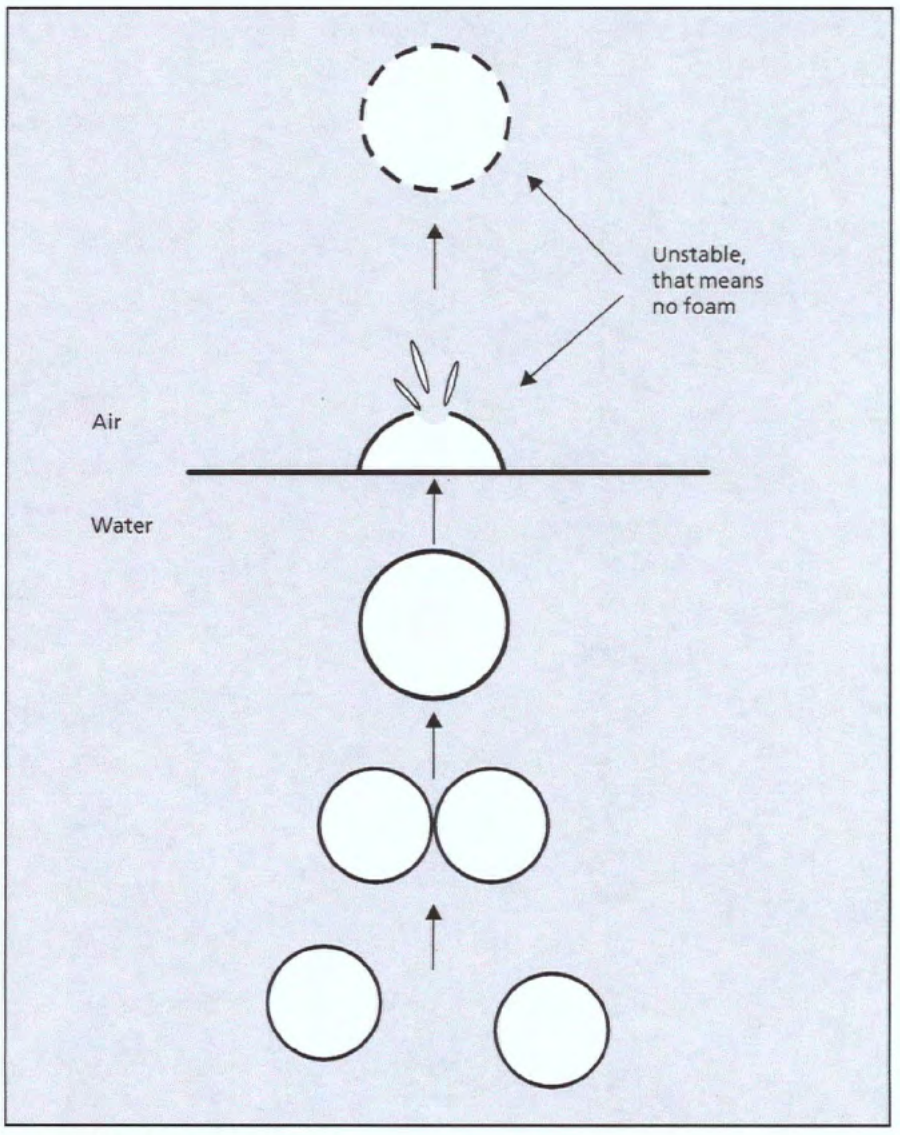


Fig. 3: Self-deaeration in pure water

However, if the water contains special dissolved or also non-dissolved components, relatively stable dispersions of air in water can be formed. The result then is the formation of foam on the water surface (see figure 4).

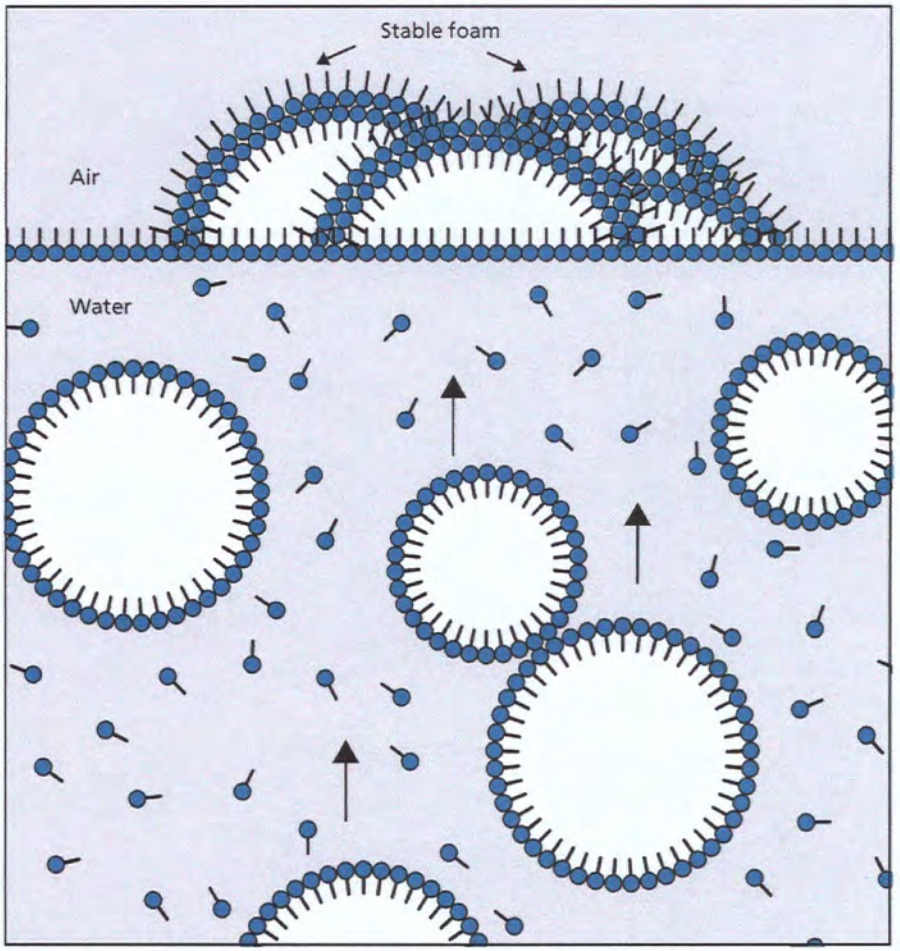


Fig. 4: Self-deaeration of water with dissolved and non-dissolved components

The durability (life, stability) of the foam produced can vary greatly. Foams that break down very quickly are not likely to cause problems. On the other hand, very stable foams may also be formed, which in the most unfavourable cases may even dry without breaking down. However, foams are generally only formed if the water contains surface-active agents (surfactants, proteins, cellulose derivatives, polyelectrolytes or polysaccharides).

Surface-active agents

(b) (4)

Surfactants

Surfactants are chemical compounds which are preferentially adsorbed on an interface when dissolved or dispersed in a liquid. The molecules of the tenside surfactants have a hydrophobic (= lipophilic, i. e. water repellent = oil attracting) organic residual part, and a hydrophilic (= lipophobic, i. e. water attracting = oil repellent) group (see figure 5).

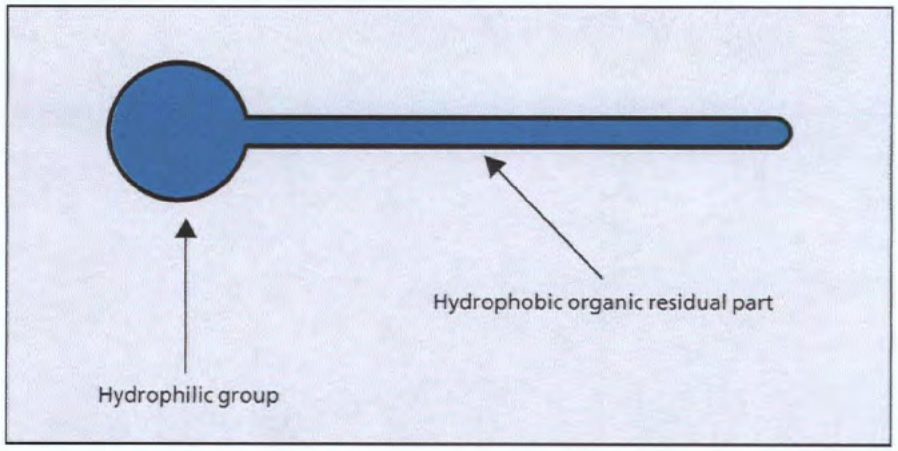


Fig. 5: Graphic representation of a surfactant molecule

Water/air interface

Formation of a monomolecular layer

The surfactant molecules settle on the water/air interface in such a way that the hydrophilic part of the molecule is in the water, and the hydrophobic part in the air. A monomolecular layer is formed – i. e. a thin film – from the surfactant molecules (see figure 6).

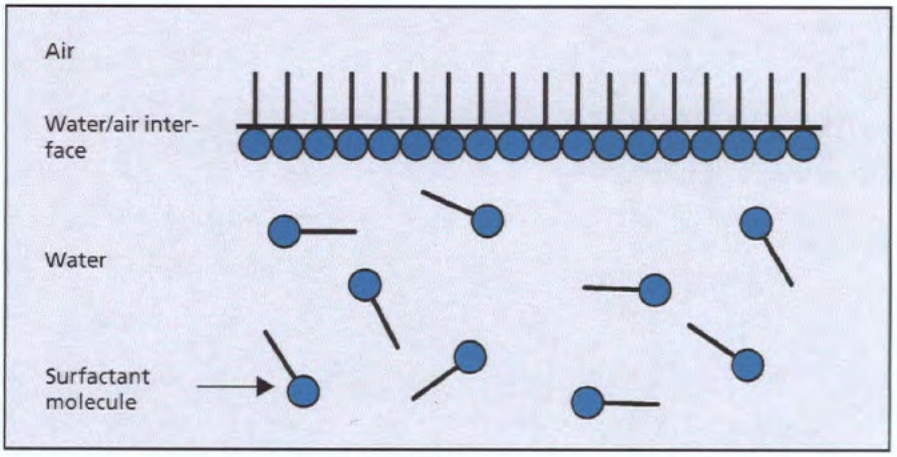


Fig. 6: Formation of a monomolecular layer of surfactant molecules

This layer (film) is the precondition for the formation of stable foams. The high concentration at the water/air interface means that frequently only very small quantities of surfactant (foaming agent) in relation to the total quantity of water are sufficient to bring about considerable foam formation.

When no surfactants are present: spherical foam

First of all, the small air bubbles dispersed in water have a spherical shape. When these small air bubbles rise to the water surface and no surfactant is present, a spherical foam is formed, which breaks down very rapidly through bubble growth (see figure 7).

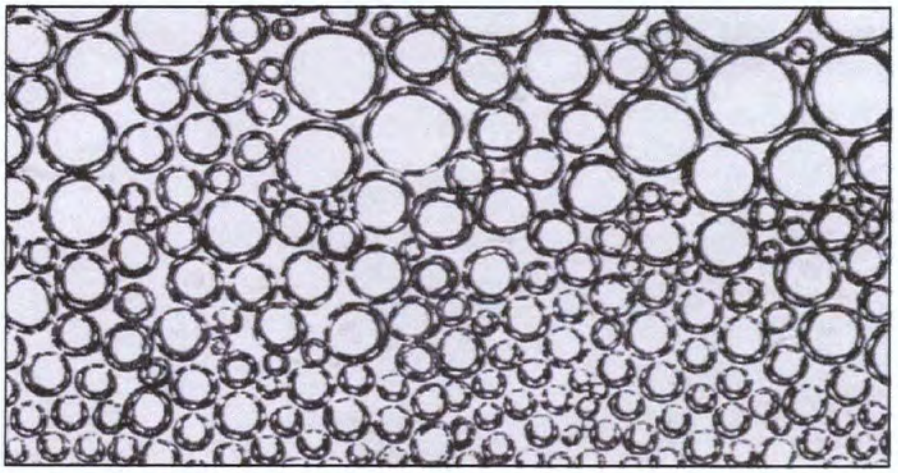


Fig. 7: Spherical foam

If, however, surfactants are dissolved in the water, the monomolecular elastic layer which is formed at the water/air interface is raised by means of the escaping gas bubbles and surrounds the foam bubble on the outside. A corresponding layer is likewise formed on the inside of the foam lamella, and in this case the hydrophobic residual parts of the surfactant molecule also project from the water (see figure 8).

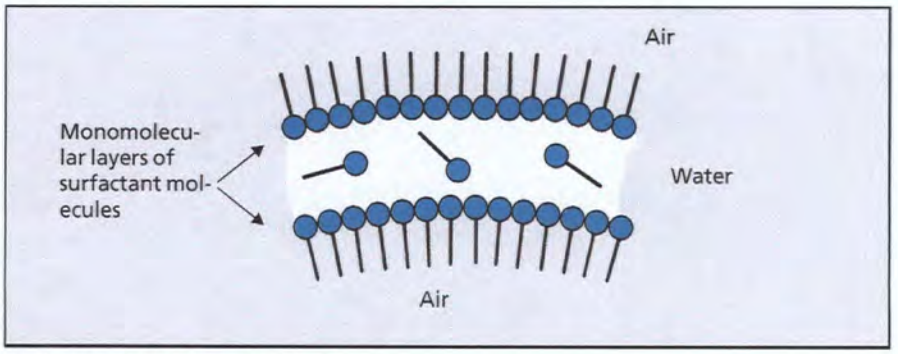


Fig. 8: Schematic section of a foam lamella

(b) (4)

Foam that has been stabilized by a surfactant is only slowly dewatered, since the water only flows off gradually under the influence of gravity and the foam lamella becomes thinner and more unstable. Like electrical charges may also contribute to the stability of the foam lamella, i. e. when the foam lamella becomes thinner the surface layers with the same charge repel. Furthermore, draining of the water between the surface layers is slowed down further due to the strong bond of the water to the hydrophilic groups of the surfactant. The stable foam bubbles then coalesce to larger bubbles, and in doing so they lose their spherical shape and a polyhedral foam is formed (see figure 9).

Dissolved surfactant: polyhedral foam

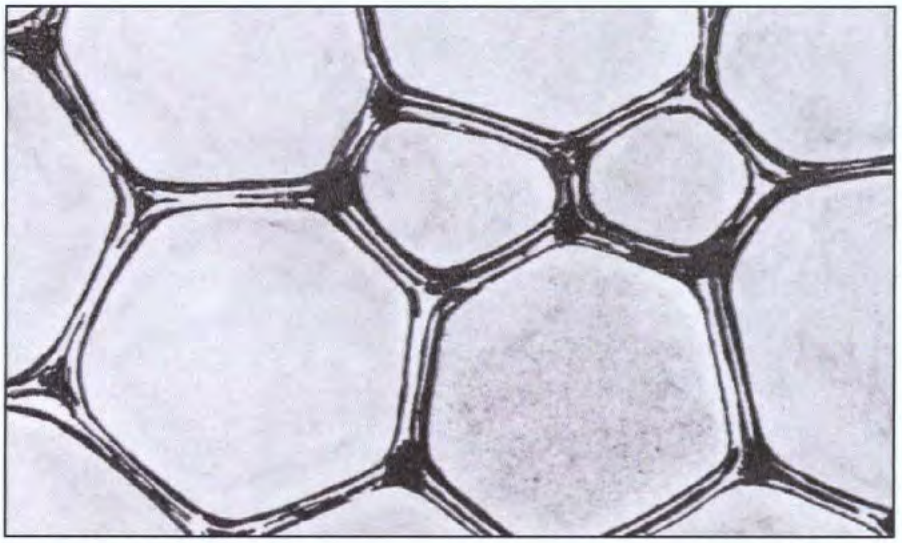


Fig. 9: Polyhedral foam

Until the maximum spreading pressure is reached additional surfactant molecules are taken up by the layer and a two-dimensional structure is produced. The critical micelle concentration is thereby reached and the spreading pressure remains constant.

Foam production

There are several methods of foam production:

1. Dispersion method: enlargement of the surface regions between the liquid and the gas by mechanical action (stirring, pumping)
2. Condensation method: production of gas bubbles from a supersaturated solution of the gas (beer or CO_2-containing solutions)
3. The finely distributed introduction of gas into a medium (biological stage of effluent treatment)

Foam stabilisation is explained by two related effects.

2.1 Gibbs´ Elastic Films and the Marangoni Effect

- **Gibbs Film Elasticity**
  As the lamella expands the surfactant concentration is reduced locally, resulting in an increase in surface tension. The high surface tension creates a restoring force which stabilises the foam (Gibbs).
- **Marangoni Effect**
  As the lamella expands the water flows back more quickly than the surfactant molecules. The water that is flowing back increases the layer thickness of the lamella and thereby stabilises the foam.

Gibbs

Marangoni

In the case of foam bubbles such local expansion occurs frequently. To this extent both effects are necessary for stabilisation.

In detail, the following forces act in thin films:

Due to the loss of liquid in the lamellae the wet foam turns into a dry one. The plateau zones absorb liquid, whereby the lamellae can become so thin that they affect one another.

Van der Waals forces, electrostatic and steric forces counteract this destabilisation. The van der Waals forces cause the two surfaces of the lamella to attract one another, so that more liquid is squeezed out. However, if the liquid contains dissolved ions which can form an electric charge layer (surfactants), the repulsion of the like charge results in stabilisation of the thin lamellae.

Similarly, dissolved macromolecular stabilisers (e. g., polyelectrolytes, proteins, saponin) can lead to mutual repulsion of the lamella surfaces through steric obstruction.

2.2 Rheological Aspects of Foam Stability

Lamella lose liquid under the influence of gravity. This reduces their stability. They are stabilised by all forces that minimise the efflux of liquid. A slight increase in viscosity in the interlaminar liquid stabilises the foam, while viscosities that are too high can prevent foam formation.

Slightly increased viscosity stabilises foam

Surface Shear Viscosity

A high surface shear viscosity should also stabilise the foam. This occurs, for example, through the addition of higher alcohols or other water-insoluble substances. If the surface shear velocity is low the foam decomposes more quickly.

The foam can also be located in a finely dispersed state between solids. Such foams can be very firm and can form a stable surface when they dry out, as is also observed in the case of floating sludge in the biological cleaning stage.

(b) (4)

Apart from surfactants, there are also high molecular organic compounds which can accumulate at the water/air interface. These include, for example:

High molecular organic compounds

- **Saponins** (in sugar beet etc.)
- **Proteins** (e. g. gelatine, casein and albumin)
- **Polysaccharides** (e. g. starch, pectins and hemi-cellulose)
- **Cellulose derivatives** (e. g. cellulose ethers and carboxy methyl cellulose)
- **Humic acids**
- **Polyelectrolytes**

In some cases extremely high stabilization of foams which have been formed can be produced by these substances. These obstinate, stable and elastic foams are so-called micro bubble foams.

Possible consequence: micro bubble foam

Apart from the dissolved substances mentioned, under certain conditions a foam can also be stabilized by finely distributed solids, which are concentrated on the surface of the foam bubbles, such as fibre fragments (e. g. cellulose, synthetic plastic and mineral fibres), pigments (and fillers), calcium soaps and metallic hydroxides. In some cases, for example in paper mills and sewage plants, any foam that is formed visibly on the surface of the aqueous phase indicates at the same time the presence of an "invisible foam," i. e. the dispersed air in the aqueous phase.

Stabilization brought about by finely distributed solids

"Invisible foam" = dispersed air

On the other hand, aqueous phases containing solids (suspensions) can contain considerable quantities of air, without any significant surface foam being formed. Although there are essentially the same substances that promote the formation of surface foam and increase the air content of the suspension, the mechanism is different.

The air content depends to a considerable extent on the interaction between the solids surface and dispersed air. The small air bubbles (diameter in the range 10 - 100 µm) may be retained or rejected, depending on the charge of the solids surface. Since this charge depends on the pH-value and the electrolytes dissolved in the water, the air content is also affected by these factors. Substances which produce a hydrophobic effect on the solids surface, e. g. resins, can also increase the air content. Figure 10 shows an enlarged section of a suspension containing air.

Air content

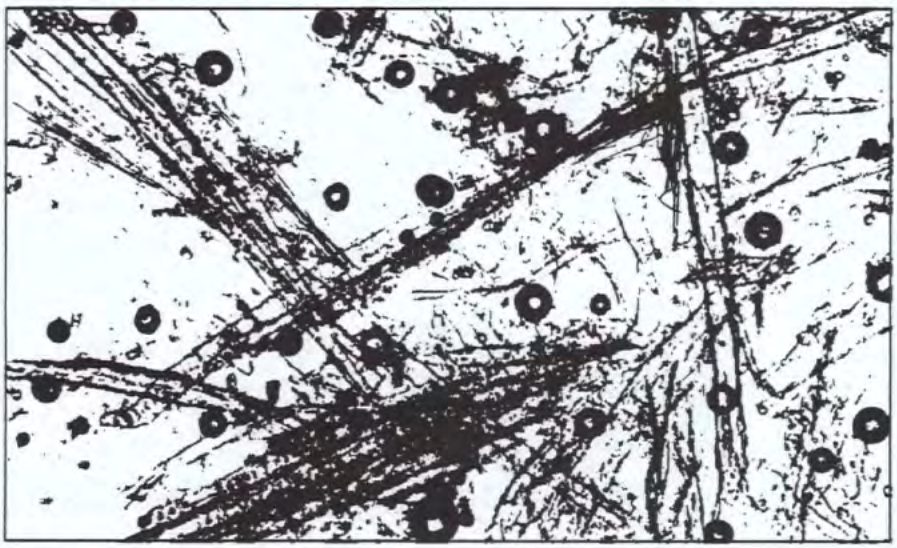


Fig. 10: Solid suspension containing air (paper industry)

The dispersed air often also causes undesirable flotation of the solid particles.

3. Defoamers

Mechanism of action of defoamers

As the lamella becomes thinner it reaches a point at which boundary layer forces prevent a further reduction in the thickness of the wall. These forces may be electrostatic or steric and come from the surface-active substances which make the foam possible.

A defoamer must be present as undissolved particles or droplets in the medium that is to be defoamed. In contrast to what was stated in many older publications, it has not been possible to establish in more recent literature any connection between the effect of the defoamer and the spreading of the oil on the water-air surface.

Often the mechanism of action is considered to be the replacement of the foaming substance in the lamella by a spread film of a new substance, which can be equated with elimination of the stabilising surface tension gradient. In some cases this mechanism does indeed appear to apply. For example, the addition of octanol results in a very rapid reduction in surface tension.

However, analyses conducted up to now have not shown any connection between the reduction in surface tension as a result of a spreading liquid in the lamella and the effect of the defoamer. It has also not been possible to prove by experiment the creation of a surface tension gradient through the spreading of the defoamer liquid. It is possible that the spreading liquid draws the underlying liquid of the foam lamella along with it, with the result that this becomes thinner and tears. This would create a kind of Marangoni spreading.

3.1 Oil and Fat Defoamers

Defoamers consist of oils, fats, polyalkylene glycols and emulsifiers and usually take the form of a clear to slightly turbid oily liquid. When they are diluted with water, a low-stability emulsion is produced. The resulting oil droplets have a defoaming effect in combination with the polyalkylene glycols. Similar principles to those described under 3.3 apply to the mechanism of action.

3.2 Polyalkylene Glycols

Polyalkylene glycol ethers and esters are effective above the cloud point

This group also includes the so-called EO-PO block polymers, the ethylene diamine EO-PO block polymers, the polyalkylene glycols based on polyols and the fatty alcohol EO-PO esters, as well as the fatty acid EO-PO esters. These are non-ionic, low-foam surfactants which have a defoaming effect in the vicinity of or above their cloud point. The cloud point is the temperature range in which the surfactants become insoluble in water. Often it is not possible to measure an exact cloud point in water because the solutions are already turbid in water at a temperature of 10 – 20 °C and become even more turbid when the temperature is increased.

The process of becoming turbid is reversible, i. e. upon cooling down the solutions become clear again, or at least clearer than they were. A polyalkylene glycol does not have a standard molecular weight, which is why it is not possible to determine a specific cloud point. Some molar weight fractions are soluble for longer, while others are soluble only for a shorter time. As the cloud point can be influenced by other chemicals, all cloud point information should only be seen as approximate values. This system is comparable with oil defoamers, as a mixture of organic phase (droplets) and water is produced in the vicinity of the cloud point. In contrast, the oil phase here consists of insoluble surfactant droplets however.

Unlike the turbidity range in water, the analytically determined cloud point is a physicochemical variable used to characterise a surfactant. This cloud point is often measured in butylene diglycol (BDG) or BDG-water mixtures, but its relevance to the effectiveness of a defoamer in application technology is limited.

3.3 Defoamers Consisting of Oils and Hydrophobic Particles

Mechanism of action of oil defoamers with and without hydrophobic particles

The use of hydrophobic particles in oil defoamers based on mineral oils or silicone oils has been described in the patent literature for a long time (1950), and the theme has also been taken up in literature since 1970. It is generally known that oils or hydrophobic particles alone have only a slight defoaming effect with respect to anionic surfactants. When used together their performance increases approximately sevenfold.

If solid particles are suspended at the liquid-liquid interface and belong to both interfaces, they possess an angle of contact to both surfaces. For a good defoaming effect a low level of wettability (high contact angle) is required. Therefore the air-water contact angle should be more than 90°.

Hydrophobic particles that can be used are, for example, waxes, stearin, paraffins, metal soaps and hydrophobised silicic acid. Quantities as small as 1 % hydrophobised silicic acid in mineral oil are effective.

The above statements concerning the spreading behaviour of oils also apply in these oil-particle mixtures, although it is certain that the particles do not have any effect on the spreading behaviour of the oils.

Although not proven experimentally, but possibly of decisive significance, is the formation of oil lenses on the lamella. These form bridges which subsequently tear.

Also the theory that hydrophobic particles absorb surfactants from the lamella and transport them to the water has been shown to be false.

In contrast, it has been shown beyond doubt that compounds form from oil droplets with hydrophobic particles.

3.3.1 Particle Geometry

Particles with sharp corners and edges are more effective than round or smooth particles, as experiments have shown. Precipitated particles often have an amorphous, undefined structure.

It has been shown that the film first has to become thinner and achieve approximately the dimensions of the particles. This process takes some time; the following film tear occurs within <0.001 s. The smaller the particles, the longer the drying of the lamella takes until it reaches the particle size. The smaller the particles, the more of them are contained in the medium for a given dose, and the probability of a film tear increases.

The thickness of the lamella lies approximately within the following orders of magnitude:

Thickness of the foam lamella

■ 1 to 10 µm	Interference colours
■ <0.1 µm	Black films
■ approx. 0.05 µm	Film tear

This means that even without the effect of the hydrophobic particles the film tears at 0.05 µm. Hydrophobic particles in defoamers should therefore be larger than 0.05 µm and smaller than 1 µm.

Alkylene oxide adducts

In contrast, the defoaming properties of the products based on alkylene oxide adducts depend on the fact that at a certain temperature – the so-called cloud point – a second phase that is richer in defoaming agents separates off. This process is induced by the higher solubility at low temperatures and lower solubility at higher temperatures. This phase has a very destructive effect on the foam. However, the optimum level of foam destruction need not necessarily lie at the temperature of the cloud point.

3.3.2 Capturing the Particles with Air Bubbles and Fusing the Particles

When the foam bubbles rise or are swirled up they can absorb hydrophobic particles from the defoamer, which ultimately results in the disintegration of the bubble. Rough particles and those with corners and edges are more likely to be captured than smooth particles. If one considers oil-particle compounds, the role of the particles appears to consist in making possible the penetration of the oil droplets into the air-water surface.

Hydrophobic materials for defoamers often have melting points of less than 100 °C. It has been shown that close to the melting point the defoaming effect of the particles is greatly reduced.

The melting point/dissolving temperatures of the hydrophobic particles lie within the range 80 – 90 °C in the oil phase. It is of no significance whether the wax melts or is dissolved in the oil.

Performance may be better shortly before the melting point because round particles have then already been deformed by hydrodynamic forces. However, at this point performance may also become worse in the case of angular particles.

3.3.3 Mechanism of Action of Hydrophobic Particles in Oils

Mechanism of action

No systematic analyses have so far been carried out on the influence of particle size on the effectiveness of oil-particle defoamers. However, if a partial mechanism is penetration into a double layer between the oil droplets and air bubbles, the particle size should lie within the same order of magnitude as the thickness of the lamella, e. g. >0.1 µm and <10 µm.

It can be assumed that oil droplets and hydrophobic particles form combined structures (compounds) which initially settle on the foam lamella as a lens. Thereafter the compounds penetrate to the other side of the lamella and form a bridge in the film. This film dries out in exactly the same way as a film without hydrophobic particles. The drying out process is the rate-determining step in the defoaming effect.

The film tear then occurs within a very short time.

(b) (4)

Observations of mineral oils and hydrophobic silicates indicate that improvements to defoamer effectiveness for a given dosage are possible by reducing the size of the compounds to at least 1 – 2 µm.

3.4 Mechanism of Action of Deaerating Agents

Hydrophobic deaerating agent particles are embedded in the interface of the air bubbles dispersed in water, where they are drawn slightly to the centre of the bubble.

If a second air bubble now touches the embedded deaerating agent particle, disequilibrium then results. The deaerating agent particle cannot take up its previous resting position, either in the first or in the second bubble. The result is that the interface in the vicinity of the deaerating agent particle is subjected to an increasing contact pressure, until both bubbles coalesce. The bubble growth continues in this way. The upthrust of a bubble, which has been formed by the coalescence of two bubbles of about the same size, is double that of the original bubbles. This results therefore in rapid deaeration.

Rapid deaeration due to bubble growth

Bubble growth is directly dependent on the number of bubble collisions per time unit. Therefore a deaerating agent is also expected to achieve its optimum effect in turbulent zones.

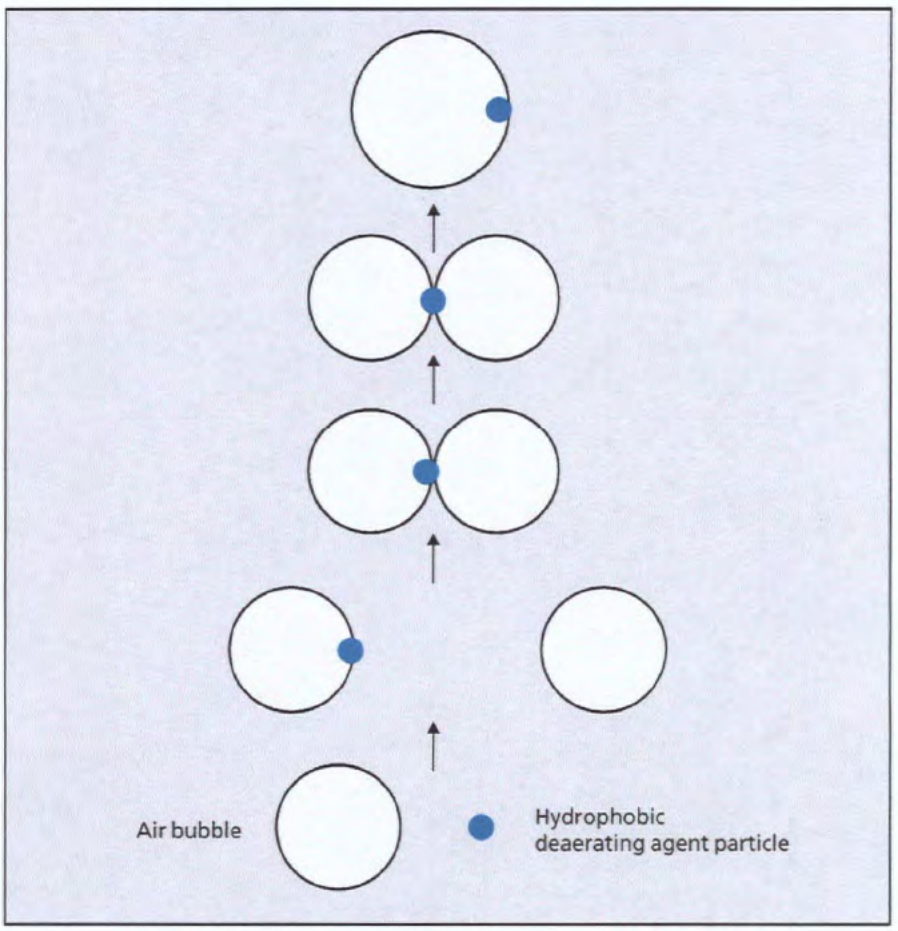


Fig. 11: Mechanism of action of a deaerating agent

3.5 Composition of Defoaming and Deaerating Agents

The following are important preconditions for the effectiveness of defoaming and deaerating agents:

- **Lower surface tension** than the medium to be defoamed (deaerated)
- **Low solubility** in the medium to be treated
- A sufficiently **fine dispersion** (formation of **particle size with optimum effect**)

Normally defoaming and deaerating agents should not react with the medium to be defoamed. There are a few exceptions to this rule, e. g. defoaming agents for the attack of phosphate rock with mineral acids. Furthermore the defoaming agent should act over a long period, i. e. the defoaming agent with the best initial effect (spontaneous effectiveness) is not necessarily the best defoaming agent. In the same way not all defoaming agents are at the same time good deaerating agents.

Not all defoaming agents are at the same time good deaerating agents

Numerous surface active substances, e. g. some non-ionic alkylene oxide adducts, are known to act as defoamers. Just as important as these are the defoaming and deaerating agents which are produced from formulations of different substances. Components of such formulations may include the following:

Surface active substances, e. g. alkylene oxide adducts

Formulations

- **Alkylene oxide adducts**
- **Hydrocarbons** (including mineral oils, white oil, paraffins)
- **Alcohols**
- **Fatty acids**
- **Fatty acid salts of multivalent cations**
- **Esters** (including fats, waxes, phosphoric esters)
- **Amides** (including amide waxes)
- **Silicone oils**
- **Silicas**

(b) (4)

Chemical composition of defoaming and deaerating agents

Defoaming and deaerating agents can be appropriately classified according to their chemical composition, as follows:

- **Fat defoamers**
  Fat defoamers are products containing a fat substance as their effective component. They may, however, also contain hydrocarbons, alcohols and emulsifiers.
- **Alkylene oxide adducts or defoaming agents containing alkylene oxide adducts**
  Defoaming agents of this type contain non-ionic alkylene oxide adducts as effective substance. They may, however, also contain hydrocarbons, alcohols, esters, water and emulsifiers.
- **Defoaming agents containing metal soaps**
  Defoaming agents containing metal soaps include fatty acid salts of multivalent cations (e. g. calcium, aluminium) as effective substance. Hydrocarbons, alcohols and esters are used as solvents or dispersing agents, and an emulsifier may also be additionally included.
- **Silicone or silicone containing defoaming agents**
  Silicone or silicone containing defoaming agents include silicone oils as effective component. Hydrocarbons, alcohols, esters, alkylene oxide adducts and emulsifiers may also be included.
- **Wax defoaming agents**
  Wax defoaming agents contain a finely dispersed wax (dispersed phase) as effective substance. Hydrocarbons, alcohols and esters are used as dispersing agents, and emulsifiers may also be additionally included.
- **Dispersion defoaming agents**
  Dispersion defoaming agents are dispersions (suspensions, emulsions) of hydrocarbons, alcohols and esters in water containing dispersing agents and generally also contain an emulsifier.
- **Sulpho-carboxylic ester defoaming agents**
  These defoaming agents contain a sulpho-carboxylic ester as the effective component. The alcohol, which has a defoaming effect, is gradually formed by hydrolysis under extreme pH conditions.

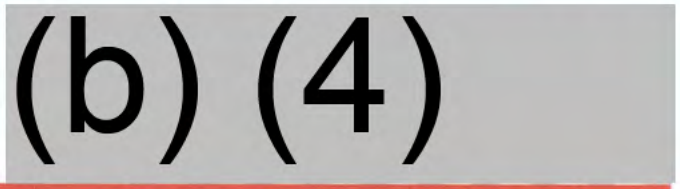
Soluble, emulsifiable and non-emulsifiable defoaming agents

Defoaming agents can also be differentiated according to their behaviour during application. There are soluble (see explanation on turbidity point of alkylene oxide adducts), emulsifiable (finely to coarsely dispersed) and non-emulsifiable defoaming agents.

Selection of the correct defoaming agent by carrying out practical foam tests in the laboratory

Many defoaming agents are very specific in their effectiveness, i. e. the most suitable defoaming agent must generally be selected empirically. Therefore, in order to select the correct defoaming agent a practical foam test must generally be carried out in the laboratory.

3.6 Recommended Fields of Application for (b) (4) Defoaming and Deaerating Agents



Mining
Salt pastes for dust binding
Chemical industry
Effluent
Bauxite extraction (white operation)
Dispersion paints
Emulsion polymerisation
Gas washing
Gelatine (solutions, emulsions)
Sewage plant (biological, mechanical)
Paint spraying cabins
Sea water desalination
Metal working oil emulsions
Methyl cellulose production
Mineral fibre industry, process water
Phosphoric acid production (wet)
Super phosphate extraction
Surfactant solutions (alkaline)
Compound fertilizer production
Washing and dry cleaning solutions
Paper and cellulose industry
Cellulose production
Pulp
Size press
Coating
Effluent
Food industry, biotechnological processes
Alkohol distillation
Alkohol fermentation
Amino acid production
Fermentation processes
Bottle cleaning plants
Yeast production
Potato processing
Effluent/wash water
Effluent
Starch production
Protein extraction
Cheese factory effluent
Dairy effluent
Pectin production (effluent)
Slaughterhouse
Effluent
Scalding plant
Starch saccharification
Sugar extraction from beet
Outside operation
Inside operation

In addition to the (b) (4) products listed here, test products are permanently being developed for special fields of application. Please contact our sales engineers or our technical application division for information concerning these products. With respect to their use in the food industry, please also note the information contained in chapter 5.

(b) (4)

4. Testing of Defoaming and Deaerating Agents in the Laboratory

There are a number of different methods of testing the effectiveness of (b) (4) products in the laboratory. Foam is produced with the medium to be defoamed or deaerated by shaking, stamping, stirring, (pump) circulating or introducing air (or gas). The (b) (4) products can be added either before or during the application of mechanical force (after foam has already been produced), or in individual cases also afterwards. It is important that all parameters are kept constant during the test.

Constant parameters

Optimal (b) (4) products for solving foam problems can be selected with laboratory tests. The precise dosing quantities required have to be determined by field tests at the plant.

4.1 General Selection Criteria for Defoaming and Deaerating Agents

In order to limit the selection from the range of possible (b) (4) products right from the start, the following criteria should be first clarified:

Criteria

- **Type of medium** (e. g. protein or surfactant-containing effluent)
- **Temperature of medium**
  (it is essential that this is also observed during the laboratory tests)
- **pH-value**
- **How is the foam produced** (e. g. by stirring or pumping)?
- **Is a long-term effect of the defoaming product necessary**
  (e. g. in a closed loop process)?
- **Should the ANTIFOAMAN (b) (4) product be used in diluted form, i. e. must it be emulsifiable?**
- **Are authorisation documents required for the defoaming product**
  (e. g. FDA, BfR, HACCP)?
- **Other requirements** (e. g. degradability, toxicology, sterilizability)

The more exact the general criteria for defoaming and deaerating agents are investigated at the place of application, the greater is the probability that the (b) (4) product which is suitable in the laboratory tests will also guarantee an optimum problem solution in later operation.

4.2 Laboratory Tests with (b) (4) Defoaming and Deaerating Agents

When carrying out laboratory tests with (b) (4) products, it should be ensured that the test apparatus is thoroughly cleaned each time after use. Unsatisfactory cleaning, i. e. residues of defoaming agent left in the apparatus, may be the cause of significant deviations, and therefore faulty assessment of the test results.

4.2.1 Shaking Method

The shaking method is very easy to carry out, and is suitable as a rapid orientation test to determine the most suitable (b) (4) product for the particular foam problem. This test can also be carried out locally. Screw cap glass jars are half filled with the medium to be defoamed; 1 drop of each different product is put onto the surface of the medium in each of the jars by means of a thin glass rod. The jars are then closed and each is shaken in the same way for the same time.

This method can only be carried out with media which foam relatively easily (otherwise either no foam is produced or 1 drop of each (b) (4) product is too high a dosage). It only shows the spontaneous effect, but not the long-term effect of an (b) (4) product.

Comparison with a blank (without addition of a defoaming product), or comparison of the jars with each other, immediately and after a certain standing time, generally shows clearly which (b) (4) products are suitable (see figure 12).

Test parameters are:

- Duration or frequency of shaking,
- Test temperature and
- Quantity of the particular (b) (4) product added.

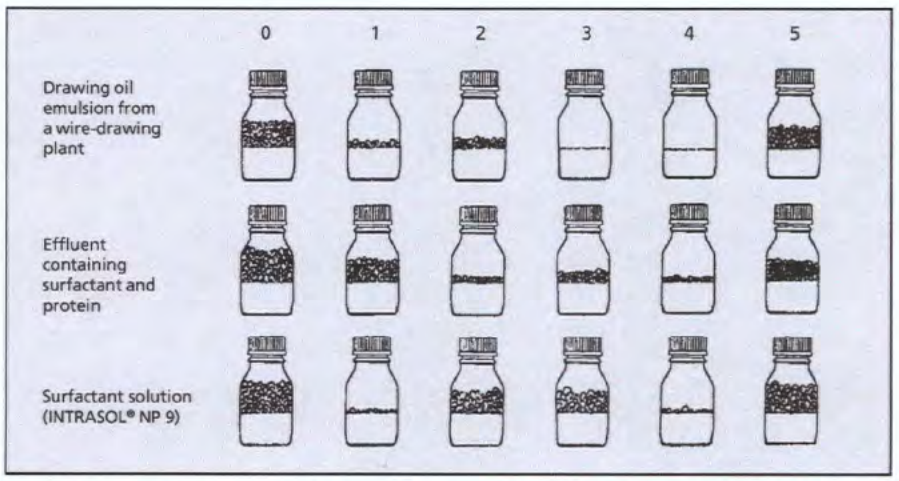


Fig. 12: Selection of the correct (b) (4) product using the shaking method (500 ml jars)

Defoaming agents:

0 Without

1 (b) (4)
Fat defoaming agent type

2 (b) (4)
Alkylene oxide adduct type

3 (b) (4)
Metallic soap containing defoaming agent type

4 (b) (4)
Wax defoaming agent type

5 (b) (4)
Dispersion defoaming agent type

(b) (4)

4.2.2 Perforated Disc Impact Method

Foam volume and foam stability

This test is carried out based on DIN 53 902. The criteria used for assessing the effectiveness of an (b) (4) defoaming agent are the foam volume and foam stability. The foam is produced by stamping onto the test medium in an upright cylinder with a perforated disc fitted onto a handle. In order to determine the foam volume and stability, the quantity of foam produced is measured immediately after stamping and after standing for a certain time.

Test parameters are:

- Test temperature and
- Quantity of the particular (b) (4) product added.

Quick testing

The perforated disc impact method is suitable for quick testing, e. g. for synthetic plastic dispersions which may be stored for several months after addition of an (b) (4) defoaming agent. In this case samples can be taken at certain intervals, and the "actual condition" with respect to foaming behaviour can be determined.

Foam behaviour

This method gives no information about the long-term effect of an (b) (4) defoaming agent.

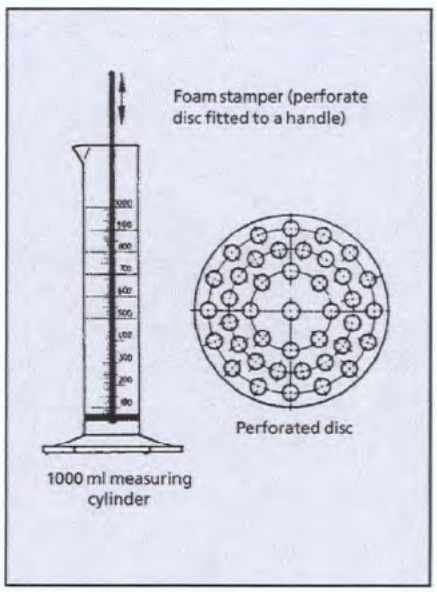


Fig. 13: Perforated disc impact method

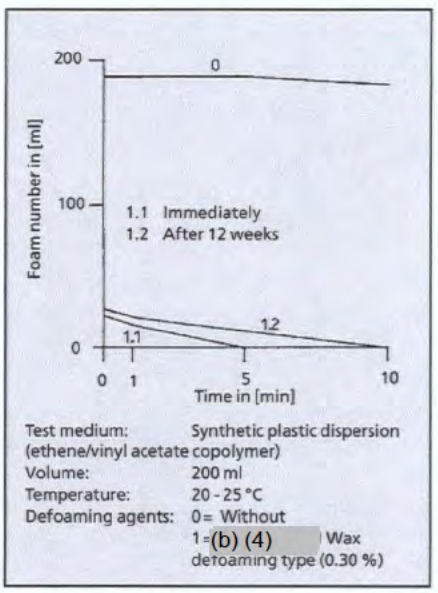


Fig. 14: Foam stability using an (b) (4) defoaming agent

4.2.3 Stirring Method

A certain quantity of the test medium to which a small quantity (generally 5 – 100 ppm) of the selected (b) (4) defoaming agent has been added, is intensively stirred (generally using a propeller stirrer, n = 1000 – 2000 min^{-1}) at the test temperature (temperature at the sampling point for the test medium). The height to which the foam rises or the extent to which it collapses after switching off the stirrer, is measured in relation to time.

Rise and collapse of foam

Test parameters are:

- Test volume,
- Stirrer type,
- Stirrer speed,
- Stirring time and
- Quantity of particular (b) (4) defoaming agent added.

The effectiveness of the (b) (4) defoaming agent depends on the shear intensity (rotations of stirrer) and the length of time the shear force is applied.

The results obtained by the stirring method can be correlated to shear sensitivity and the long-term effect of ar (b) (4) roduct in a given test medium.

Shear sensitivity and long-term effect

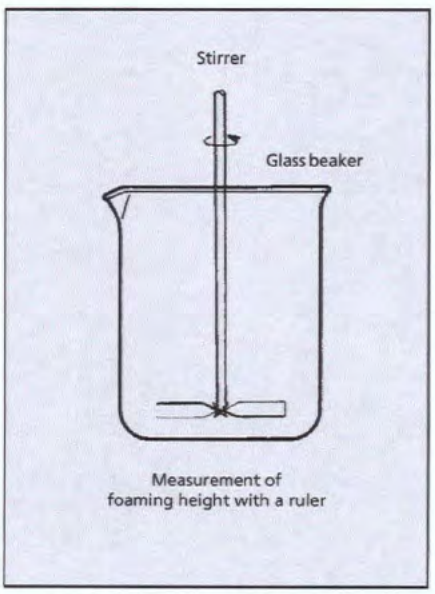


Fig. 15: Stirring method (without heating)

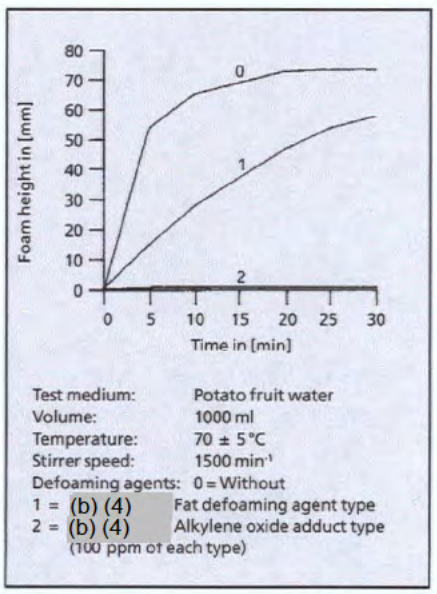


Fig. 16: Effectiveness of various (b) (4) products tested using the stirring method

(b) (4)

4.2.4 Pump Circulation Method

In the pump circulation method foaming is produced by circulating the test medium at the test temperature (temperature at the sampling point of the test medium). Various types of pumps can be used, e. g. centrifugal, tubular and diaphragm. The height of the foam or the extent to which the foam collapses after switching off the pump is measured in relation to time.

Rise and collapse of foam

Test parameters are:

- Test volume,
- Height of fall (from tube outlet to surface of test medium),
- Pump type,
- Pump performance,
- Pumping time and
- Quantity of particular (b) (4) defoaming agent added.

The shear force applied in this method is different to that in the stirring method. The pump circulation method gives results which can be correlated to shear sensitivity (during pumping), and the long-term effect of an (b) (4) defoaming agent in a certain test medium.

Shear sensitivity and long-term effect

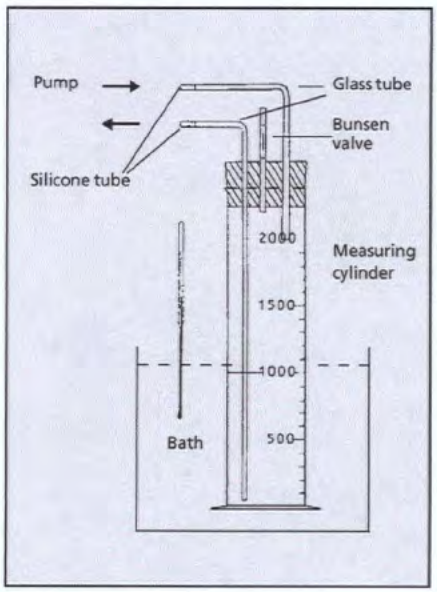


Fig. 17: Pump circulation method

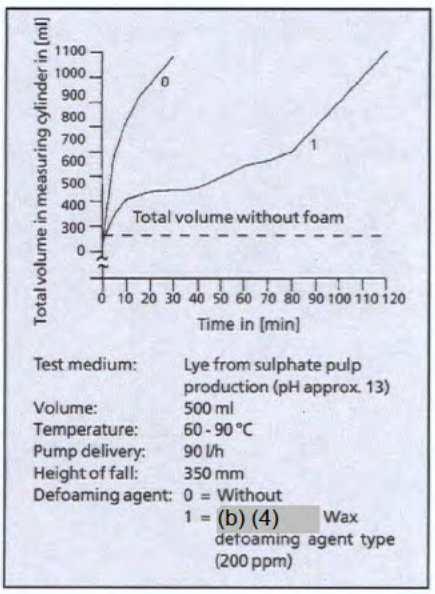


Fig. 18: Long-term effect of an (b) (4) product tested using the pump circulation method

4.2.5 Air (or Gas) Injection Method

In this test air or gas is blown through a sinter plate at the lower end of a vertically standing tube, which is partly filled with the test medium, and the height or drop of the foam level is measured in relation to time. The particular (b) (4) defoaming agent can be put on the foam immediately before the beginning of the test or during the test. If the product is put on an already existing foam, its spontaneous attack can be very well observed.

Test parameters are:

- Test volume,
- Test temperature (only limited),
- Volume of air (or gas) introduced per time unit,
- Duration of air (or gas) injection and
- Quantity of particular (b) (4) defoaming agent added.

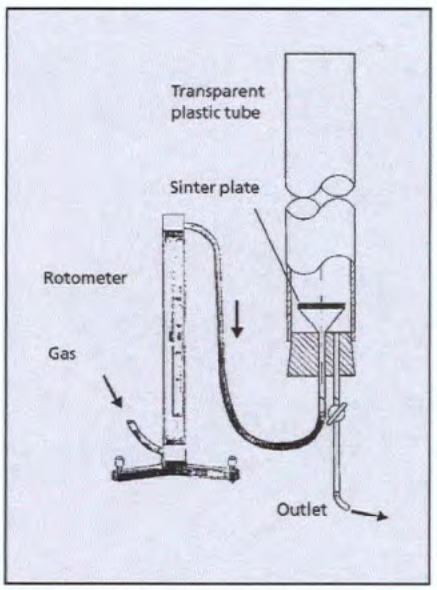


Fig. 19: Air (or gas) injection method

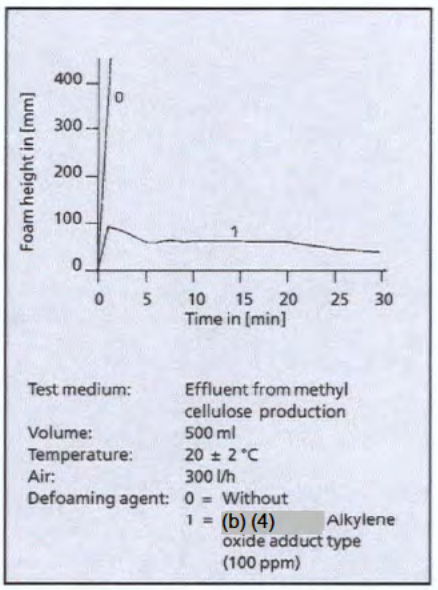


Fig. 20: Long-term effect of an (b) (4) defoaming agent tested using the air injection method

4.2.6 Special Test Methods

Special test methods have been developed for testing the effectiveness of defoaming products in a few processes in the chemical industry, e. g. wet attack of phosphate rock with mineral acids. In this process the attacked rock mixture foams mainly because of gases formed during the attack process.

(b) (4)

5. Defoaming Agents for Food and Animal Feed

Requirements

For the production of a commodity that comes into contact with food or a processing auxiliary used in the production of a foodstuff, special requirements are placed on process auxiliaries.

Examples of Listings, Laws and State Recommendations:

■ USA	FDA paragraphs
■ Europe	The CoE's Paper Resolution
■ Germany	BfR recommendations

Examples of our own Safety Certificates:

■ USA	Opinion Letter
■ Europe	HACCP analysis and/or migration analyses

Before use as a process or processing auxiliary in the production of foodstuffs, a checklist has to be completed. Analysis is carried out on the basis of the details provided concerning whether the statutory preconditions for use in the food industry have been fulfilled.

6. Solution for your Foam Problems

Our laboratory experts will be pleased to help you in selecting the suitable test method. For tests in our laboratory a representative sample (approx. 10 – 20 l) of the medium to be defoamed should be submitted. Taking into account the general selection criteria stated under section 4.1, our technical staff will propose the suitable (b) (4) defoamer for a plant trial.

(b) (4)

(b) (4)

® Registered trademark.

All statements, information and data presented herein are believed to be accurate and reliable but are not to be taken as a guarantee, express warranty or implied warranty of merchantability or fitness for a particular purpose, or representation, express or implied, for which seller assumes legal responsibility, and they are offered solely for your consideration, investigation and verification. Statements or suggestions concerning possible use of this product are made without representation or warranty that any such use is free of patent infringement and are not recommendations to infringe on any patent.

(b) (4)

T-3

DSM Nutritional Products
45 Waterview Boulevard
Parsippany
NJ 07054
United States of America

phone +1 800 526 0189
fax +1 973 257 8675

Dr. Andrea Krause
Center for Veterinary Medicine
Ingredient Safety Team (HFV 224)
7519 Standish Place
Rockville, Maryland 20855

Date
February 8, 2013

Sodium Benzoate Use in an Enzyme Preparation

Dear Dr. Krause

DSM is responding to your phone call of 20 December 2012 in which you requested additional information to substantiate the use of sodium benzoate in Ronozyme® HiPhos (L) at a concentration of 0.15%. I have received confirmation from our manufacturing partner that the quantity of sodium benzoate added to the liquid enzyme preparation is indeed 1.5 grams per kilogram of finished goods.

Sodium Benzoate is listed as a GRAS substance for human food at 21 CFR 184.1733 (42 Fed. Reg. 14653, March 15, 1977) based upon SOGS report #7 of 1973. At 21 CFR 184.1733 it is noted that the "*Current usage results in a maximum level of 0.1 percent in food. (The Food and Drug Administration has not determined whether significally different conditions of use would be GRAS.*"

In 1976 the commissioner of the FDA determined that a number of substances that had been identified as being GRAS for human food were also GRAS for animal food. In the case of Sodium Benzoate which is regulated for use in animal food as a chemical preservative at 21 CFR 582.3733 the current usage of the human food regulation was transcribed as "*...a level not exceeding 0.1 percent in accordance with good manufacturing or feeding practice.*"

At 21 CFR 582.1 (b) (2) good manufacturing or feeding practice is further defined as "*The quantity of a substance that becomes a component of animal food as a result of its use in the manufacturing, processing, or packaging of food, and which is not intended to accomplish any physical or other technical effect in the food itself, shall be reduced to the extent reasonably possible.*"

Ronozyme® HiPhos (L) is an ingredient used in the manufacture of food for poultry. The material is not a final food. It is not intended to be fed directly to animals. Ronozyme® HiPhos (L) is in essence a dilute protein solution with a moderate pH that is susceptible to mold growth. Labeling for the product instructs the user to add 25 to 4000 grams to 2000 Kg for finished feed.

When the enzyme product is preserved with 0.15% sodium benzoate the product maintains the enzyme activity and low microbiological load expected for commercial distribution and use. The sodium benzoate

DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

in our product is not intended to accomplish any physical or technical effect in the food itself. The results of storage studies provided in our dossier substantiate the practicality of the current formulation. We have determined that the use of 0.15 % sodium benzoate in the manufacture of our product is necessary for preservation.

When Ronozyme® HiPhos (L) is used at the highest use level noted in our dossier, 4000 FTY / Kg of finished food, the concentration of sodium benzoate in the finished food due to the use of the enzyme preparation would be 0.3 mg/Kg or 0.00003% well below the maximum level for animal food.

Ronozyme® HiPhos (L) has 20,000 FTY /g
4000 FTY / (20,000 FTY/g) = 0.2 g of Ronozyme HiPhos (L)
0.2 g of Ronozyme® HiPhos (L) x (1.5 g sodium benzoate/Kg of Ronozyme® HiPhos (L)) = 0.0003 g or 0.3 mg
0.3 mg/Kg = 0.00003 %

Per the NRC ***Predicting Feed Intake of Food Producing Animals*** (National Academies Press, 1987):
Chickens consume about 0.1 Kg of feed per day and weigh about 2 Kg. The exposure to sodium benzoate for a chicken would therefore be 0.03 mg/day or 0.015 mg/Kg of body weight.
Turkeys consume about 0.5 Kg of feed per day and weigh about 8 Kg. The exposure to sodium benzoate for a turkey would therefore be 0.15 mg/day or 0.019 mg/Kg of body weight.

Ronozyme® HiPhos (L) was used for the toxicology studies presented in the dossier. No adverse effects were noted in these studies even at levels greatly in excess of the highest proposed use level. The results of these studies indicate that the use of sodium benzoate at 0.15% wt/wt of the enzyme preparation does not present a safety risk.

The EPA risk assessment for Benzoic Acid, see attached file, discusses several animal studies where it is noted that no adverse effects were reported in rats at doses as high as 1% of the diet, equivalent to 50 mg/Kg bw/day. A lifetime study in mice at >3350 mg/Kg bw/day revealed no abnormal histopathology in any of the 11 organs evaluated. Both no adverse effect exposure levels are thousands of times greater than that possible with the use of Ronozyme® HiPhos at the highest suggested use level.

Because the exposure to sodium benzoate due to its use in Ronozyme HiPhos (L) is below the exposure anticipated in the regulation and is several thousand times less than the no effect level from toxicological investigations, DSM believes that the use of sodium benzoate at 0.15% in the animal food ingredient, Ronozyme® HiPhos, is a safe and appropriate use of the additive in compliance with the regulations.

Kind regards

James La Marta, Ph.D.

RECEIVED
FEB 14 2013
By

James La Marta, Ph.D.
Senior Manager Regulatory Affairs

Benzoic acid (CASRN 65-85-0)

view QuickView

MAIN CONTENTS

List of IRIS Substances
Search IRIS by Keyword go
IRIS Summaries/Toxicological Reviews
Entire IRIS Website

Reference Dose for Chronic Oral Exposure (RfD) go

0355

Benzoic acid; CASRN 65-85-0

Human health assessment information on a chemical substance is included in the IRIS database only after a comprehensive review of toxicity data, as outlined in the IRIS assessment development process. Sections I (Health Hazard Assessments for Noncarcinogenic Effects) and II (Carcinogenicity Assessment for Lifetime Exposure) present the conclusions that were reached during the assessment development process. Supporting information and explanations of the methods used to derive the values given in IRIS are provided in the guidance documents located on the IRIS website.

STATUS OF DATA FOR Benzoic acid

File First On-Line 09/07/1988

Category (section)	Status	Last Revised
Oral RfD Assessment (I.A.)	on-line	07/01/1993
Inhalation RfC Assessment (I.B.)	no data	
Carcinogenicity Assessment (II.)	on-line	05/01/1991

_I. Chronic Health Hazard Assessments for Noncarcinogenic Effects

_I.A. Reference Dose for Chronic Oral Exposure (RfD)

Substance Name — Benzoic acid
CASRN — 65-85-0
Last Revised — 07/01/1993

The oral Reference Dose (RfD) is based on the assumption that thresholds exist for certain toxic effects such as cellular necrosis. It is expressed in units of mg/kg-day. In general, the RfD is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. Please refer to the Background Document for an elaboration of these concepts. RfDs can also be derived for the noncarcinogenic health effects of substances that are also carcinogens. Therefore, it is essential to refer to other sources of information concerning the carcinogenicity of this substance. If the U.S. EPA has evaluated this substance for potential human carcinogenicity, a summary of that evaluation will be contained in Section II of this file.

__I.A.1. Oral RfD Summary

Critical Effect	Experimental Doses*	UF	MF	RfD
		1	1	4E+0 mg/kg/day
No adverse effects observed Human daily per capita intakes FDA, 1973; Selected Committee on Review of the GRAS List	NOAEL: 34 mg/day benzoic acid and 328 mg/day for sodium benzoate (converted to 312 mg/day benzoic acid) LOAEL: none			

*Conversion Factors -- 328 mg/day sodium benzoate x [122.12 (MW benzoic acid)/144.11 (MW sodium benzoate)] = 278 mg/day benzoic acid. 278 mg/day benzoic acid from sodium benzoate + 34 mg/day benzoic acid = 312 mg/day; assuming adult human body weight of 70 kg, the exposure dose is 312 divided by 70 = 4.4 mg/kg/day.

__I.A.2. Principal and Supporting Studies (Oral RfD)

FDA (Food and Drug Administration). 1973. Evaluation of the Health Aspects of Benzoic Acid and Sodium Benzoate as Food Ingredients. DHEW, Washington, DC. Report No. SCOGS-7. NTIS PB-223837/6.

Early studies (Gerlach, 1909) indicate that laboratory animals are inappropriate models for studying the toxicity of benzoic acid in humans (FDRL, 1972) (see Additional Comments). Based on data regarding the amounts of benzoic acid and sodium benzoate produced as a food preservative, FDA (1973) estimated a daily per capita intake of 0.9-34 mg for benzoic acid and 34-328 mg for sodium benzoate. At these levels, there are no reports of toxic effects in humans. These compounds have Generally Recognized as Safe (GRAS) status by FDA. Therefore, the upper ranges can be considered NOAELs for benzoic acid and sodium benzoate. In the stomach, both benzoic acid and sodium benzoate exist in their ionized form, benzoate, which is absorbed rapidly and completely by the GI tract. Therefore, exposure to sodium benzoate is comparable to exposure to benzoic acid if molecular weight differences are corrected for; here, 328 mg sodium benzoate is equivalent to 278 mg benzoic acid. Adding 278 to the daily intake for benzoic acid of 34 mg yields a total of 312 mg benzoic acid (see Conversion Factors). If no uncertainty factor is used, the RfD is 312 mg/day for a 70 kg human or 4 mg/kg/day.

__I.A.3. Uncertainty and Modifying Factors (Oral RfD)

UF — An uncertainty factor of 10 for the protection of sensitive subgroups was considered unnecessary; although reactions to benzoate and structurally related compounds do occur, an uncertainty factor of 10 would be of little value to the sensitive individuals.

MF — None

__I.A.4. Additional Studies/Comments (Oral RfD)

Sodium benzoate appeared to have no maternal toxicity, fetal toxicity, or teratogenicity in mice, rats, hamsters, or rabbits when given orally (FDRL, 1972). The highest doses tested were 175.0 in mice and rats, 300.0 in hamsters, and 250.0 mg/kg/day in rabbits.

The only chronic oral data available involve administration of benzoic acid to rats and mice (Shtenberg and Ignat'ev, 1970; Ignat'ev, 1965; Marquardt, 1960). A dose of 40 mg/kg/day for 17 months was associated with decreased resistance to stress in mice and possibly with reduced food and water intake in rats after 18 months (Shtenberg and Ignat'ev, 1970). However, another report from this laboratory (Ignat'ev, 1965) indicated that 80 mg/kg/day in rats for 18 months was not associated with adverse effects on body weight,

survival, or gross or microscopic pathology. If 40 mg/kg/day in mice in the study by Shtenberg and Ignat'ev (1970) is considered to be the LOAEL, application of an uncertainty factor of 1000 would result in an RfD of 0.04 mg/kg/day or 2.8 mg/day, which is near the lower end of the range of the estimated daily human exposure to benzoic acid (not including exposure to sodium benzoate). The lower RfD based on animal data is not unexpected, however, since application of uncertainty factors is intentionally conservative in the absence of human data. Since human data are available in this case, it is not appropriate to use the animal data for the RfD.

Other long-term dietary studies (Marquardt, 1960) showed decreased food intake and body weight in rats fed 1.5% benzoic acid (750 mg/kg/day); at a dose of 1.0% in the diet (50 mg/kg/day) there were no signs of toxicity or adverse reproductive effects.

Gerlach (1909) reported no externally visible effects in humans ingesting benzoic acid at 0.5-1.0 g/day for 44 consecutive days or for 82/86 or 88/92 days. Assuming a human body weight of 70 kg, this level corresponds to a dose of 14 mg/kg/day. Wiley and Bigelow (1908), however, observed irritation, discomfort, weakness, and malaise in humans given oral bolus doses of less than or equal to 1.75 g/day over a 20-day period (25 mg/kg/day). The RfD (4 mg/kg/day) is well below these doses.

__I.A.5. Confidence in the Oral RfD

Study — Medium
Database — Medium
RfD — Medium

Medium confidence is placed in the FDA (1973) estimate of per capita intake. Medium confidence in the database reflects the inappropriateness of using animal data as the basis of the RfD for humans and the lack of reported effects in humans at the estimated intakes. Thus, confidence in the RfD is medium.

__I.A.6. EPA Documentation and Review of the Oral RfD

Source Document — U.S. EPA, 1987

Limited peer review and extensive Agency-wide review 1987.

Other EPA Documentation — None

Agency Work Group Review — 09/17/1987

Verification Date — 09/17/1987

Screening-Level Literature Review Findings — A screening-level review conducted by an EPA contractor of the more recent toxicology literature pertinent to the RfD for Benzoic acid conducted in August 2003 did not identify any critical new studies. IRIS users who know of important new studies may provide that information to the IRIS Hotline at hotline.iris@epa.gov or 202-566-1676.

__I.A.7. EPA Contacts (Oral RfD)

Please contact the IRIS Hotline for all questions concerning this assessment or IRIS, in general, at (202) 566-1676 (phone), (202)566-1749 (FAX) or hotline.iris@epa.gov (internet address).

_I.B. Reference Concentration for Chronic Inhalation Exposure (RfC)

Substance Name — Benzoic acid
CASRN — 65-85-0

Not available at this time.

_II. Carcinogenicity Assessment for Lifetime Exposure

Substance Name — Benzoic acid
CASRN — 65-85-0
Last Revised — 05/01/1991

Section II provides information on three aspects of the carcinogenic assessment for the substance in question; the weight-of-evidence judgment of the likelihood that the substance is a human carcinogen, and quantitative estimates of risk from oral exposure and from inhalation exposure. The quantitative risk estimates are presented in three ways. The slope factor is the result of application of a low-dose extrapolation procedure and is presented as the risk per (mg/kg)/day. The unit risk is the quantitative estimate in terms of either risk per ug/L drinking water or risk per ug/cu.m air breathed. The third form in which risk is presented is a drinking water or air concentration providing cancer risks of 1 in 10,000, 1 in 100,000 or 1 in 1,000,000. The rationale and methods used to develop the carcinogenicity information in IRIS are described in The Risk Assessment Guidelines of 1986 (EPA/600/8-87/045) and in the IRIS Background Document. IRIS summaries developed since the publication of EPA's more recent Proposed Guidelines for Carcinogen Risk Assessment also utilize those Guidelines where indicated (Federal Register 61(79):17960-18011, April 23, 1996). Users are referred to Section I of this IRIS file for information on long-term toxic effects other than carcinogenicity.

_II.A. Evidence for Human Carcinogenicity

__II.A.1. Weight-of-Evidence Characterization

Classification — D; not classifiable as to human carcinogenicity

Basis — No human data and inadequate data from animal bioassays.

__II.A.2. Human Carcinogenicity Data

None.

__II.A.3. Animal Carcinogenicity Data

Inadequate. In a lifetime study, Toth (1984) administered sodium benzoate (of 99% purity) to 50 male and 50 female 5 week-old albino Swiss mice at a level of 2% in the drinking water. Control groups consisted of 100 mice/sex. The dose level was selected based on results of a subchronic study in which levels of 4 and 8% were considered to be too toxic. The 2% level was equivalent to sodium benzoate doses of 4133 mg/kg/day for males and 3973 mg/kg/day for females. Based on average measured daily water consumptions of 6.2 mL for males and 5.9 mL for females and an assumed average body weight of 0.03 kg. The equivalent benzoic acid doses, adjusted for moleculer weight differences between sodium benzoate and benzoic acid, are 3502 mg/kg/day and 3367 mg/kg/day for males and females, respectively. Histopathologic examinations of all mice included 11 organs and all gross lesions. The treatment had no apparent effect on survival or tumor incidence.

As part of a 5-generation reproduction study, Shtenberg and Ignat'ev (1970) administered test compounds in a paste in daily doses of 40 mg/kg benzoic acid combined with 80 mg/kg sodium bisulfite in a paste before feeding an otherwise unspecified basic diet to a group of 50 white cross-bred mice/sex for 17 months. Another group received benzoic acid only; no further details were given. An unspecified number of control animals received only basic diet. Malignant tumors (not otherwise specified) occurred in 8/100 treated mice and 1/8 mice in the third generation of the treated group. Tumor incidences were not reported for untreated mice.

__II.A.4. Supporting Data for Carcinogenicity

Dinerman and Ignat'ev (1966) reported that a 3-month exposure to 0.2% benzoic acid in the diet increased the susceptibility of mice to the development of carcinomas following intraperitoneal inoculation with Erlich ascites carcinoma cells. Tumors developed in 62/90 (68.8%) of benzoic acid- treated mice and in 16/49 (32.6%) of the control mice.

Benzoic acid and sodium benzoate have been tested for mutagenicity or genotoxicity in prokaryotes (McCann et al., 1975), eukaryotes (Litton Bionetics, Inc., 1974), and several mammalian test systems (Litton Bionetics, Inc., 1974, 1975; Oikawa et al., 1980). No positive results have been reported.

_II.B. Quantitative Estimate of Carcinogenic Risk from Oral Exposure

Not available.

_II.C. Quantitative Estimate of Carcinogenic Risk from Inhalation Exposure

Not available.

_II.D. EPA Documentation, Review, and Contacts (Carcinogenicity Assessment)

__II.D.1. EPA Documentation

Source Document — U.S. EPA, 1987

The 1987 Health and Environmental Effects Document has received OHEA review.

__II.D.2. EPA Review (Carcinogenicity Assessment)

Agency Work Group Review — 03/01/1989

Verification Date — 03/01/1989

Screening-Level Literature Review Findings — A screening-level review conducted by an EPA contractor of the more recent toxicology literature pertinent to the cancer assessment for Benzoic acid conducted in August 2003 did not identify any critical new studies. IRIS users who know of important new studies may provide that information to the IRIS Hotline at hotline.iris@epa.gov or 202-566-1676.

__II.D.3. EPA Contacts (Carcinogenicity Assessment)

Please contact the IRIS Hotline for all questions concerning this assessment or IRIS, in general, at (202) 566-1676 (phone), (202)566-1749 (FAX) or hotline.iris@epa.gov (internet address).

_III. [reserved]
_IV. [reserved]
_V. [reserved]

_VI. Bibliography

Substance Name — Benzoic acid
CASRN — 65-85-0
Last Revised — 08/01/1989

_VI.A. Oral RfD References

FDA (Food and Drug Administration). 1973. Evaluation of the Health Aspects of Benzoic Acid and Sodium Benzoate as Food Ingredients. DHEW, Washington, DC. Report No. SCOGS-7. NTIS PB-223 837/6.

FDRL (Food and Drug Research Labs., Inc.). 1972. Teratologic Evaluation of FDA 71-37 (Sodium Benzoate). p.\75-79.

Gerlach, V. 1909. VII. Summary of the results. In: Physiological Activity of Benzoic Acid and Sodium Benzoate, V. Gerlach, Ed. Verlag von Heinrich Staadt, Wiesbaden. p.\90-92. (Cited in Informatics, Inc., 1972)

Ignat'ev, A.D. 1965. Experimental information contributing to a hygienic characterization of the combined effect produced by some food presentations. Vop. Pitan. 24(3): 61-68. (Cited in Informatics, Inc., 1972)

Informatics, Inc. 1972. GRAS (Generally Recognized as Safe) Food Ingredients: Benzoic Acid and Sodium Benzoate. p. 75-79.

Shtenberg, A.J. and A.D. Ignat'ev. 1970. Toxicological evaluation of some combinations of food preservatives. Food Cosmet. Toxicol. 8(4): 369-380.

U.S. EPA. 1987. Health and Environmental Effects Document for Benzoic Acid. Prepared by the Office of Health and Environmental Assessment, Environmental Criteria and Assessment Office, Cincinnati, OH for the Office of Solid Waste and Emergency Response, Washington, DC.

Wiley, H.M. and W.D. Bigelow. 1908. Influence of benzoic acid and benzo- ates on digestion and health. Bulletin 84, pt. IV, Bureau of Chemistry, U.S. Dept. Agriculture. (Cited in Informatics, Inc., 1972)

_VI.B. Inhalation RfD References

None

_VI.C. Carcinogenicity Assessment References

Dinerman, A.A and A.D. Ignat'ev. 1966. Effect of certain food preservatives on the development of tumors in mice. Gig. Sanit. 31(9): 38-42. (Eng. trans.)

Litton Bionetics, Inc. 1974. Mutagenic Evaluation of Compound FDA 71-37, Sodium Benzoate. Report No. LBI 2446-297, FDA, Washington, DC, PB-245-453/6.

Litton Bionetics, Inc. 1975. Mutagenic Evaluation of Compound FDA 73-70, Benzoic Acid Certified A.C.S. Report No. LBI-2468-376; FDABF-GRAS-376 PB-245- 500/4.

McCann, J., E. Choi, E. Yamasaki and B.N. Ames. 1975. Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. Proc. Natl. Acad. Sci. 72: 5135-5139.

Oikawa, A., H. Tohda, M. Kanai, M. Miwa and T. Sugimura. 1980. Inhibitors of poly(adenosine diphosphate ribose) induced sister chromatid exchanges. Biochem. Biophys. Res. Commun. 97(4): 1311-1316.

Shtenberg, A.J. and A.D. Ignat'ev. 1970. Toxicological evaluation of some combinations of food preservatives. Food Cosmet. Toxicol. 8(4): 369-380.

Toth, B. 1984. Lack of tumorigenicity of sodium benzoate in mice. Fund. Appl. Toxicol. 4(3): 494-496.

U.S. EPA. 1987. Health and Environmental Effects Document for Benzoic Acid. Prepared by the Office of Health and Environmental Assessment, Environmental Criteria and Assessment Office, Cincinnati, OH for the Office of Solid Waste and Emergency Response, Washington, DC.

_VII. Revision History

Substance Name — Benzoic acid
CASRN — 65-85-0

Date	Section	Description
09/07/1988	I.A.	Oral RfD summary on-line
05/01/1989	II.	Carcinogen assessment now under review
07/01/1989	I.A.	Principal study clarified
07/01/1989	VI.	Bibliography on-line
08/01/1989	II.	Carcinogen summary on-line
08/01/1989	VI.C.	Carcinogen references added
01/01/1991	I.A.	Text edited
01/01/1991	II.	Text edited
05/01/1991	II.A.3.	Text edited
06/01/1991	I.A.1.	Conversion Factor text clarified
01/01/1992	I.A.7.	Secondary contact changed
01/01/1992	IV.	Regulatory Action section on-line
07/01/1993	I.A.6.	Source Doc. year corrected; Other EPA Doc. clarified
04/01/1997	III., IV., V.	Drinking Water Health Advisories, EPA Regulatory Actions, and Supplementary Data were removed from IRIS on or before April 1997. IRIS users were directed to the appropriate EPA Program Offices for this information.
10/28/2003	I.A.6., II.D.2.	Screening-Level Literature Review Findings message has been added.

_VIII. Synonyms

Substance Name — Benzoic acid
CASRN — 65-85-0
Last Revised — 09/07/1988

65-85-0
benzenecarboxylic acid
Benzoic acid
carboxybenzene
dracylic acid
phenyl carboxylic acid
phenylformic acid

T-4

DSM Nutritional Products
45 Waterview Boulevard
Parsippany
NJ 07054
United States of America

phone +1 800 526 0189
fax +1 973 257 8675

Mr. Geoffrey Wong
Center for Veterinary Medicine
Ingredient Safety Team (HFV 224)
7519 Standish Place
Rockville, Maryland 20855
CC: Dr. Andrea Krause
Dr. Rial Christensen

February 22, 2013

Questions regarding DSM's GRAS Notification for Ronozyme® HiPhos

Dear Mr. Wong and Drs. Kause & Christensen

During our phone discussion of 11 February you requested the following information:

1) Dr. Christensen requested more information about possible open reading frames and a more detailed explanation of the unlabeled diagram on page 20 of the dossier. The response from our partner is in Attachment 1

2) CVM was interested in knowing why (b) (4) was added to the fermentation media

   (b) is an essential enzyme cofactor for the production organism and is added as part of a trace mineral mixture. Attachment 2 provides an overview of (b) utilization in microbial metabolism.

3) Explain the purpose of Sodium Thiosulfate in the 'M' formulation as it relates to 21 CFR 582.6807.

   Please see attachment 3a for an explanation and 3b is the EPA report cited in the explanation.

4) Provide Certificates of Analysis for three lots of any one of the three formulations

   Attachment 4 contains the certificates for three lots of Ronozyme HiPhos (M)

5) Provide the supplier's specifications for Zinc Acetate, Sodium Thiosulfate and Corn Steep Liquor

   Attachment 5 contains the specification sheets from the suppliers of the three materials

DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

6) Describe the packaging that will be utilized for the products

Attachment 6 is a brief description of the packaging used for each of the product forms.

7) Change the statement 'feed / food grade' currently found in the dossier to 'suitable for use in feed'.

I confirm that the requested change in terminology has been made in the dossier.

Kind regards

James La Marta, Ph.D.

James La Marta, Ph.D.
Senior Manager Regulatory Affairs

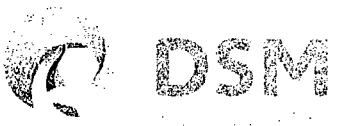
DSM

Attachment 1

Open Reading Frames in the genome of the Ronozyme® HiPhos production organism

The diagram is a theoretical illustration of integration of two copies of the expression plasmid into the genome by non – homologous single recombination. The illustration shows integration of a circular plasmid at a random position in the genome and a random position in the plasmid by a single recombination event. During such an event the DNA is integrated in as a linear fragment at the location in the genome where the recombination happened. If more than one copy is integrated, the copies are integrated in a head to tail fashion in multiple copies. Typically 20 or more copies are integrated into the genome of Aspergillus oryzae using the selection markers that Novozymes use. It cannot be excluded that new theoretical open reading frames (ORF) are formed when the DNA integrates. However for expression and accumulation of the corresponding protein translation product to happen, a functional promoter is needed in front of the ORF and the translation product need to be able to fold into a structure that is not destined for degradation by the quality system of the fungus. If a misfolded protein is formed it is instantly ubiquitinylated and destined for degradation in the proteasome.

Should a stable protein be formed from an ORF generated by the integration event, possible negative toxicological effects of such a protein would be detected during the toxicology studies.



Attachment 2

Pages FDA/CVM1828-1850 have been removed in accordance with copyright laws.
Please see:

(b) (4)

DSM

Attachment 3a

Sodium Thiosulfate use in Ronozyme® HiPhos (L)

Sodium Thiosulfate is listed as a GRAS substance for use in human food at 21 CFR 184.1807 (43 Fed. Reg. 22938, May 30, 1978 as amended at 49 Fed. Reg. 5613, February 4, 1984) based upon SOGS report #52 of 1975. In the SCOGS report it is noted that "*Experimental animal studies show that sodium thiosulfate is well tolerated.*" The two food categories listed are alcoholic beverage and table salt.

Sodium Thiosulfate is regulated for use in animal food as a chemical sequestrant at 21 CFR 582.6807 where it states that "*This substance is generally recognized as safe when used in salt in accordance with good manufacturing or feeding practices.*" with a tolerance of 0.1 percent.

At 21 CFR 582.1 (b) (2) good manufacturing or feeding practice is further defined as "*The quantity of a substance that becomes a component of animal food as a result of its use in the manufacturing, processing, or packaging of food, and which is not intended to accomplish any physical or other technical effect in the food itself, shall be reduced to the extent reasonably possible.*"

Ronozyme® HiPhos (M) is an ingredient used in the manufacture of food for poultry. The material is not a final food. It is not intended to be fed directly to animals. Ronozyme® HiPhos (M) is in essence a dilute protein solution adsorbed on a solid carrier. Labeling for the product instructs the user to add 5 to 80 grams to 1000 Kg of finished feed.

When the enzyme product is stabilized with 0.25% sodium thiosulfate the product maintains the enzyme activity expected for commercial distribution and use. The sodium thiosulfate in our product is not intended to accomplish any physical or technical effect in the food itself. The results of storage studies provided in our dossier substantiate the practicality of the current formulation. We have determined that the use of 0.25 % sodium thiosulfate in the manufacture of our product is necessary for stability.

When Ronozyme® HiPhos (M) is used at the highest use level noted in our dossier, 4000 FYT / Kg of finished food, the concentration of sodium thiosulfate in the finished food due to the use of the enzyme preparation would be 0.02 g/Kg or 0.00002% well below the maximum level for animal food.

4000 FYT / 50,000 FYT /g of Ronozyme® HiPhos (M) = 0.08 g of Ronozyme® HiPhos (M)
0.08 g X 0.25 % sodium thiosulfate = 0.2 mg of Sodium Thiosulfate
0.2 mg of Sodium thiosulfate /Kg of feed = 0.00002 % or 0.2 ppm

Per the NRC ***Predicting Feed Intake of Food Producing Animals*** (National Academies Press, 1987):
Chickens consume about 0.1 Kg of feed per day and weigh about 2 Kg. The exposure to sodium thiosulfate for a chicken would therefore be 0.02 mg/day or 0.01 mg/Kg of body weight.
Turkeys consume about 0.5 Kg of feed per day and weigh about 8 Kg. The exposure to sodium benzoate for a turkey would therefore be 0.1 mg/day or 0.0125 mg/Kg of body weight.

Ronozyme® HiPhos (M) was used for several efficacy studies presented in the dossier.(Annexes 00001790, 00000960, 00000959, 00001628, 00002585, and 00003287) No adverse effects were noted in these studies even at the highest proposed use level. The results of these studies indicate that the use of sodium thiosulfate at 0.25% wt/wt of the enzyme preparation did adversely affect the animals.

The EPA issued an exemption for the establishment for a tolerance for sodium thiosulfate on December 6, 2001 citing several animal studies, including ones reviewed by FDA, where it is noted that the acute oral dose LD50 was 5,050 mg/kg BW. No adverse maternal or developmental effects were reported in mice at 550 mg/Kg bw/day, in rats at 400 mg/kg bw/day and in rabbits at 580 mg/kg bw/day.

Because the exposure to sodium thiosulfate due to its use in Ronozyme HiPhos (M) under good manufacturing practices is well below the exposure anticipated 21 CFR 582.6810 and is several thousand times less than the no adverse effect level reported in the developmental toxicity investigations; DSM believes that the use of sodium thiosulfate as a sequestrant at 0.25% in the animal food ingredient, Ronozyme® HiPhos (M), is a safe and appropriate use of the additive.

DSM

Attachment 3b

sodium thiosulfate Exemption from the Requirement of a Tolerance 12/01

ENVIRONMENTAL PROTECTION AGENCY

40 CFR Part 180

[OPP-301196; FRL-6811-6]
RIN 2070-AB78

Sodium thiosulfate; Exemption from the Requirement of a Tolerance

AGENCY: Environmental Protection Agency (EPA).

ACTION: Final rule.

SUMMARY: This regulation establishes an exemption from the requirement of a tolerance for residues of sodium thiosulfate when used as an inert ingredient (dechlorinator) in or on growing crops, or when applied to raw agricultural commodities after harvest. Eden Bioscience submitted a petition to EPA under the Federal Food, Drug, and Cosmetic Act, as amended by the Food Quality Protection Act of 1996 requesting an exemption from the requirement of a tolerance. This regulation eliminates the need to establish a maximum permissible level for residues of sodium thiosulfate.

DATES: This regulation is effective December 21, 2001. Objections and requests for hearings, identified by docket control number OPP-301196, must be received by EPA on or before February 19, 2002.

ADDRESSES: Written objections and hearing requests may be submitted by mail, in person, or by courier. Please follow the detailed instructions for each method as provided in Unit VIII. of the SUPPLEMENTARY INFORMATION. To ensure proper receipt by EPA, your objections and hearing requests must identify docket control number OPP-301196 in the subject line on the first page of your response.

FOR FURTHER INFORMATION CONTACT: By mail: Kathryn Boyle, Registration Division (7505C), Office of Pesticide Programs, Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460; telephone number: (703) 305-6304; and e-mail address: boyle.kathryn@epa.gov.

SUPPLEMENTARY INFORMATION:

I. General Information

A. Does this Action Apply to Me?

You may be affected by this action if you are an agricultural producer, food manufacturer, or pesticide manufacturer. Potentially affected categories and entities may include, but are not limited to:

Categories	NAICS codes	Examples of potentially affected entities
Industry	111	Crop production
	112	Animal production
	311	Food manufacturing
	32532	Pesticide manufacturing

This listing is not intended to be exhaustive, but rather provides a guide for readers regarding entities likely to be affected by this

action. Other types of entities not listed in the table could also be affected. The North American Industrial Classification System (NAICS) codes have been provided to assist you and others in determining whether or not this action might apply to certain entities. If you have questions regarding the applicability of this action to a particular entity, consult the person listed under FOR FURTHER INFORMATION CONTACT.

B. How Can I Get Additional Information, Including Copies of this Document and Other Related Documents?

1. Electronically. You may obtain electronic copies of this document, and certain other related documents that might be available electronically, from the EPA Internet Home Page at http://www.epa.gov/. To access this document, on the Home Page select ``Laws and Regulations,'' ``Regulations and Proposed Rules,'' and then look up the entry for this document under the ``Federal Register--Environmental Documents.'' You can also go directly to the Federal Register listings at http://www.epa.gov/fedrgstr/.
A frequently updated electronic
version of 40 CFR
part 180 is available at http://www.access.gpo.gov/nara/cfr/cfrhtml_00/Title_40/40cfr180_00.html, a beta site currently under development.

2. In person. The Agency has established an official record for this action under docket control number OPP-301196. The official record consists of the documents specifically referenced in this action, and other information related to this action, including any information claimed as Confidential Business Information (CBI). This official record includes the documents that are physically located in the docket, as well as the documents that are referenced in those documents. The public version of the official record does not include any information claimed as CBI. The public version of the official record, which includes printed, paper versions of any electronic comments submitted during an applicable comment period is available for inspection in the Public Information and Records Integrity Branch (PIRIB), Rm. 119, Crystal Mall #2, 1921 Jefferson Davis Hwy., Arlington, VA, from 8:30 a.m. to 4 p.m., Monday through Friday, excluding legal holidays. The PIRIB telephone number is (703) 305-5805.

II. Background and Statutory Findings

In the Federal Register of September 6, 2000 (65 FR 54015) (FRL-6738-4), EPA issued a notice pursuant to section 408 of the Federal Food, Drug, and Cosmetic Act (FFDCA), 21 U.S.C. 346a, as amended by the Food Quality Protection Act (FQPA) (Public Law 104-170) announcing the filing of a pesticide petition (PP 0E6177) by Eden Bioscience, 11816 Creek Parkway North, Bothell, Washington, 98011-8205. This notice included a summary prepared by the petitioner. There were no comments received in response to the notice of filing.

The petition requested that 40 CFR 180.1001(c) be amended by establishing an exemption from the requirement of a tolerance for residues of sodium thiosulfate penthydrate (CAS Reg. No. 10102-17-7). The petition requested only the use of sodium thiosulfate pentahydrate; however, sodium thiosulfate is also available in an anhydrous form. The two chemical substances differ only in the attachment of the water molecules. The petition specified that sodium thiosulfate should be used at a concentration of 1 to 6% of the formulated product.

The sodium thiosulfate will be used as a pretreatment for the water in tank mixes to remove chlorine or other reactant species, thus functioning as a dechlorinator or reducing agent. When mixed with chlorine-containing water, sodium thiosulfate reacts with the chlorine according to the equation $Na_2S_2O_3$ + $4Cl_2 + 5H_2O$ $2NaHSO_4 + 8HCl$.
Sodium thiosulfate also reacts with hydrochloric acid (produced in the previous reaction) to form breakdown products such as sulfur, salt and water: $Na_2S_2O_3 + 2HCl$ $2NaCl$ $+ H_2O + S + SO_2$.

Section 408(b)(2)(A)(i) of the FFDCA allows EPA to establish an exemption from the requirement for a tolerance (the legal limit for a pesticide chemical residue in or on a food) only if EPA determines that the tolerance is ``safe.'' Section 408(b)(2)(A)(ii) defines ``safe'' to mean that ``there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including

all anticipated dietary exposures and all other exposures for which there is reliable information.'' This includes exposure through drinking water and in residential settings, but does not include occupational exposure. Section 408(b)(2)(C) requires EPA to give special consideration to exposure of infants and children to the pesticide chemical residue in establishing a tolerance and to ``ensure that there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residue. . . .''

EPA performs a number of analyses to determine the risks from aggregate exposure to pesticide residues. First, EPA determines the toxicity of pesticides. Second, EPA examines exposure to the pesticide through food, drinking water, and through other exposures that occur as a result of pesticide use in residential settings.

III. Inert Ingredient Definition

Inert ingredients are all ingredients that are not active ingredients as defined in 40 CFR 153.125 and include, but are not limited to, the following types of ingredients (except when they have a pesticidal efficacy of their own): Solvents such as alcohols and hydrocarbons; surfactants such as polyoxyethylene polymers and fatty acids; carriers such as clay and diatomaceous earth; thickeners such as carrageenan and modified cellulose; wetting, spreading, and dispersing agents; propellants in aerosol dispensers; microencapsulating agents; and emulsifiers. The term ``inert'' is not intended to imply nontoxicity; the ingredient may or may not be chemically active. Generally, EPA has exempted inert ingredients from the requirement of a tolerance based on the low toxicity of the individual inert ingredients.

IV. Toxicological Profile

Consistent with section 408(b)(2)(D) of FFDCA, EPA has reviewed the available scientific data and other relevant information in support of this action and considered its validity, completeness and reliability and the relationship of this information to human risk. EPA has also considered available information concerning the variability of the sensitivities of major identifiable subgroups of consumers, including infants and children. The nature of the toxic effects caused by sodium thiosulfate are discussed in this unit. The information submitted in support of this petition included portions of the Food and Drug Administration (FDA) generally recognized as safe (GRAS) determination (``Evaluation of the Health Aspects of Sodium Thiosulfate as a Food Ingredient''), articles from open literature, and an acute oral toxicity study.

A. Medical Uses

There are medical uses of sodium thiosulfate. It has been used as an antidote for acute cyanide poisoning (intravenous injection), and is an ingredient in various dermally-applied lotion formulations used to treat acne and ringworm.

B. GRAS Determination

Sodium thiosulfate pentahydrate has been classified as GRAS by the FDA when used as a formulation aid or reducing agent in alcoholic beverages (not to exceed 0.00005%) and table salt (not to exceed 0.1%). A GRAS determination means general recognition of safety by experts qualified by scientific training and experience to evaluate the safety of the substance for the specified use pattern. As noted by the limitations stated above, sodium thiosulfate has a very limited use pattern. EPA will use the information evaluated as part of the FDA GRAS determination to inform the Agency's decision.

In its 1975 Evaluation, FDA reported the following information on the sodium thiosulfate absorption and metabolism: Sodium thiosulfate is a normal constituent of human body fluids and is excreted in the urine of man and higher animals. Quantitative studies have demonstrated the consistent presence of 2 to 17 milligrams (mg) of thiosulfate sulfur in 24-hour urine specimens of healthy young adults. Variations in excretion of thiosulfate are related to the extent of protein metabolism, activity of the intestinal flora, and the sulfur-amino

acid content of the diet. The sulfur-containing amino acids of dietary protein are the source of the endogenous thiosulfate pool. Orally administered thiosulfate that is absorbed from the gastrointestinal tract is excreted in the urine unchanged or after oxidation to sulfate. >From 5 to 70% of an oral dose of sodium thiosulfate is considered to be absorbed from the gastrointestinal tract of man and the remainder to be excreted in the feces.

According to the Evaluation, sodium thiosulfate was found to cause no mutagenic effects.

The Evaluation also included a summary of the results of developmental studies on rats, mice, and hamsters. It was determined there was no effect on nidation, maternal or fetal survival, or fetal development.

C. Open Literature Articles

Three of the articles from open literature were reviewed to determine if the articles could supply information to the Agency on the genotoxicity of sodium thiosulfate. There is no indication of any mutagenic activity associated with exposure to sodium thiosulfate.

D. Acute Oral Toxicity Study

An acute oral toxicity study in the rat performed with sodium thiosulfate pentahydrate was submitted. The study was classified as acceptable, toxicity category IV. The LD_{50} is greater than 5,050 milligrams/kilograms (mg/kg) (males and females combined).

E. Developmental Toxicity

As part of the information submitted in support of the petition, the petitioner submitted the final reports for the rat, mouse, and hamster developmental studies that were discussed in the FDA Evaluation (dated 1972), as well as the final report for a rabbit developmental toxicity study (dated 1974). These studies were performed using the anhydrous form of sodium thiosulfate. Due to the passage of almost 30 years, as well as the changes in laboratory techniques that have occurred during this time, the data tables in the reports were reviewed to determine if any additional information were contained in the tables.

1.Mouse. Animals were tested at the following dose levels: Negative control, positive control, 5.5, 25.5, 118 or 550 mg/kg/day over a 10-day period from day 6 through day 15 of gestation. There was no indication of any effect on maternal or fetal survival, or in incidences of visceral or skeletal abnormalities. The male/female ratio of the fetuses were calculated to be, respectively, 1.08, 0.93, 0.74, 0.90, 0.88, or 0.68. The ratios at the lowest and highest dose levels are lower than the other ratios.

2. Rat. Animals were tested at the following dose levels: Negative control, positive control, 4.0, 19.0, 86.0, or 400 mg/kg/day over a 10-day period from day 6 through day 15 of gestation. There was no indication of any effect on maternal or fetal survival, or in incidences of visceral or skeletal abnormalities. The male/female ratio of the fetuses were calculated to be, respectively, 0.84, 0.78, 0.84, 0.98, 0.92, or 0.73. There is an indication of skewing (a lowering) in these ratios at the highest dose level and in the positive control.

3.Hamster. Animals were tested at the following dose levels: negative control, positive control, 4.0, 19.0, 86.0, or 400 mg/kg/day over a 5-day period from day 6 through day 10 of gestation. There was no indication of any effect on maternal or fetal survival, or in incidences of visceral or skeletal abnormalities. The male/female ratio of the fetuses were calculated to be, respectively, 0.52, 0.54, 0.59, 0.47, 0.40, or 0.53. These ratios (including those from the controls) are very unusual.

4. Rabbit. The results of the rabbit developmental study were not considered in the FDA Evaluation. Animals were tested over a 13-day period from day 6 through day 18 of gestation. There was no indication of any effect on maternal or fetal survival, or in incidences of visceral or skeletal abnormalities at the highest dose level of 580 mg/kg/day. There was no indication of any effect on the male/female ratio of the fetuses since the ratio ranged from 1.13 to 1.26.

F. Information from the Internet

To ascertain whether additional information on sodium thiosulfate were available, the Agency also searched the Tox Net website at the National Library of Medicine (http://www.toxnet.nlm.nih.gov). This website contained only information on sodium thiosulfate anhydrous (CAS. Reg. No. 7772-98-7). The Tox Net website classified sodium thiosulfate as moderately toxic, and generally supported the information presented in the petition. The excerpts and summaries indicated that sodium thiosulfate is not mutagenic.No internet information indicated concerns for carcinogenicity or developmental/ reproductive toxicity. One study which investigated the ability of sodium thiosulfate to cross the placenta in sheep, concluded that maternally-administered sodium thiosulfate (50 mg/kg) does not increase fetal plasma thiosulfate concentrations. No information on sodium thiosulfate was available on the National Toxicology Program website, the Agency for Toxic Substances and Disease Registry website, or the Agency's Integrated Risk Information System website. The TSCATs database (http://esc.syrres.com/efdb/TSCATS.htm) did not contain any summaries of any developmental or reproductive studies conducted with sodium thiosulfate.

G. Toxicity of Sodium Thiosulfate

Overall, sodium thiosulfate presents as a chemical with slight to moderate toxicity. It is Category IV for acute oral toxicity (the lowest classification), and there are no indications of mutagenicity. The available developmental data indicates no effect on maternal or fetal survival or increase in incidences of visceral or skeletal abnormalities. The sex ratios (the male/female ratio of the fetuses) should cluster close to 1, indicating equal numbers of males and females. This is evident in the range of ratios in the rabbit study. However, the Agency's re-evaluation of the summary data for the rat and mouse developmental data (two out of four species) suggest the possibility that various doses of sodium thiosulfate may be associated with an apparent skewing (a lowering) of the sex ratio. However, it was also most unusual that this skewing occurred not only for certain dose levels, but also for a positive control. The sex ratios for the hamster are very unusual. Therefore, there is an uncertainty as to what these ratios mean. But, there is the possibility of technician error in sex identification. In the three studies included in the FDA Evaluation (rat, mice, and hamster), the description of the studies included the following: All fetuses were examined grossly for the presence of external congenital abnormalities. One-third of the fetuses of each litter underwent detailed visceral examinations employing 10X magnification. ``The remaining two-thirds were cleared and examined for skeletal defects.'' Thus, there was no chance to correct any mis-sexing. The rabbit study, in which there was no effect on the male/ female ratio of the fetuses, was performed in a different manner: ``All fetuses underwent a detailed gross examination for the presence of external congenital abnormalities.'' All were examined for visceral abnormalities. ``All fetuses were then cleared and examined for skeletal defects.'' Thus, the examination of all fetuses apparently allowed for greater accuracy in sexing.

V. Aggregate Exposures

In examining aggregate exposure, FFDCA section 408 directs EPA to consider available information concerning exposures from the pesticide residue in food and all other non-occupational exposures, including drinking water from ground water or surface water and exposure through pesticide use in gardens, lawns, or buildings (residential and other indoor uses).

EPA establishes exemptions from the requirement of a tolerance only in those cases where the risks from aggregate exposure to pesticide chemical residues under reasonably foreseeable circumstances will pose no appreciable risks to human health. In order to determine the risks from aggregate exposure to pesticide inert ingredients, the Agency considers the toxicity of the inert in conjunction with possible exposure to residues of the inert ingredient through food, drinking water, and through other exposures that occur as a result of pesticide use in residential settings. If EPA is able to determine that a finite

tolerance is not necessary to ensure that there is a reasonable certainty that no harm will result from aggregate exposure to the inert ingredient, an exemption from the requirement of a tolerance may be established.

A. Dietary Exposure

For the purposes of assessing potential exposure under this exemption, EPA considered that sodium thiosulfate could be present in all raw and processed agricultural commodities and drinking water, and that non-occupational non-dietary exposure was possible.

1. Food. Protein, which is composed of various amino acids, is required for human survival. Sodium thiosulfate is produced in the human body during the metabolism of sulfur-containing amino acids. There is an effective self-regulating mechanism to rid the body of excess sodium thiosulfate through excretion in the urine. As previously stated, sodium thiosulfate is considered to be GRAS for a very specific use pattern. In the 1975 Evaluation, it was estimated that the per capita consumption of sodium thiosulfate was 12 micrograms (□g) per day. Considering the use of sodium thiosulfate in pesticide products, as a dechlorinator when mixed with certain proteins such as harpin protein, and given the reactive nature (as a reducing agent) of sodium thiosulfate, this use pattern should not significantly increase the amount of sodium thiosulfate in the food supply above those amounts permitted by FDA.

2. Drinking water exposure. Thiosulfate can be produced naturally by the reaction of elemental sulfur with sulfite ion in boiling water. Therefore, thiosulfate occurs naturally in such environments as hot springs, geysers, and marine hydrothermal vents. It can also occur in nature as the result of the biological or chemical oxidation of sulfide, and thus can be found in freshwater and marine sediments, and salt marshes.

Considering that thiosulfate can be metabolized by sulfate-reducing bacteria, and given its ability to react with chlorine (to act as a reducing agent), sodium thiosulfate is unlikely to occur in drinking water.

B. Other Non-Occupational Exposure

The medicinal uses of sodium thiosulfate are also regulated by FDA. There are other industrial uses of sodium thiosulfate which include use as a photographic fixing agent. Sodium thiosulfate is also used to remove chlorine from water used in aquariums.

C. Exposure Estimates

As previously stated, it was estimated that the per capita consumption of sodium thiosulfate was 12 □g per day. This was based on the amount of sodium thiosulfate used by the food industry and assuming a population of 210 million. (The Agency acknowledges that this exposure estimate is almost 30 years old.) If this were converted to mg/kg/day using a 60 kg (female) body weight, then the exposure could be estimated as 0.0002 mg/kg/day. The highest dose levels in each of the developmental toxicity studies (mouse, rat, hamster, and rabbit) were respectively 550, 400, 400, and 580 mg/kg/day. No effects were noted at these levels. The Agency has not attempted to use a safety factor analysis for sodium thiosulfate; however, the 0.0002 mg/kg/day is orders of magnitude lower than the highest dose levels from any of the developmental toxicity studies. Thus, the reported uses of sodium thiosulfate, its use as a GRAS substance and its use as an inert ingredient (a dechlorinator) should result in human exposure far below any dose level that could possibly produce an adverse effect.

VI. Cumulative Effects

Section 408 (b)(2)(D)(v) of FFDCA requires that, when considering whether to establish, modify, or revoke a tolerance or tolerance exemption, the Agency consider ``available information'' concerning the cumulative effects of a particular chemical's residues and ``other substances that have a common mechanism of toxicity.'' Sodium thiosulfate is produced in the human body during the metabolism of sulfur-containing amino acids. There is an effective self-regulating mechanism (excretion) to rid the body of excess sodium thiosulfate, so

cumulative effects are unlikely as a result of exposure to sodium thiosulfate and a substance sharing a common mechanism of toxicity, assuming such a substance exists. The Agency has not made any conclusions as to whether or not sodium thiosulfate shares a common mechanism of toxicity with any other chemicals, since cumulative effects for sodium thiosulfate and other substances are unlikely.

VII. Determination of Safety for U.S. Population

Based on the low-moderate toxicity of sodium thiosulfate and the low potential for exposure from the EPA regulated uses of sodium thiosulfate, as well as the FDA GRAS uses, the Agency has determined that aggregate exposure to sodium thiosulfate under reasonably foreseeable circumstances will pose no appreciable risks to human health. Accordingly, EPA concludes that there is a reasonable certainty of no harm to the U.S. population from aggregate exposure to residues of sodium thiosulfate and that a tolerance is not necessary.

VIII. Determination of Safety for Infants and Children

FFDCA section 408 provides that EPA shall apply an additional tenfold margin of safety for infants and children in the case of threshold effects to account for prenatal and postnatal toxicity and the completeness of the data base unless EPA concludes that a different margin of safety will be safe for infants and children. Due to the expected low toxicity of sodium thiosulfate, EPA has not used a safety factor analysis to assess the risk. For the same reasons the additional tenfold safety factor is unnecessary. The Agency has determined that there is a reasonable certainty of no harm to infants and children from aggregate exposure to residues of sodium thiosulfate and that a tolerance is not necessary.

IX. Other Considerations

A. Endocrine Disruptors

FQPA requires EPA to develop a screening program to determine whether certain substances, including all pesticide chemicals (both inert and active ingredients), ``may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or such other endocrine
effect.'' EPA has been working with interested stakeholders to develop a screening and testing program as well as a priority setting scheme. As the Agency proceeds with implementation of this program, further testing of products containing sodium thiosulfate for endocrine effects may be required.

B. Analytical Method(s)

An analytical method is not required for enforcement purposes since the Agency is establishing an exemption from the requirement of a tolerance without any numerical limitation.

C. Existing Exemptions

There are no existing exemptions for sodium thiosulfate anhydrous or sodium thiosulfate pentahydrate.

D. International Tolerances

The Agency is not aware of any country requiring a tolerance for sodium thiosulfate anhydrous or sodium thiosulfate pentahydrate nor have any CODEX Maximum Residue Levels (MRLs) been established for any food crops at this time.

X. Conclusions

Based on the information in this preamble, EPA concludes that there is a reasonable certainty of no harm from aggregate exposure to residues of sodium thiosulfate anhydrous or sodium thiosulfate pentahydrate. Accordingly, EPA finds that exempting sodium thiosulfate anhydrous or sodium thiosulfate pentahydrate from the requirement of a tolerance will be safe.

XI. Objections and Hearing Requests

Under section 408(g) of the FFDCA, as amended by the FQPA, any person may file an objection to any aspect of this regulation and may also request a hearing on those objections. The EPA procedural regulations which govern the submission of objections and requests for hearings appear in 40 CFR part 178. Although the procedures in those regulations require some modification to reflect the amendments made to the FFDCA by the FQPA of 1996, EPA will continue to use those procedures, with appropriate adjustments, until the necessary modifications can be made. The new section 408(g) provides essentially the same process for persons to ``object'' to a regulation for an exemption from the requirement of a tolerance issued by EPA under new section 408(d), as was provided in the old FFDCA sections 408 and 409. However, the period for filing objections is now 60 days, rather than 30 days.

A. What Do I Need to Do to File an Objection or Request a Hearing?

You must file your objection or request a hearing on this regulation in accordance with the instructions provided in this unit and in 40 CFR part 178. To ensure proper receipt by EPA, you must identify docket control number OPP-301196 in the subject line on the first page of your submission. All requests must be in writing, and must be mailed or delivered to the Hearing Clerk on or before February 19, 2002.

1. Filing the request. Your objection must specify the specific provisions in the regulation that you object to, and the grounds for the objections (40 CFR 178.25). If a hearing is requested, the objections must include a statement of the factual issues(s) on which a hearing is requested, the requestor's contentions on such issues, and a summary of any evidence relied upon by the objector (40 CFR 178.27). Information submitted in connection with an objection or hearing request may be claimed confidential by marking any part or all of that information as CBI. Information so marked will not be disclosed except in accordance with procedures set forth in 40 CFR part 2. A copy of the information that does not contain CBI must be submitted for inclusion in the public record. Information not marked confidential may be disclosed publicly by EPA without prior notice.

Mail your written request to: Office of the Hearing Clerk (1900), Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460. You may also deliver your request to the Office of the Hearing Clerk in Rm. C400, Waterside Mall, 401 M St., SW., Washington, DC 20460. The Office of the Hearing Clerk is open from 8 a.m. to 4 p.m., Monday through Friday, excluding legal holidays. The telephone number for the Office of the Hearing Clerk is (202) 260-4865.

2. Tolerance fee payment. If you file an objection or request a hearing, you must also pay the fee prescribed by 40 CFR 180.33(i) or request a waiver of that fee pursuant to 40 CFR 180.33(m). You must mail the fee to: EPA Headquarters Accounting Operations Branch, Office of Pesticide Programs, P.O. Box 360277M, Pittsburgh, PA 15251. Please identify the fee submission by labeling it ``Tolerance Petition Fees.''

EPA is authorized to waive any fee requirement ``when in the judgement of the Administrator such a waiver or refund is equitable and not contrary to the purpose of this subsection.'' For additional information regarding the waiver of these fees, you may contact James Tompkins by phone at (703) 305-5697, by e-mail at tompkins.jim@epa.gov, or by mailing a request for information to Mr. Tompkins at Registration Division (7505C), Office of Pesticide Programs, Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460.

If you would like to request a waiver of the tolerance objection fees, you must mail your request for such a waiver to: James Hollins, Information Resources and Services Division (7502C), Office of Pesticide Programs, Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460.

3. Copies for the Docket. In addition to filing an objection or hearing request with the Hearing Clerk as described in Unit VIII.A., you should also send a copy of your request to the PIRIB for its inclusion in the official record that is described in Unit I.B.2. Mail your copies, identified by docket control number OPP-301196, to: Public Information and Records Integrity Branch, Information Resources and Services Division (7502C), Office of Pesticide Programs, Environmental

Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460. In person or by courier, bring a copy to the location of the PIRIB described in Unit I.B.2. You may also send an electronic copy of your request via e-mail to: opp-docket@epa.gov. Pleaseuse an ASCII file format and avoid the use of special characters and any form of encryption. Copies of electronic objections and hearing requests will also be accepted on disks in WordPerfect 6.1/8.0 or ASCII file format. Do not include any CBI in your electronic copy. You may also submit an electronic copy of your request at many Federal Depository Libraries.

B. When Will the Agency Grant a Request for a Hearing?

A request for a hearing will be granted if the Administrator determines that the material submitted shows the following: There is a genuine and substantial issue of fact; there is a reasonable possibility that available evidence identified by the requestor would, if established resolve one or more of such issues in favor of the requestor, taking into account uncontested claims or facts to the contrary; and resolution of the factual issues(s) in the manner sought by the requestor would be adequate to justify the action requested (40 CFR 178.32).

XII. Regulatory Assessment Requirements

This final rule establishes an exemption from the tolerance requirement under FFDCA section 408(d) in response to a petition submitted to the Agency. The Office of Management and Budget (OMB) has exempted these types of actions from review under Executive Order 12866, entitled Regulatory Planning and Review (58 FR 51735, October 4, 1993). Because this rule has been exempted from review under Executive Order 12866 due to its lack of significance, this rule is not subject to Executive Order 13211, Actions Concerning Regulations That Significantly Affect Energy Supply, Distribution, or Use (66 FR 28355, May 22, 2001). This final rule does not contain any information collections subject to OMB approval under the Paperwork Reduction Act (PRA), 44 U.S.C. 3501 et seq., or impose any enforceable duty or contain any unfunded mandate as described under Title II of the Unfunded Mandates Reform Act of 1995 (UMRA) (Public Law 104-4). Nor does it require any special considerations under Executive Order 12898, entitled Federal Actions to Address Environmental Justice in Minority Populations and Low-Income Populations (59 FR 7629, February 16, 1994); or OMB review or any Agency action under Executive Order 13045, entitled Protection of Children from Environmental Health Risks and Safety Risks (62 FR 19885, April 23, 1997). This action does not involve any technical standards that would require Agency consideration of voluntary consensus standards pursuant to section 12(d) of the National Technology Transfer and Advancement Act of 1995 (NTTAA), Public Law 104-113, section 12(d) (15 U.S.C. 272 note). Since tolerances and exemptions that are established on the basis of a petition under FFDCA section 408(d), such as the exemption in this final rule, do not require the issuance of a proposed rule, the requirements of the Regulatory Flexibility Act (RFA) (5 U.S.C. 601 et seq.) do not apply. In addition, the Agency has determined that this action will not have a substantial direct effect on States, on the relationship between the national government and the States, or on the distribution of power and responsibilities among the various levels of government, as specified in Executive Order 13132, entitledFederalism (64 FR 43255, August 10, 1999). Executive Order 13132 requires EPA to develop an accountable process to ensure ``meaningful and timely input by State and local officials in the development of regulatory policies that have federalism implications.'' ``Policies that have federalism implications'' is defined in the Executive Order to include regulations that have ``substantial direct effects on the States, on the relationship between the national government and the States, or on the distribution of power and responsibilities among the various levels of government.'' This final rule directly regulates growers, food processors, food handlers and food retailers, not States. This action does not alter the relationships or distribution of power and responsibilities established by Congress in the preemption provisions of FFDCA section 408(n)(4). For these same reasons, the Agency has determined that this rule does not have any ``tribal implications'' as described in Executive Order 13175, entitled Consultation and

Coordination with Indian Tribal Governments (65 FR 67249, November 6, 2000). Executive Order 13175, requires EPA to develop an accountable process to ensure ``meaningful and timely input by tribal officials in the development of regulatory policies that have tribal implications.'' ``Policies that have tribal implications'' is defined in the Executive Order to include regulations that have ``substantial direct effects on one or more Indian tribes, on the relationship between the Federal government and the Indian tribes, or on the distribution of power and responsibilities between the Federal government and Indian tribes.'' This rule will not have substantial direct effects on tribal governments, on the relationship between the Federal government and Indian tribes, or on the distribution of power and responsibilities between the Federal government and Indian tribes, as specified in Executive Order 13175. Thus, Executive Order 13175 does not apply to this rule.

XIII. Submission to Congress and the Comptroller General

The Congressional Review Act, 5 U.S.C. 801 et seq., as added by the Small Business Regulatory Enforcement Fairness Act of 1996, generally provides that before a rule may take effect, the agency promulgating the rule must submit a rule report, which includes a copy of the rule, to each House of the Congress and to the Comptroller General of the United States. EPA will submit a report containing this rule and other required information to the U.S. Senate, the U.S. House of Representatives, and the Comptroller General of the United States prior to publication of this final rule in the Federal Register. This final rule is not a ``major rule'' as defined by 5 U.S.C. 804(2).

List of Subjects in 40 CFR Part 180

Environmental protection, Administrative practice and procedure, Agricultural commodities, Pesticides and pests, Reporting and recordkeeping requirements.

Dated: December 6, 2001.
Peter Caulkins,
Acting Director, Registration Division, Office of Pesticide Programs.

Therefore, 40 CFR chapter I is amended as follows:

PART 180--[AMENDED]

1. The authority citation for part 180 continues to read as follows:

Authority: 21 U.S.C. 321(q), 346(a) and 371.

2. In Sec. 180.1001, the table in paragraph (c) is amended by adding alphabetically the following inert ingredient to read as follows:

Sec. 180.1001 Exemptions from the requirement of a tolerance.

* * * * *
(c) * * *

Inert ingredients	Limits	Uses
* * * * * * *		
Sodium thiosulfate anhydrous (CAS Reg. No.7772-98-7 or sodium thiosulfate pentahydrate,CAS Reg. No. 10102-17-7)	Not to exceed 6% of theformulated product	Dechlorinator, reducing agent
* * * * * * *		

* * * * *

To Top

For more information relative to pesticides and their use in New York State, please contact the PMEP staff at:

5123 Comstock Hall
Cornell University
Ithaca, NY 14853-0901
(607) 255-1866

Cornell University

This site is supported, in part, by funding from the Northeastern IPM Center

Questions regarding the development of this web site should be directed to the PMEP Webmaster

DSM

Attachment 4

novozymes®
Rethink Tomorrow

July 10, 2009

CERTIFICATE OF ANALYSIS

RONOZYME HiPhos (M)

Batch number:	PPQ28656	PPQ28683	PPQ28684
Analysis name	**Result**	**Result**	**Result**
Phytase activity, FYT(B)/g	**60000**	**60400**	**62400**
Total viable count/g	**3600**	**4800**	**1400**
Coliform/g	**<10**	**<10**	**<10**
E.coli/25g	**ND**	**ND**	**ND**
Salmonella/25g	**ND**	**ND**	**ND**

Novozymes
Quality Management
Bjørn Sønder
(MSc., Chemical Engineering)

Novozymes A/S
Krogshøjvej 36
DK-2880 Bagsværd
Denmark

Telephone:
+45 8824 9999
Telefax:

E-mail:

Internet:
www.novozymes.com

CVR number:
10 00 71 27

Attachment 5

SPECIFICATIONS **Ref: C70-104R40**

SOLULYS 048E

DEFINITION :

Corn steep liquor.
Concentrated solution of soluble products extracted from maize during the soaking process prior to fractionation of the kernel following the wet milling process.
CAS n$: 66071-94-1 EINECS : 266-113-4

SPECIFICATIONS :

APPEARANCE	Thick brown liquid	
DRY SUBSTANCE	(b) (4)	
pH IN SOLUTION		
TOTAL ACIDITY (EXPRESSED AS LACTIC ACID)		(*)
REDUCING SUGARS		(*)
AMINO NITROGEN		(*)
TOTAL NITROGEN		(*)
ASH		(*)
PHOSPHORUS (AS P)		(*)

(*) : ON DRY MATTER

TYPICAL VALUES :

PROTEIN CONTENT (N x 6,25)

STORAGE :

Chemical stability : 12 months
Solulys048 E may settle during storage.

MCL.MMC: (b) (4)

QUALITY ASSURANCE / INDUSTRY February 14, 2013

(b) (4)

Data Sheet

Date 10.03.2008

Chemical Name	Sodium thiosulfate
Formula	$Na_2S_2O_3$
CAS-No	7772-98-7

Product Group: 15523

Sodium thiosulfate, anhydrous

Quality: acc. spec.

Description: white, powder

General Product Information:

Solubility in water: 44.6 g/l (20°C)

Other qualities available: Special parameters and limits are available on request.

Storage: Well closed in a dry and cool place (< 25°C / < 65% air humidity) in original packaging.

Shelf life: Can be kept for 3 years, if stored in a dry and cool place (< 25°C / < 65 % air humidity) in original packaging.

Package Information:

Material:	**Package:**	**Packages:**
155230136001	25 kg	Paper bag
	special customers packaging possible.	

Safety: See safety data sheet.

(b) (4)

(b) (4)

Data Sheet

Date 10.03.2008

Chemical Name	Sodium thiosulfate
Formula	$Na_2S_2O_3$
CAS-No	7772-98-7

Specifications

Parameter	Specification
Appearance of solution	clear, colourless
Identity	complies
Sulphates and sulphites	correlates to test
Sulphides	correlates to test
Loss on drying (Ph Eur)	<= 1,00 %
Assay (Ph Eur)	99,0 - 101,0 %
Heavy metals (as Pb)	<= 10,0 mg/kg
pH	6,00 - 8,40
Lead	<= 10,0 mg/kg
Selenium	<= 10,0 mg/kg

Company name and address

(b) (4)

(b) (4)

(b) (4)

Product specification sheet

Zinc Acetate 2-hydrate Pharmaceutical

Product code: 40069, 48964

PRODUCT PROPERTIES

Appearance		white crystals
Assay	%(m) min.	99.5
Insoluble in water	ppm max.	30
Chloride (Cl)	ppm max.	5
Sulphate (SO4)	ppm max.	50
Copper (Cu)	ppm max.	5
Iron (Fe)	ppm max.	3
Lead (Pb)	ppm max.	10
Arsenic (As)	ppm max.	2
pH of 5% solution		6.0 - 6.6
Alkali and alkaline earths	%(m) max.	0.2
Substances reducing permanganate		conform test

Pharmaceutical grade conforms to the latest USP

Analytical methods available on request.

For more detailed information please see Product Data Sheet and Material Safety Data Sheet.

(b) (4)

DSM

Attachment 6

Packaging of Ronozyme® HiPhos products

Ronozyme® HiPhos (L) is a liquid enzyme preparation that is packaged in new, clean, food grade, 200 L polyethylene plastic drums or in new, clean, food grade, 1000 L polyethylene plastic totes that are surrounded by a wire frame.

Ronozyme® (CT) and (M) are free-flowing powders that are packaged in 20 Kg multi-walled paper bags with a food grade polyethylene liner or 1000 Kg woven fiber 'supersacks' with a food grade polyethylene liner.

T-5

DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

DSM Nutritional Products
45 Waterview Boulevard
Parsippany
NJ 07054
United States of America

phone +1 973 257-8325
fax +1 973 257 8414

Mr. Geoffrey Wong
Center for Veterinary Medicine
Ingredient Safety Team (HFV 224)
7519 Standish Place
Rockville, Maryland 20855
CC: Dr. Andrea Krause

April 10, 2013

Re: Question regarding DSM's GRAS Notice for Ronozyme® HiPhos

Dear Mr. Wong and Dr. Krause

Thank you for the opportunity to provide additional information about our GRAS Notice. DSM Nutritional Products is responding to the center's request for a statement addressing the suitability of the substances utilized in the manufacture and formulation of our product, Ronozyme HiPhos.

DSM Nutritional Products has determined that all the substances utilized in the manufacture and formulation of Ronozyme HiPhos are suitable for use in animal food in compliance with a regulation of the Food and Drug Administration, a listing in the Official Publication of the American Association of Feed Control Officials or has been determined by DSM Nutritional Products to be generally recognized as safe (GRAS), for the intended use. DSM has determined that Nickel Chloride, Sodium Benzoate, Sodium Thiosulfate and Potato Maltodextrin to be generally recognized as safe for their intended use in the manufacture and formulation of Ronozyme HiPos.

Please do not hesitate to contact me if you have any additional questions regarding this matter.

Kind regards,

DSM Nutritional Products

Alberto Davidovich, DVM, Ph.D.
Director, Regulatory Affairs