



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

DSM Nutritional Products

45 Waterview Boulevard
Parsippany
NJ 07054
United States of America

phone +1 973 257 8347
fax +1 973 257 8414

June 27, 2014

GRAS Notification of Canthaxanthin (Carophyll Red®) 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, DSM Nutritional Products is hereby submitting a Notice of the Generally Recognized As Safe use of Canthaxanthin (Carophyll Red®) as a nutritive antioxidant in the food of breeding chickens.

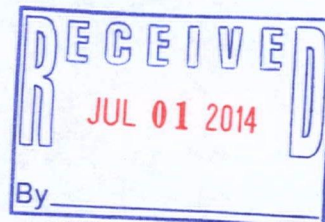
DSM Nutritional Products gathered the appropriate information on the safety and utility of the notified substance which was provided to an independent panel of experts in the field for their evaluation. The enclosed dossier contains the identity, manufacturing for the substance and the commercial forms, and the safety and efficacy study data that was provided to the panel as well as the panel's signed conclusion statement. Also included are copies of the pertinent literature and the peer reviewed publications addressing the safety of Canthaxanthin (Carophyll Red®) and its performance in poultry foods indicative of those normally fed in the United States.

DSM Nutritional Products has concluded that Canthaxanthin (Carophyll Red®) 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 the GRAS Notification are available to the Food and Drug Administration for review and copying upon request during normal business hours.

Sincerely DSM Nutritional Products,

James La Marta, Ph.D.
Senior Manager Regulatory Affairs



DSM1493-001



**THE SAFETY AND THE
GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF
THE USE OF CANTHAXANTHIN
(β -CAROTENE-4,4'-DIONE)
IN FOOD FOR POULTRY BREEDERS**

By

DSM Nutritional Products

Elkin Amaya
Kaiseraugst, Switzerland

James La Marta
Parsippany, New Jersey, USA

Table of Contents

1. EXECUTIVE SUMMARY	7
1.1 INTRODUCTION.....	8
1.2 GRAS Exemption Claim	9
1.3 Name and address of Notifier.....	10
1.4 Name and Address of Manufacturer	10
1.5 Name and Address of the Exclusive Distributor.....	10
2. IDENTITY OF THE NOTIFIED SUBSTANCE.....	11
2.1 Common or usual name.....	11
2.2 Standard of Canthaxanthin Identity in the US	11
2.3 Characterization of the substance	12
2.3.1 Characterization of the active ingredient (Canthaxanthin)	12
2.3.1.1 <i>Description</i>	12
2.3.1.2 <i>Relevant properties</i>	13
2.3.1.3 <i>Manufacturing</i>	14
<i>Raw Materials and Reagents</i>	14
<i>Canthaxanthin Manufacturing Process</i>	14
<i>Canthaxanthin Impurities</i>	15
<i>Process Control</i>	16
2.3.2 Characterization of CAROPHYLL® Red	16
2.3.2.1 <i>Description</i>	16
2.3.2.2 <i>Packaging</i>	17
2.3.2.3 <i>Relevant properties</i>	17
2.3.2.4 <i>Manufacturing</i>	17
<i>Ingredients and approval status</i>	17
<i>CAROPHYLL® Red 10% Manufacturing Process</i>	18
2.4 Physico-chemical and technological properties of the additive.....	19
2.5 Stability.....	19
2.5.1.1 <i>Stability of the additive</i>	19
2.5.1.2 <i>Stability of the additive used in premixtures</i>	19
2.5.1.3 <i>Stability of the additive used in feed</i>	20
2.5.2 Homogeneity.....	20
2.6 Analytical methods	21
2.6.1 Methods of analysis for Canthaxanthin crystalline.....	21
2.6.2 Analytical methods for the determination of Canthaxanthin in different matrixes.....	22
2.6.3 Method for the determination of the antioxidant activity of Canthaxanthin.....	23
2.6.3.1 <i>Introduction</i>	23
2.6.3.2 <i>TBARS as an Analytical assay</i>	23
2.6.3.3 <i>Comparison of methods used for TBARS determination in poultry tissues</i>	23
2.6.3.4 <i>Proposed method</i>	24

3.	CONDITIONS OF USE AND CANTHAXANTHIN UTILITY	26
3.1	Proposed use	26
3.2	Utility	26
3.2.1	Background	26
3.2.2	Antioxidant activity of carotenoids	28
3.2.3	Fate of dietary Canthaxanthin in poultry layers and offspring	33
3.2.3.1	<i>Canthaxanthin deposition in poultry</i>	33
3.2.3.2	<i>Maternal transfer of Canthaxanthin to poultry offspring</i>	35
3.2.4	Canthaxanthin use in "Breeder Layers"	37
3.2.4.1	<i>Needs of the poultry breeding industry</i>	37
3.2.4.2	<i>Susceptibility of egg yolk and embryo tissues to lipid oxidation</i>	37
3.2.4.3	<i>Antioxidant protection of egg yolk and embryo tissues by Canthaxanthin</i>	39
3.3	Studies with canthaxanthin (CAROPHYLL® Red) as a nutritive antioxidant that supports the development of chicks	41
3.3.1	Introduction	41
3.3.2	Studies	43
3.3.2.1	<i>Surai et al. 2003. Effect of Canthaxanthin content of the maternal diet on the antioxidant system of the developing chick</i>	43
Objective		43
Methods		43
General Results		43
Specific results on antioxidant activity		43
Conclusion		44
3.3.2.2	<i>Robert et al. 2007. Effects of Canthaxanthin supplementation in the ROSS breeder diet on oxidative stress of chicks</i>	45
Objective		45
Methods		45
General Results		45
Specific results on antioxidant activity		45
Conclusion		46
3.3.2.3	<i>Zhang et al. 2011. Influence of Canthaxanthin on broiler breeder reproduction, chick quality, and performance</i>	47
Objective		47
Methods		47
General Results		47
Specific results on antioxidant activity		47
Conclusion		49
3.3.2.4	<i>Rosa et al. 2012. Effects of Canthaxanthin on the Productive and Reproductive Performance of Broiler Breeders</i>	50
Objective		50
Methods		50
General Results		50
Specific results on antioxidant activity		50
Conclusion		51
3.3.3	Discussion and Conclusions	52
4.	SAFETY	55
4.1	Introduction	55
4.2	Target Animal Safety	58
4.2.2	Absorption, Distribution, Metabolism, Excretion in poultry	58
4.2.3	Tolerance Studies	60

4.2.5	Conclusions on Target Animal Safety	62
4.3	Safety of Canthaxanthin for consumers	63
4.3.1	Introduction	63
4.3.2	Absorption, Distribution, Metabolism, and Excretion	64
4.3.2.1	<i>Summary of the ADME data</i>	64
4.3.2.2	<i>Animal Studies</i>	65
4.3.2.3	<i>Human Studies</i>	70
4.3.3	Acute Toxicity	72
4.3.3.1	<i>Acute Oral Toxicity</i>	72
4.3.3.2	<i>Acute Dermal Toxicity</i>	72
4.3.3.3	<i>Acute Inhalation Toxicity</i>	72
4.3.4	Irritation	72
4.3.4.1	<i>Summary</i>	72
4.3.4.2	<i>Eye Irritation</i>	72
4.3.4.3	<i>Skin Irritation</i>	73
4.3.4.4	<i>Skin Sensitisation</i>	73
4.3.5	Repeated Dose Toxicity	74
4.3.5.1	<i>Summary</i>	74
4.3.5.2	<i>Studies in mice</i>	75
4.3.5.3	<i>Studies in rats</i>	76
4.3.5.4	<i>Studies in dogs</i>	78
4.3.5.5	<i>Studies in monkeys</i>	80
4.3.6	Genotoxicity	82
4.3.6.1	<i>Summary</i>	82
4.3.6.2	<i>Studies</i>	82
4.3.7	Carcinogenicity	86
4.3.7.1	<i>Summary</i>	86
4.3.7.2	<i>Studies</i>	86
4.3.8	Reprotoxicity and Developmental Toxicity	96
4.3.8.1	<i>Summary</i>	96
4.3.8.2	<i>Reprotoxicity</i>	96
4.3.8.3	<i>Developmental Toxicity / Teratogenicity</i>	98
4.3.9	Specific Investigations	100
4.3.9.1	<i>Mechanistic studies on Canthaxanthin crystalline deposits</i>	100
4.3.9.2	<i>Animal models for retinal Canthaxanthin deposition</i>	100
4.3.9.3	<i>Canthaxanthin crystals in human retina</i>	101
4.3.9.4	<i>Induction of xenobiotic metabolizing enzymes</i>	106
4.3.9.5	<i>Immuno-modulatory activities</i>	110
4.3.10	Overall Summary	110
4.3.10.1	<i>Absorption, Distribution, Metabolism, and Excretion</i>	110
4.3.10.2	<i>Acute Toxicity</i>	112
4.3.10.3	<i>Effects on Skin and Eye</i>	112
4.3.10.4	<i>Repeated Toxicity</i>	112
4.3.10.5	<i>Genotoxicity and Mutagenicity</i>	113
4.3.10.6	<i>Carcinogenicity</i>	113
4.3.10.7	<i>Reprotoxicity and Developmental Toxicity</i>	114
4.3.10.8	<i>Immuno-modulatory activities</i>	114
4.3.10.9	<i>Canthaxanthin deposition in the eye</i>	114
4.3.10.10	<i>General conclusions</i>	115
4.3.11	Acceptable Daily Intake	116
4.3.11.1	<i>ADI in the US</i>	116
4.3.11.2	<i>Other ADI values</i>	117
4.3.12	Consumer Exposure to Canthaxanthin	117
4.3.12.1	<i>Fate of culled eggs from poultry breeders</i>	117

4.3.12.2	<i>Calculation from proposed new use as antioxidant</i>	118
4.3.12.3	<i>Cumulative exposure to Canthaxanthin from current and proposed uses:</i>	119
4.4	Overall Safety conclusions	120
5.	ENVIRONMENTAL SAFETY	121
6.	ANNEXES	122
6.	REFERENCES	125
7.	GRAS EXPERT PANEL OPINION	140
7.1	Background	140

List of Tables

Table 2-1	Canthaxanthin Specifications (CFR 21 § 73.75)	12
Table 2-2	Chemical description of Canthaxanthin.	12
Table 2-3	Canthaxanthin specifications and batch to batch variation	13
Table 2-4	Relevant Physical and Chemical properties of Canthaxanthin	13
Table 2-5	Canthaxanthin content and batch to batch variation of impurities	15
Table 2-6	CAROPHYLL® Red products composition (weight proportion %).	16
Table 2-7	Batch to batch variation for Canthaxanthin content and impurities in CAROPHYLL® Red 10%	16
Table 2-8	CAROPHYLL® Red 10% physical state and batch to batch variation	17
Table 2-9	Shelf life of Canthaxanthin in CAROPHYLL® Red 10%	19
Table 2-10	Stability of CAROPHYLL® Red 10% in a complete poultry premix	20
Table 2-12	Content uniformity of pelleted broiler compounded feed containing CAROPHYLL® Red 10%	21
Table 2-13	Overview of analytical methods for Canthaxanthin determination in different matrixes.	22
Table 2-14	Analytical methods for antioxidant activity of Canthaxanthin	25
Table 3-1	Proposed mode of use of Canthaxanthin (CAROPHYLL® Red)	26
Table 3-2	CXN effect on omega-3 PUFAs content of poultry meat	31
Table 3-3	Yolk and Embryo tissues susceptibility to lipid peroxidation in vitro	32
Table 3-4	CXN, MDA and PUFA (22:4n-6) in egg yolk from commercial layers supplemented with CXN	32
Table 3-5	CXN content an antioxidant status of Gull egg yolks supplemented with CXN in feed	36

Table 4-1 Safety Evaluations for Canthaxanthin in the US and the EU.....	56
Table 4-2 Distribution of Canthaxanthin and its metabolites in chicken tissues (Tyczkowski et al. 1988).....	60
Table 4-3 Studies on target animal safety from Roche's 1971 Color Additive Petition for Canthaxanthin.....	60
Table 4-4 Studies with poultry breeders fed Canthaxanthin at different doses in feed.	61
Table 4-5 Theoretical Exposure from consuming culled eggs from breeder hens.....	118
Table 4-6 Canthaxanthin deposition in eggs and tissues of egg breeders, supplemented with Canthaxanthin at 0, and 6 mg/kg feed for 24 weeks, expressed as mean \pm SEM.....	119
Table 4-7 Theoretical exposure from consuming edible breeder tissues*.....	119
Table 4-8 Cumulative canthaxanthin intake and safety margin.....	119

List of Figures

Figure 2-1 Structural formula of Canthaxanthin	12
Figure 2-2 Canthaxanthin Manufacturing Principle.....	14
<u>Figure 2-3 Canthaxanthin Manufacturing Process</u>	15
Figure 2-4 Flow diagram of the production of CAROPHYLL [®] Red 10%.....	18
Figure 3-3-1 Linearity of the dose response of dietary CXN to CXN in egg yolk*.....	34
Figure 3-3-2 Linear regression: CXN content in egg yolks from "Breeding Layers" fed CXN	35
Figure 3-3 Antioxidant system in the egg	40
Figure 3-4 Surai et al. 2003***. Susceptibility to lipid peroxidation (TBARS) in livers from Offspring of hens fed with or without CAROPHYLL [®] Red - decreased oxidative stress.....	44
Figure 3-5 Zhang et al. 2011**. Susceptibility to lipid peroxidation (TBARS) in yolk and serum from Offspring of hens fed with or without CAROPHYLL [®] Red - decreased oxidative stress....	49
Figure 3-6 Rosa et al. 2012*. Susceptibility to lipid peroxidation (TBARS) in yolk and serum from Offspring of hens fed with or without CAROPHYLL [®] Red - decreased oxidative stress....	51
Figure 3-7 Chick Embryo Development (Source: Cobb 2012).....	52
Figure 3-8 Overview of four studies: Susceptibility to lipid peroxidation (TBARS) in offspring from hens fed with or without CAROPHYLL [®] Red and decreased oxidative stress.....	53
Figure 3-9 Overview of four studies: Susceptibility to lipid peroxidation (TBARS) in Offspring from hens fed with or without CAROPHYLL [®] Red - Oxidative stress of CXN Treatments respect to Control (%)......	54
Figure 4-1 Pathway for the metabolism of Canthaxanthin in chickens (Tyczkowski et al. 1988)	59

1. EXECUTIVE SUMMARY

DSM Nutritional Products convened an independent panel of recognized experts (hereafter referred to as the GRAS Panel), qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients. As described in Title 21 of the Code of Federal Regulations (21CFR§570.30), the GRAS Panel has concluded, based upon scientific procedures, that the proposed use of Canthaxanthin (B-Carotene-4,4-Dione, trade name CAROPHYLL® Red) in poultry breeder diets, as a nutritive antioxidant to support the development of chicks is Generally Recognized As Safe. Canthaxanthin will be used in breeder diets at a level no greater than 6 mg/kg feed; 60 mg/kg feed as the commercial formulation CAROPHYLL® Red.

Canthaxanthin is currently authorized as a Color Additive in the US, where its safety and use has been confirmed by the FDA for the following applications per 21 CFR 73.75: to enhance the yellow color of broiler chicken skin at 4.41 mg/kg of complete feed; to enhance the pink to orange-red color of flesh of salmonids fish at not more than 80 mg/kg of feed; for use as a general food color at not more than 30 mg/lb of solid and semi-solid food and not more than 30 mg/pint of liquid food; and per 21 CFR 73.1075, for coloring ingested drugs generally in amounts consistent with good manufacturing practice. DSM does not intend to exceed the current statutory limits for use of Canthaxanthin as a color additive and merely seeks to expand its permitted use to poultry breeder diets as a nutritive antioxidant to support the development of chicks. All other ingredients in CAROPHYLL® Red are also suitable for use in animal food in the USA.

A comprehensive search of scientific literature for safety and toxicity information on Canthaxanthin was conducted by DSM Nutritional Products through November, 2013 and are provided in the Annexes as literature searches 1, 2 and DSM 2013. The following databases were searched: NCBI Pubmed, SciFinder, Scopus, TOXNET, OECD eChem Portal, RTECS, IPCS INCHEM, NTP (National Toxicology Program), ILSI (International Life Sciences Institute) and BIBRA toxicity profiles and EFSA and JECFA publications. All relevant publications were reviewed, summarized and incorporated into the GRAS dossier, assimilated by The (b) (4) Group, and submitted to the Expert Panel in 2011. Copies of the literature were available for review by the GRAS Panel. The GRAS Panel also received information pertaining to the method of manufacture, product specification, analytical data, intended use levels in specified food formulation rations, anticipated residues and resulting consumption estimates from all intended uses, safety studies conducted with Canthaxanthin and any other relevant data on safety and tolerance-related information.

The members of the Expert Panel were

(b) (4)

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. Subsequent to the GRAS determination by the Panel in 2011, additional scientific literature on the safety and proposed use of canthaxanthin and CAROPHYLL® Red became available in the public domain. This new data

supports the GRAS Panel's conclusion that the proposed use as a nutritive antioxidant is safe and GRAS as presented in this updated dossier.

1.1 INTRODUCTION

The purpose of this document is to provide technical and scientific information supporting the conclusion that canthaxanthin (CAROPHYLL® Red) as a nutritive antioxidant in poultry breeders' food to support the development of chicks, is Generally Recognized as Safe (GRAS) based upon scientific procedures. DSM Nutritional Products Ltd. is the manufacturer and distributor of CAROPHYLL® Red and other carotenoid materials for use in human and animal nutrition around the world.

CAROPHYLL® Red is a carotenoid preparation that contains Canthaxanthin, (CAS number 514-78-3, B- Carotene-4,4'-dione) as the active ingredient. Canthaxanthin is an approved color additive that has been in the human food supply in the United States since 1969 and in animal feed since 1985.

For its use as a color additive in animal nutrition, Canthaxanthin is currently marketed in the US under the trade name CAROPHYLL® Red 10%, which is a (b) (4) form containing a target content of 10% Canthaxanthin. The same CAROPHYLL® Red product form is to be used in poultry breeder diets as a nutritive antioxidant to support the development of chicks. CAROPHYLL® Red use in breeder diets is not aimed to color egg yolk of animal tissues. In order to differentiate the color and antioxidant functionalities and corresponding animal classes, relevant labeling will be provided for CAROPHYLL® Red.

Additional CAROPHYLL® Red product forms may be developed in the future with feed grade ingredients according to marketing needs. Carophyll is CAROPHYLL® Red and canthaxanthin are produced by DSM Nutritional Products Ltd. at its plant in (b) (4) under GMP (21 CFR 110) via a (b) (4) process.

The safety of canthaxanthin as a nutritional antioxidant is the same as canthaxanthin as a color additive because it is manufactured to the same specification as the approved color additive in the US. Furthermore, all other ingredients in CAROPHYLL® Red are approved for use in animal food by FDA or the Association of American Feed Control Officials (AAFCO). Canthaxanthin has been the subject of various safety reviews by a number of authorities worldwide, including the European Food Safety Authority, FDA's Center for Food Safety And Applied Nutrition and the Center of Veterinary Medicine. An Acceptable Daily Intake of 150 mg/person/day has been established for humans by the U.S. FDA.

The functionality of canthaxanthin as an antioxidant source has been widely demonstrated in in vivo and in vitro trials. Likewise, the antioxidant capacity of canthaxanthin via CAROPHYLL® Red fed to poultry as a nutritive antioxidant has been demonstrated in in vivo studies. Relevant published literature is summarized in this document.

The proposed new use of canthaxanthin in CAROPHYLL® Red as a nutritional antioxidant is exempt from the color additive definition as allowed for at 21 CFR 70.3 (g) because the target species (poultry breeders) are solely aimed for the production of fertile eggs and chicks, not for

the production of table eggs or pigmented yolk. Although color is certainly imparted to culled eggs and spent breeder hen tissues that may be added to the food supply, those materials would constitute a very small percentage of the annual commercial tonnage of poultry products. This means that any color imparted to the processed foods made with ingredients containing the aforementioned breeder materials is clearly unimportant insofar as the appearance, value, marketability, or consumer acceptability is concerned. See section 4.3.12 for a more detailed discussion.

1.2 GRAS Exemption Claim

DSM Nutritional Products hereby notifies FDA of its conclusion that Canthaxanthin and the commercial form, CAROPHYLL® Red, are exempt from the definition of a "food additive" and thus from the premarket approval requirements outlined in section 201(s) of the Federal Food, Drug and Cosmetic Act because its use in animal feed at levels up to 6 milligrams per Kg of feed (60 mg/kg feed for CAROPHYLL® Red) as a nutritive antioxidant in poultry breeders' food to support the development of chicks, is generally recognized as safe (GRAS) by qualified experts, as shown through scientific procedures, corroborated by a history of safe use, substantial equivalence to canthaxanthin approved as a color additive, and extensive safety data.

To make the GRAS conclusion, DSM Nutritional Products compiled information regarding the nature of the substance, specifications, manufacturing, proposed conditions of use and technical evidence of safety into a comprehensive technical dossier (GRAS dossier) and sought the opinion of a GRAS Panel specifically convened for the purpose of reviewing the information therein to determine whether there is consensus among qualified experts that the use of the CAROPHYLL® Red form of Canthaxanthin as described entails a reasonable certainty of no harm and is generally recognized as safe.

All data and information that are the basis for the GRAS conclusion are available for FDA's review and copying at reasonable times at DSM Nutritional Products, 45 Waterview Blvd, Parsippany, NJ 07054, and will be sent to FDA upon request.

Signature: 

Date: 27 June 2014

James La Marta, Ph.D., CFS
Senior Manager Regulatory Affairs
DSM Nutritional Products
45 Waterview Blvd
Parsippany, NJ 07054

DSM1493-001



1.3 Name and address of Notifier

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

Person responsible for the dossier:

James La Marta, Ph.D., CFS
45 Waterview Boulevard
Parsippany, New Jersey 07054
Tel: 973-257-8325

1.4 Name and Address of Manufacturer

DSM Nutritional Products (b) (4)
(b) (4)

1.5 Name and Address of the Exclusive Distributor

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

2. IDENTITY OF THE NOTIFIED SUBSTANCE

2.1 Common or usual name

“Canthaxanthin” is the common name of the active substance that is the subject of this GRAS notification and is also noted as “CXN” within the dossier.

CAROPHYLL[®] Red is the trade name of the preparations manufactured by DSM Nutritional Products Ltd., containing Canthaxanthin as the active substance. DSM has produced three different canthaxanthin preparations:

- CAROPHYLL[®] Red
- CAROPHYLL[®] Red 10%
- CAROPHYLL[®] Red 15%

These product forms are all formulated and manufactured using the same principles and thus have similar nutritional and functional features.

“CAROPHYLL[®] Red 10%” is the preferred form of canthaxanthin to be placed in the US market for use in poultry breeder food, consistent with the proposed conditions described in this GRAS notice. This GRAS use of canthaxanthin will be in addition to the currently approved use of canthaxanthin in the US as a color additive for broiler and salmonids pigmentation regulated at 21 CFR 73.75.

Other stable Canthaxanthin formulations may be made with ingredients suitable for use in animal food to satisfy future market needs.

2.2 Standard of Canthaxanthin Identity in the US

Canthaxanthin is currently authorized as a Color Additive in the US, where its safety and efficacy has been confirmed by the FDA for the following applications:

- “to enhance the yellow color of broiler chicken skin at a maximum of 4.41 mg/kg of complete feed to supplement other known sources of xanthophyll and associated carotenoids to accomplish the intended effect”. (21 CFR § 73.75)(Color Additive Petition (CAP) submitted in 1971, by Hoffman-La Roche).
- “to enhance the pink to orange-red color of flesh of salmonids fish at not more than 80 mg/kg of feed salmonids fish”. (21 CFR 73.75) (CAP approved in 1999 submitted by BASF)
- “General food color at not more than 30 mg/lb of solid and semi-solid food and not more than 30 mg/pint of liquid food”. (21 CFR 73.75) (CAP approved in 1969, submitted by Hoffman-La Roche)
- may be safely used for coloring ingested drugs generally in amounts consistent with good manufacturing practice (21 CFR § 73.1075).

Canthaxanthin can lawfully be added as a color additive in the US, only if the applicable standards of identity (Specifications), as described in Title 21 of the Code of Federal Regulations (21 CFR § 73.75), are met (Table 2-1). The canthaxanthin that is the subject of this GRAS notification meets the same purity standards as the Canthaxanthin already approved and used as a color additive.

Table 2-1 Canthaxanthin Specifications (CFR 21 § 73.75)

Parameter	Specification
Physical state	solid
1% solution in chloroform	Complete and clear
Melting range (decomposition)	207 -212°C (corrected)
Loss on drying	max 0.2%
Residue on ignition	max 0.2%
Total carotenoids other than trans Canthaxanthin	max 5%
Lead	max 10 ppm
Arsenic	max 3 ppm
Mercury	max 1 ppm
Assay	96 to 101%

2.3 Characterization of the substance

2.3.1 Characterization of the active ingredient (Canthaxanthin)

2.3.1.1 Description

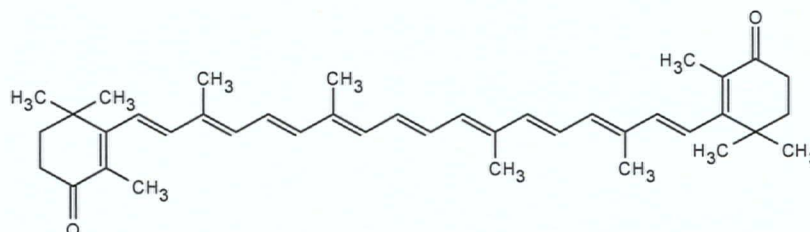
The chemical description of Canthaxanthin used in CAROPHYLL® Red is presented in Table 2-2 and its structural formula in

Figure 2-1.

Table 2-2 Chemical description of Canthaxanthin.

Generic name	Canthaxanthin
International chemical name (IUPAC nomenclature)	β-Carotene-4,4'-dione
Synonyms	all-trans-Canthaxanthin, β-Carotene-4,4'-dione, all-trans
Chemical abstract service number (CAS)	514-78-3
EINECS number	208 187 2
Empiric Formula	C40H52O2
Molecular Mass	564.85 g/mol

Figure 2-1 Structural formula of Canthaxanthin



Certificates of analysis for typical Canthaxanthin batches (Annex DSM 2011a) demonstrate consistency of production lots to US specifications (CFR 2010a). Canthaxanthin in CAROPHYLL® Red is characterized as presented in Table 2-3.

Table 2-3 Canthaxanthin specifications and batch to batch variation

Parameter	Units	Specification (CFR 2010a)	Lot 1 UE01101001	Lot 2 UE01101002	Lot3 UE01101003
Physical state		solid	solid	solid	solid
1% solution in chloroform		Complete and clear	complete and clear	complete and clear	complete and clear
Melting range (decomposition)	°C	207 -212 (corrected)	corresponds	corresponds	corresponds
Loss on drying	%	max 0.2	0.1	0.2	0.1
Residue on ignition	%	max 0.2	0.04	0.01	0.02
Total carotenoids other than trans CXN	%	max 5	2.86	2.95	2.79
Lead	ppm	max 10	< 2	< 2	< 2
Arsenic	ppm	max 3	< 1	< 1	< 1
Mercury	ppm	max 1	< 1	< 1	< 1
Assay	%	96 to 101	98.7	98.3	98.0

2.3.1.2 Relevant properties

Physical and chemical properties of Canthaxanthin used in CAROPHYLL® Red are presented in Table 2-4.

Table 2-4 Relevant Physical and Chemical properties of Canthaxanthin

Properties	Value	Method/Data/ Reference
Physical state	Color: Violet - Form: powder	Visual
Electrostatic properties (volume resistivity)	(1.5 ± 0.6) · 10 ⁷ Ω·m	Megohmmeter M1500P, Sefelec
Melting point	207°C with decomposition	OECD N° 102 (OECD 1995a)
Density	Solid 1.0582 + 0.0004 g/cm ³ @ 25.9°C Bulk 0.323 + 0.001 g/cm ³ @ 25.9°C	(Annex DSM 2010a)
Solubility in water at 20°C	< 0.053mg/l	OECD No. 105 (OECD 1995b)
Solubility in chloroform	soluble	Ph.Eur. <2.2.1>
Solubility in ethanol	Insoluble	(EP 2005)
Kow Kd/Koc	14.1 (calculated)	(Annex DSM 2011b)
UV spectrum	(b) (4)	
Mass spectrum		
Infrared spectrum		
NMR spectrum		
Possible Isomers	Cis/trans-isomers: all-trans, 9-cis, 13-cis, 15-cis	

2.3.1.3 Manufacturing

The principle for Canthaxanthin synthesis by DSM is based on the (b) (4)

An optimized manufacturing process is currently carried out, meeting Canthaxanthin specifications and purity as currently described in the Code of Federal Regulations (21CFR § 73.75). (b) (4)

Raw Materials and Reagents

(b) (4)

Canthaxanthin Manufacturing Process

(b) (4)

(b) (4)



Canthaxanthin Impurities

Analysis for typical canthaxanthin batches demonstrate consistency of production lots. Impurities other than available in the relevant CFR specifications are presented in Table 2-5.

Table 2-5 Canthaxanthin content and batch to batch variation of impurities

Parameter	Lot 1 UE00803010	Lot 2 UE00803011	Lot3 UE00803012	Average
-----------	---------------------	---------------------	--------------------	---------

(b) (4)



Process Control

Control of the suitability of the process is ultimately confirmed by compliance of each batch of Canthaxanthin to product specifications. A sample label of Canthaxanthin (b) (4) is provided in Annex DSM 2012.

2.3.2 Characterization of CAROPHYLL® Red

2.3.2.1 Description

The composition of CAROPHYLL® Red 10%, the product form currently available in the US market, and that of other Canthaxanthin preparations discussed in this GRAS Notice are presented in (Table 2-6). Certificates of analysis for three batches of CAROPHYLL® Red 10% demonstrate consistency of production lots (Annex DSM 2010c). Test results for Canthaxanthin content and impurities in three batches of CAROPHYLL® Red 10% are given in

Table 2-7.

Table 2-6 CAROPHYLL® Red products composition (weight proportion %).

Ingredient	CAROPHYLL® Red 10%*	CAROPHYLL® Red	CAROPHYLL® Red 15%**
Canthaxanthin	10.00	10.00	15.00
(b) (4)			

**Also called Jia Li Hong 15% (in Chinese language).

Table 2-7 Batch to batch variation for Canthaxanthin content and impurities in CAROPHYLL® Red 10%

Parameter	Unit	Lot 1 UE0A810061	Lot 2 UE0A810062	Lot3 UE0A810063
Canthaxanthin content	%	10.3	10.4	10.0
Arsenic	ppm	<1	<1	<1
Lead	ppm	<1	<1	<1
Mercury	ppm	<1	<1	<1
(b) (4)				

*Within VICH-GL18 limits

2.3.2.2 Packaging

CAROPHYLL® Red 10% is packaged in new, clean, food grade multi-walled bags composed of a outer layer of (b) (4). Each bag holds 20 Kg / 44 Lbs. of product.

2.3.2.3 Relevant properties

Physical state of three batches of CAROPHYLL® Red 10% are given in Table 2-8 (Annex DSM 2010d).

Table 2-8 CAROPHYLL® Red 10% physical state and batch to batch variation

Parameter	Limits/Test	Lot 1 UE00804015	Lot 2 UE0A910103	Lot3 UE0A910104	Average
Appearance/Particle shape	free-flowing powder	free-flowing powder	free-flowing powder	free-flowing powder	
Color	red-violet	red-violet	red-violet	red-violet	
Loss on drying	<8 %	(b) (4)			5.7
Particle Size Distribution	>100% -Ø850µm	(b) (4)			100
	>90% -Ø425µm	(b) (4)			98.3
	<30% - Ø150µm	(b) (4)			10
Powder fines (%)	Particles<0.05mm	(b) (4)			0.0
Density (kg/liter)	bulk/tapped	(b) (4)			0.6/0.8
Dusting Potential (Heubach test*)	product on filter (mg)	(b) (4)			68.5
	product on filter (ppm)	-	-	-	2740**
	active in dust (%)	-	-	-	3.17
	active on filter (ppm)	-	-	-	87

*The product is rated as very low dusting.
 ** Calculated value from product on filter (mg)

2.3.2.4 Manufacturing

Ingredients and approval status

Ingredient	Safety Data Sheet	Ingredient Specifications	Registration Status allowing ingredient use in CAROPHYLL® Red 10%
(b) (4)			

Ingredient	Safety Data Sheet	Ingredient Specifications	Registration Status allowing ingredient use in CAROPHYLL® Red 10%
(b) (4)			
A material safety datasheet with additional information on composition and ingredients in CAROPHYLL® Red 10% is provided (Annex DSM 2011d).			

CAROPHYLL® Red 10% Manufacturing Process

(b) (4)



A sample label of CAROPHYLL[®] Red 10% is provided in Annex DSM 2012a.

2.4 Physico-chemical and technological properties of the additive

2.5 Stability

Because the crystalline form of Canthaxanthin is sensitive to oxidation, light and heat, it is mandatory to market this carotenoid as a stabilized preparation, in this case CAROPHYLL[®] Red 10% from DSM Nutritional Products. This way, formulated products placed in the market shall be stable during storage and when added to premixes and/or feed.

2.5.1.1 Stability of the additive

Shelf life of CAROPHYLL[®] Red 10%, at two different test conditions, is presented in Table 2-9. Canthaxanthin has excellent stability (shelf life) in CAROPHYLL[®] Red 10%, where at least 90.2% of the added content was retained after a storage period of 36 months at 15°C. Further stability data under accelerated conditions, at 40°C and 75%RH, is also presented (Annex DSM 2010e). Based upon this stability data, the shelf life is defined to be 18 months from the day of production provided that the product is kept under the storage conditions DSM Nutritional Products recommends (below 15°C) (Annex DSM 2011c).

Table 2-9 Shelf life of Canthaxanthin in CAROPHYLL[®] Red 10%

Storage Period	Retention of initial value (%)					
	@ 15°C ± 2°C			@ 40°C ± 2°C / 75%RH ± 5%		
Months	Lot 1 MG05001	Lot 2 MG05002	Average	Lot 1 MG05001	Lot 2 MG05002	Average
1	-	-		(b) (4)		97.1
3	(b) (4)		96.6			94.1
6			95.5			94.1
9			95.6	-	-	
12			93.6	-	-	
18			95.5	-	-	
24			97.6	-	-	
36			92.1	-	-	

2.5.1.2 Stability of the additive used in premixtures

Stability of a preparation of Canthaxanthin, CAROPHYLL[®] Red 10%, was tested in a complete poultry premixture (minerals + vitamins). Test results show that after initial processing, 98.1% of the added Canthaxanthin content was still found in the premixture containing the test product. These results indicate that practically no losses were found after mixing the products into the premixtures. Further results show that Canthaxanthin retention after storage for 3 months at 25°C was 94.9% of the added content (it is common practice in the industry not to store premixtures longer than three months). Thus, the storage stability of the test product was good. It is concluded that Canthaxanthin in CAROPHYLL[®] Red 10% is stable during mixing and at a storage time of 3 months @ 25°C in complete premixtures. Stability results for CAROPHYLL[®]

Red 10% in premixture are presented in Table 2-10, which also includes comparative stability data for the CAROPHYLL® Red product form (Annex DSM 2007b), demonstrating similar behavior of the Canthaxanthin preparations.

Table 2-10 Stability of CAROPHYLL® Red 10% in a complete poultry premix

Test Product	CAROPHYLL® Red 10% (Lot: VTP0334)	
CXN added to premix (g/kg)	1.05	
Canthaxanthin	Found* (g/kg)	Retained %
After mixing	1.03	98.1
1-month, 25°C	1.06	101.2
1-month, 35°C	0.98	93.7
3-months, 25°C	1.00	94.9

2.5.1.3 Stability of the additive used in feed

Stability of CAROPHYLL® Red 10% was tested in mash and pelleted poultry compounded feeds. In the mash feed, retentions were in the range of 106-109% of added amount, indicating an excellent stability of the test products during processing and storage. Based on these findings, we conclude that CAROPHYLL® Red 10% is stable in mash feed when stored for 3 months. Similar results were also achieved with CAROPHYLL® Red. In pelleted feed, and after feed processing at temperatures up to 90°C, 100% retention was found. During a storage period of 3 months, losses were small, i.e. 1% for CAROPHYLL Red 10%. Based on these findings, we conclude that CAROPHYLL Red 10% is stable in stored feed pelleted up to 90°C. Similar results were also achieved with CAROPHYLL® Red. Stability results during processing and storage of feed are presented in **Error! Reference source not found.**, which also includes stability data for CAROPHYLL® Red form (Annex DSM, 2007c).

Table 2-11 Stability of CAROPHYLL® Red 10% and CAROPHYLL® Red in mash and pelleted poultry compounded feed.

	Test Product		CAROPHYLL® Red 10%	CAROPHYLL® Red
		CXN added to feed	mg/kg	20.6
Mash Feed	Canthaxanthin Found			
	Initial	mg/kg	22.4	22.2
	3-months, 25°C	mg/kg	21.0	22.2
	Canthaxanthin Retention			
	Initial	%	109.0	105.7
	3-months, 25°C	%	106.0	105.7
Pelleted Feed	Canthaxanthin Found			
	Initial	mg/kg	22.6	21.5
	1-month, 35°C	mg/kg	20.1	18.4
	3-months, 25°C	mg/kg	20.3	19.3
	Canthaxanthin Retention			
	Initial	%	108.7	102.3
	1-month, 35°C	%	97.5	87.6
3-months, 25°C	%	98.5	91.9	

2.5.2 Homogeneity

To ensure proper efficacy and safety of the product, Canthaxanthin preparations must provide homogenous mixing in premixtures and feedstuffs. Based on our experience, CAROPHYLL® Red can be homogeneously mixed in premix and feedstuffs.

Content uniformity of a broiler pelleted feed containing three batches of CAROPHYLL® Red 10%, at a dose rate of 5 mg/kg feed, was examined (Annex DSM 2010f). Homogeneity test results for feed batches containing the test product are given in the Table 2-11.

Canthaxanthin distribution in feed, expressed as coefficients of variation, were on average 5.9%, and varied between 3.3 and 7.5% (CAROPHYLL® Red 10% was added to the premix and the premix then added to feed). Thus, the product was uniformly contained in all three feed batches. We conclude that CAROPHYLL® Red 10% is easy to uniformly mix into premixtures and compounded feeds.

Table 2-11 Content uniformity of pelleted broiler compounded feed containing CAROPHYLL® Red 10%

Sample	CXN Found, mg / kg			Average
	Lot 1 UE00804015	Lot 2 UE0A810078 (b) (4)	Lot 3 UE0A810079	
a				
b				
c				
d				
e				
f				
g				
h				
CV	6.9%	7.5%	3.3%	5.9%

2.6 Analytical methods

Analytical methods for quantification of Canthaxanthin content in the crystalline material, and Canthaxanthin determination in different matrixes such as formulated products (i.e. CAROPHYLL® Red 10%), premixes, feed and animal tissues, have been available for many years and widely used by the feed industry.

2.6.1 Methods of analysis for Canthaxanthin crystalline

Parameter	Method / Description	
	1971 CAP	State of the art methods (Currently used at DSM)
Physical state		Visual
1% solution in chloroform	Methods submitted with the 1971 Color Additive application for control of Canthaxanthin specifications (Annex Roche 1971)	In a 50 ml volumetric flask, dissolve and dilute to 50.0ml 490 to 510 mg with chloroform. Measure turbidity with the turbidimeter report in Formazin Turbidity Unit (FTU). Note: The solution should be clear and contain no undissolved particles (verify the transparency in front of a lamp on the remaining solution in the flask of 50 ml).
Melting range (decomposition)		OECD N° 102 Apparatus: Mettler FP804T – Initial temperature: 195°C – Temperature increase: 2°C/min – Final temperature: 217°C – Sample weight: 4-5 mg

Parameter	Method / Description
Loss on drying	(b) (4)
Residue on ignition	Weigh 0.9 to 1.1 g and proceed according to method MEG/QCA12(Annex DSM 2008c)
Lead, Arsenic, Mercury	Spectrophotometric method ICP/QCA11 (Annex DSM 2011g)
Assay + Total carotenoids other than trans Canthaxanth	UV: spectrophotometric method SUV/QCA07 (Annex DSM 2009c) HPLC: Calculated content via the sum of the peaks of all-trans-CXN and cis-CXN - method CPL/QCA27 (Annex DSM 2010j)

2.6.2 Analytical methods for the determination of Canthaxanthin in different matrixes.

A collaborative effort led by the "Task Force Analytics Carotenoids" of the European Federation of Feed Additives Producers (FEFANA), has recently re-assessed the analytical methods for carotenoids, including those for Canthaxanthin content in different matrixes and tissues. The methods resulting from this exercise are provided in (Table 2-12) .

Table 2-12 Overview of analytical methods for Canthaxanthin determination in different matrixes.

Parameter	Method / Description
Determination of CXN in CAROPHYLL® Red 10%	(FEFANA 2011a) Determination of Canthaxanthin by Photometry at the Isobestic Wavelength of 426 nm. The powdery or water dispersible formulations are digested in water with protease. After dilution with acetone the mass fraction of Canthaxanthin is determined photometrically at the isobestic wavelength of 426 nm. This analytical method is designed for the determination of the content of Canthaxanthin in the range of 5 to 25 g/100 g. Because of measuring at an isobestic wavelength the result for the content of Canthaxanthin does not depend on the ratio of the geometrical isomers present in the product forms.
Determination of CXN in premixes and feed	(FEFANA, 2011b) Determination of Stabilized Canthaxanthin in Premixes and Feedstuffs. The assay comprises an enzymatic digestion of the formulation followed by extraction with ethanol and dichloromethane. The extract is injected into an isocratic normal-phase HPLC system that is able to resolve the all-E isomer and the main Z isomers of Canthaxanthin. The Z isomers of Canthaxanthin are quantified on basis of the response of all-E Canthaxanthin. The lower specific absorbance of the main Z-isomers (9Z and 13Z Canthaxanthin) is taken into account by correction with an experimentally determined relative response factor.
Determination of CXN in tissues	(FEFANA 2010) Determination of Canthaxanthin in Poultry tissue. Poultry tissues like broiler skin or fat are extracted by homogenization in acetone. The extract is filtered and evaporated at reduced pressure using a rotary evaporator or a flow of nitrogen at 50°C. The residue is dissolved in a mixture of n-hexane and acetone and analyzed by normal-phase HPLC.
Determination of CXN in egg yolk	(FEFANA 2011c) This method specifies the determination of total Canthaxanthin in egg yolk by High Performance Liquid Chromatography (HPLC). Egg yolk is diluted with water. The emulsion is mixed with ethanol and extracted by shaking with n-heptane. The carotenoids are analyzed by injecting an aliquot of the n-heptane phase into a normal-phase HPLC using a detection wavelength of 466 nm.

2.6.3 Method for the determination of the antioxidant activity of Canthaxanthin

2.6.3.1 Introduction

Secondary lipid oxidation products include aldehydes like Malondialdehyde (MDA), ketones and fatty acids. The aldehydes especially can form Schiff bases with phosphatidylserin and ethanolamine as well as proteins which can cause even further damage to membranes and proteins.

Unfortunately, the reaction pathways of radicals and their detoxification via various routes in biological systems are far from being understood. Several analytical assays have been established during the last decades to monitor oxidative stress in biological systems and positive effects of antioxidants or compound/extracts with antioxidant properties. None of the compounds assayed by these tests are generally scientifically accepted as valid biomarkers since the complexity of a biological system in flux can hardly be described with one single parameter. Nevertheless, much effort has been put into standardization of analytical methods to ensure reproducibility and comparability of analytical data.

2.6.3.2 TBARS as an Analytical assay

The thiobarbituric acid reactive substances assay (TBARS) measures a distinct secondary lipid peroxidation product Malondialdehyde (MDA) with a simple spectrophotometric test. MDA reacts with thiobarbituric acid at low pH to produce a red colored complex with an absorbance at 532 nm. The test is widely used for analysis of lipid peroxidation in plasma, serum and tissue samples. There are even some test kits commercially available which show good reproducibility and precision. Nevertheless, MDA is not recognized as a validated biomarker for oxidative stress in humans or animals.

Although MDA is a lipid peroxidation marker the compound itself is very water soluble which makes it easy to apply the test to plasma or serum samples.

The test has to be adapted for lipid matrices e.g. meat, liver, egg yolk or other food matrices. Some authors describe massive interferences of the spectrophotometric assay with matrix compounds. Several modifications of the method have been published but no general extraction protocol or validation data of the method is currently available. Despite the lack of a valid extraction protocol, the method delivers reasonable reproducibility and precision in meat and tissue assays.

2.6.3.3 Comparison of methods used for TBARS determination in poultry tissues

The available methods can be divided into direct analysis methods and methods with prior extraction and/or included forced oxidation. For simple matrices like plasma or serum commercial validated test kits are available (i.e. MDA Detection Kit, Nanjing Inching Bioengineering Institut) which produce highly reproducible results using standardized conditions (Zhang et al. 2011). Other tissue samples like liver, muscle, fat etc. require specific extraction procedures to minimize interferences during subsequent spectrophotometric determination of

the MDA adduct. Whereas aqueous samples can be directly used for derivatization, lipid matrices like egg yolk or liver need an extraction step. Aqueous suspensions of lipid containing matrices can react with thiobarbituric acid but the colored complex has to be extracted from the suspension with butanol.

The analysis method of Surai (Surai et al. 1996), which is based on the method of Ohkawa et al. (1979), uses phosphate buffer for homogenizing of tissue samples and a treatment with ferrous sulphate for 60 min at 37 °C to enhance lipid oxidation. The treatment with ferrous ions increases the formation of MDA and is in this way an indirect determination of the antioxidant status of the tissue. An aliquot of the treated sample is derivatized for spectrophotometric analysis (Surai et al. 1996) or for selective HPLC analysis (Surai et al. 2000).

The TBARS method is widely used for the determination of lipid peroxidation in meat, liver, egg yolk, plasma and other biological samples. Since it uses a direct lipid oxidation product it generally shows good correlation to lipid oxidation events and also correlates to sensorial parameters. The method does not show the total antioxidant capacity nor does it reflect stability of the foodstuff nor does the assay allow general biologic interpretation of the data (e.g. individual dietary recommendations).

The only available validated protocol is the commercial MDA kit (Zhang et al. 2011) which can only be used for plasma/serum samples. Neither the direct spectrophotometric method nor the described HPLC methods were fully validated in inter-laboratory trials for tissue samples. But since the recently published analytical methods are using the same protocol for extraction and derivatization, data sets on the same matrix can be compared.

A recent publication (Papastergiadis et al. 2012) evaluated the applicability of the TBARS method in stability trials in various food matrices. They showed that for unprocessed samples (e.g. oils, uncooked meat and fish products) the spectrophotometric and the HPLC method gave comparable results. Only for processed food stuff the spectrophotometric methods resulted in higher values due to interfering reaction products.

The Surai method (Surai et al. 1996) includes an enhanced oxidation step. The procedure does not show the lipid oxidation status but the “antioxidative capacity” of the tissue using the specified conditions. The test is therefore capable of showing minimal effects of antioxidant activity but cannot be compared to TBARS results which have been produced without an enhanced oxidation step.

2.6.3.4 Proposed method

The antioxidant function of Canthaxanthin has been thoroughly reviewed. A number of analytical methods are available and demonstrate its effectiveness for this purpose. The outcome of this review supports the measurement of Thiobarbituric Acid Reactive Substances (TBARS), as the method of preference to confirm the antioxidant activity of Canthaxanthin in tissues such as egg yolk, liver and plasma/serum. The reviewed and proposed method resulting from this exercise is

DSM1493-001



provided below. It is based on Surai's method (Surai et al. 2003) and utilizes the higher sensitivity and selectivity of the HPLC-FLD method compared to the spectrometric assay.

Table 2-13 Analytical methods for antioxidant activity of Canthaxanthin.

Parameter	Method / Description
Antioxidant activity in egg yolk, plasma/serum and liver	(Annex Duesterloh 2012) The report describes an HPLC-FLD method for the determination of thiobarbituric acid reactive substances (TBARS) in biological sample materials (muscle, liver, egg yolk, plasma) after incubation with ferrous sulphate to enhance oxidation. The analytical HPLC-FLD method shows good linearity in the range of 3.6-180 µg/100 mL MDA. The limit of detection and quantitation were estimated to be 0.13 µg/100 mL and 0.44 µg/100 mL MDA, respectively. Analysis of a reagent blank showed no interferences at the retention time of the TBA-MDA adduct in the chromatogram. The method is suitable to detect small antioxidant effects and can be used to show efficacy of various antioxidants in animal trials.

3. CONDITIONS OF USE AND CANTHAXANTHIN UTILITY

3.1 Proposed use

Canthaxanthin (CXN), in the stabilized form "CAROPHYLL® Red" for GRAS purposes, is to be used as a use in poultry breeder diets as a nutritive antioxidant to support the development of chicks.

Table 3-1 Proposed mode of use of Canthaxanthin (CAROPHYLL® Red)

Animal categories	Recommended dose	Mode of Use	Utility
"Poultry breeders" (breeding hens)	6 mg Canthaxanthin / kg feed = 60 mg CAROPHYLL® Red / kg feed	fed daily via premix or direct addition to each feed batch, for targeted deposition in egg yolk	Nutritive antioxidant to support the development of chicks

Commercial poultry" for production of table eggs for human consumption, as well as the use of canthaxanthin for egg yolk pigmentation are out of the scope of the GRAS use.

Use statement: Canthaxanthin has been concluded to be GRAS for use as a nutritive antioxidant to support the development of chicks; it is not intended to be used as a color additive. The use of canthaxanthin in the feed of laying hens for the production of table eggs for human consumption and as a color additive for egg yolk pigmentation are outside of the scope of this GRAS Notice.

3.2 Utility

3.2.1 Background

In nature, carotenoids are responsible for a variety of bright colors in fall leaves, flowers (narcissus, marigold), fruits (pineapple, citrus fruits, paprika), vegetables (carrots, tomatoes), insects (ladybird), bird plumage (flamingo, cock-of-the-rock, ibis, canary) and marine animals (crustaceans, salmon) (Pfander 1992). These pigments provide different colors from light yellow to dark red and when complexed with proteins they can produce green and blue colorations (Ong and Tee 1992). Carotenoids are exclusively responsible for egg yolk color and probably play specific roles in avian embryonic development (Surai 2002).

Canthaxanthin (CXN) belongs to a group of carotenoid pigments known as xanthophylls or oxycarotenoids. It occurs in nature, for example in some edible mushrooms, green and blue-green algae, bacteria, crustaceans and fish such as carp, golden mullet, sea bream and trush wrasse (SCAN 2002).

Canthaxanthin, the active substance in CAROPHYLL® Red, is one of the more than 750 carotenoids which have been identified in nature. It was first isolated from an edible mushroom, a chanterelle species of which the designation *Cantharellus cinnabarinus* led to the name of this

diketo-carotenoid as Canthaxanthin. Canthaxanthin is also widely distributed in bacteria, algae, crustaceans and the feathers of birds (Britton et al. 2004). Canthaxanthin was also found to be a coloring agent of beaks and legs of zebra finches (McGraw and Toomey 2010) and plumage of yellow-crowned bishop and southern red bishop (Prager et al. 2009).

Canthaxanthin was first synthesized from beta-carotene in the late 1950's (Isler 1971), and being nature-identical it was subsequently chosen as a promising candidate for coloring of food, feed and pharmaceuticals in the orange/red spectrum. It was introduced to the market by Hoffman - La Roche in 1962. Because of its coloring properties, and under the trade mark CAROPHYLL® Red (DSM Nutritional Products Ltd), Canthaxanthin product forms have been extensively used by the poultry industry for over 40 years.

In the US, Canthaxanthin is currently authorized as a Color Additive, where its efficacy has been confirmed by FDA for the following applications:

- *"to enhance the yellow color of broiler chicken skin at not more than 4.41 mg/kg of complete feed to supplement other known sources of xanthophyll and associated carotenoids to accomplish the intended effect". (21 CFR § 73.75)* (Color Additive Petition (CAP) submitted in 1971 by Hoffman-LaRoche)
- *"to enhance the pink to orange-red color of flesh of salmonids fish at not more than 80 mg/kg of feed salmonids fish". (21 CFR § 73.75)* (CAP approved in 1999 submitted by BASF).

Moreover, Canthaxanthin and/or CAROPHYLL® Red are generally used and authorized worldwide as color additives for animal food and for addition to salmonids and poultry feeds. In the EU, which has carried out the most recent evaluations of efficacy and safety (SCAN 2002; EFSA 2007, 2010, 2013), the key regulated uses (EC 2008) in animal nutrition that relate to current US approvals are as follows:

- 25 mg Canthaxanthin/kg feed for broilers and other poultry than laying hens
- 8 mg Canthaxanthin/kg feed for laying hens
- 25 mg Canthaxanthin/kg feed for salmonids

While coloring is an important property of carotenoids, these groups of substances also play a key biological role as antioxidants in plant and animal tissues. The proposed GRAS use of canthaxanthin from "CAROPHYLL® Red" is aimed to fulfil the same antioxidant role that this carotenoid plays in nature, not as a color.

An important experimental in vitro property of carotenoids in general is the quenching of singlet oxygen. Canthaxanthin is among those carotenoids, which are the most potent quenchers. The calculated *k*, value for Canthaxanthin was 50% higher than that of beta-carotene but less than for lycopene or Astaxanthin (DiMascio et al. 1989).

Another important property of the carotenoids is the ability to inhibit lipid peroxidation. Canthaxanthin suppressed lipid oxidation to a lesser extent than Astaxanthin but to a greater extent than beta-carotene or zeaxanthin (Jorgensen and Skibsted 1993). Canthaxanthin was more efficient than beta-carotene or zeaxanthin in slowing free radical oxidation of methyl linoleate in solution. Rate of autocatalytic oxidation was also slower with Canthaxanthin. The author suggests that Canthaxanthin and Astaxanthin are more effective antioxidants than beta-carotene (Terao 1989).

Dietary Canthaxanthin inhibited ferrous ion catalyzed peroxidation in biological membranes prepared from chick tissue when moderate rates of peroxidation were imposed. Protective effects of Canthaxanthin were small and independent of Canthaxanthin-induced increases in alpha-tocopherol. The authors suggest that dietary carotenoids may protect against peroxidation or carcinogenesis by an indirect mechanism involving alterations in tissue levels of alpha-tocopherol (Mayne and Parker 1989).

Canthaxanthin inhibited lipid peroxidation in C3H/10T1/2 cells ($P = 0.0001$) (Zhang et al. 1991). Canthaxanthin equalled alpha-tocopherol in effectiveness as a radical-trapping antioxidant in biological membranes prepared from rat liver microsomal homogenates and strongly suppressed Malondialdehyde (MDA) formation and produced a distinct induction period (Palozza and Krinsky 1992).

Canthaxanthin acted as a chain-breaking antioxidant in the peroxidation of membranous phospholipids, when radical generators to initiate peroxidation within phosphatidylcholine liposomes and chick plasma were used (Lim et al. 1992). When investigating normal and tumor thymocytes from mice, Canthaxanthin was a more potent antioxidant than beta-carotene against lipid peroxidation induced by t-butyl hydroperoxide (Palozza et al. 1996).

In the case of wild birds and commercial poultry, Canthaxanthin has been documented to perform this antioxidant role, having a protective effect on biological tissues susceptible of oxidation. Based on its inherent function as antioxidant, and with consideration of recent scientific findings, DSM Nutritional Products has identified an innovative way to support the US poultry industry by feeding Canthaxanthin to poultry breeders.

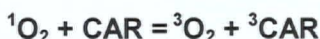
3.2.2 Antioxidant activity of carotenoids

The antioxidant properties of Canthaxanthin and its potential effects in the poultry eggs and on embryonic development of the chick have recently been reviewed (Surai 2012a, Surai 2012b). The antioxidant potential of carotenoids was first described by Monaghan and Schmitt (1932). The discovery by Foote and Denny (1968) that carotenoids, such as β -carotene, lycopene, zeaxanthin, lutein and Canthaxanthin, could quench singlet oxygen (1O_2) was an important advance in understanding the effectiveness of carotenoid pigments in preventing damage in photobiological systems (Foote et al. 1970). Sixteen years later the mechanism of quenching lipid radicals in biological membranes by carotenoids was proposed (Burton and Ingold 1984).

Antioxidant properties of carotenoids include scavenging singlet oxygen and peroxy radicals (Krinsky 1989; Terao et al. 1992), sulfur radicals (Chopra et al. 1993) as well as thiyl, sulfonyl and NO₂ radicals (Everett *et al.*, 1996) and provide protection of lipids from superoxide and hydroxyl radical attack (Krinsky and Deneke 1982). The mechanism of protection of biological systems against damage due to ¹O₂ by carotenoids includes both a physical component as well as a chemical reaction between a carotenoid and the excited oxygen molecule (Krinsky 1989a).

The deactivation of ¹O₂ by carotenoids results predominantly from physical quenching, a process involving transfer of excited energy from ¹O₂ to the carotenoids and resulting in the formation of ground state oxygen ³O₂ and triplet excited carotenoid ³Car* (Stahl and Sies 1993). Instead of further chemical reactions, the carotenoid returns to ground state dissipating its energy by interaction with the surrounding solvent. Therefore, carotenoids can actively quench singlet oxygen (¹O₂) and prevent lipid peroxidation caused by singlet oxygen and they can intercept the propagation step of lipid peroxidation *in vitro* (Rice-Evans et al. 1997).

The physical quenching reaction involves the transfer of the energy from high-energy state molecules, such as ¹O₂, to the carotenoid (CAR) with a formation of the carotenoid triplet (Bast et al. 1998):



In the subsequent reaction the carotenoid dissipates its energy as heat and returns to the basic state:



Since the carotenoids remain intact during physical quenching of ¹O₂ or excited sensitizers, they can be reused several fold in such quenching cycles. Among the various carotenoids, xanthophylls (such as Canthaxanthin) as well as carotenes proved to be efficient quenchers of singlet oxygen interacting with reaction rates that approach diffusion control (Foote and Denny 1968; Conn et al. 1991).

The efficacy of carotenoids for physical quenching is related to the number of conjugated double bonds present in the molecule which determines their lowest triplet energy level. Maximum protection is afforded by carotenoids which have 9 or more double bonds (Krinsky 1989a) and Canthaxanthin is one of those effective carotenoids.

When the reaction between ¹O₂ and carotenoids takes place through chemical scavenging, oxidative products of carotenoids are formed, but this reaction is considered as a very minor side reaction (Edge et al. 1997) and the antioxidant impact of this chemical reaction is negligible.

Carotenoids are able to react with a range of free radicals (R*) and in this case three possible mechanisms are considered:

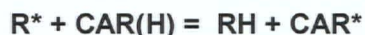
- electron transfer with a formation of carotenoid radical cation:



- addition reaction with the formation of a carotenoid-adduct radical which can react with another radical to form a non-radical product:



- hydrogen abstraction with a formation of the neutral carotenoid radical:



In accordance with widely accepted views, the addition reaction and/or hydrogen abstraction are the more probable reactions between free radicals and carotenoids (Kennedy and Liebler 1991; Kennedy and Liebler, 1992).

The relative reduction potentials of a variety of carotenoids have been established by monitoring the reaction of carotenoid radical anion (CAR1(*-)) with another carotenoid (CAR2) in hexane and benzene (Edge *et al.* 2007). This work illustrated that the presence of a carbonyl group causes the reducing ability to decrease. Indeed, the radical cations are strong oxidizing agents and the authors showed that the radical anions are very strong reducing agents.

Canthaxanthin can stop peroxy free radical chain propagation by trapping the radical in its conjugated polyene system (Ruiz *et al.* 1999). Akhtar *et al.* (1998) fed tocopherol, Canthaxanthin or oleoresin paprika alone or in combination to rainbow trout. When vitamin E and Canthaxanthin were both included in the diet, a strong antioxidant effect was found and this was attributed to the concept that these two compounds use different mechanisms to control lipid oxidation. Alpha-tocopherol is a hydrogen donor and can donate the hydrogen from its C-6 carbon while Canthaxanthin captures the peroxy free radical in its conjugated polyene system (Akhtar *et al.* 1998).

In a liposome system Canthaxanthin delayed the formation of thiobarbituric acid-reactive substances (TBARS) in a concentration-dependent manner (Akhtar *et al.* 1998a). Rainbow trout (*Oncorhynchus mykiss*) were fed a diet containing either fish oil or rapeseed oil, with or without 200 mg/kg Canthaxanthin (Baron *et al.* 2009). Results showed that Canthaxanthin effectively protected both protein and lipid against oxidation during frozen storage.

The effects of β -carotene and Canthaxanthin on lipid peroxidation and antioxidative enzyme activities in rats fed a high-cholesterol, high-fat diet were investigated (Shih *et al.* 2008). Wistar rats were divided into six groups. Negative control group (group NC) received a high-fat (150 g/kg) diet; cholesterol control group (group CC) received a high-cholesterol (10 g/kg), high-fat diet. The other four groups were fed a high-cholesterol, high-fat diet supplemented with crystal beta-carotene (group BC), beta-carotene beadlet (group BB), Canthaxanthin beadlet (group CXN) or alpha-tocopherol (group AT). It was shown that rats fed a CXN-enriched diet had significantly lower plasma TBARS concentration in comparison to the control group. In erythrocytes, glutathione peroxidase activities were significantly greater in the CXN group than

in the control group. Moreover, compared with group CC, catalase activities were significantly greater in the CXN group. In livers, SOD and glutathione reductase activities were significantly greater in the CXN group, than in group CC. Compared with group CC, hepatic retinol and alpha-tocopherol concentrations were significantly greater in the CXN group. These findings suggest that Canthaxanthin altered the pro-oxidation and antioxidation balance and suppressed cholesterol-induced oxidative stress *via* modulation of both the antioxidant system and cholesterol metabolism.

Similarly, SOD activity was increased in plasma of newly hatched chicks obtained from CXN-enriched eggs (Zhang et al. 2011). In the liver of chickens fed a CXN-enriched diet vitamin E concentrations significantly increased (Mayne and Parker 1989)

Taken together, the results show that carotenoids express their antioxidant properties *in vivo*. The efficiency of the antioxidant defense provided by carotenoids depends on many factors including stress conditions, method of oxidative stress detection, concentrations of carotenoid used, model system employed, oxygen tension and interaction with other antioxidants (Rock 1997; Rice-Evans et al. 1997; Edge et al. 1997). It has been suggested that depending on the redox potential of the carotenoid molecules and oxygen tension, carotenoid concentration and interactions with other antioxidants these pigments could show antioxidant or pro-oxidant properties (Palozza 1998).

Under physiological conditions, however, all those factors are usually favorable for the antioxidant activity of carotenoids. Therefore, carotenoids, such as Canthaxanthin, are efficient quenchers of singlet oxygen and are also effective scavengers of free radicals. Indeed, in biological systems carotenoids could be considered as an integral part of the antioxidant systems operating inside the membranes. Furthermore, results from a published study demonstrate a very strong modifying effect of Canthaxanthin with respect to the dynamic and structural properties of lipid membranes (Sujak et al. 2005).

When Se and vitamin E deficient chicken diet was supplemented with CXN (0.5 g CXN/kg diet), liver homogenates exhibited significantly ($P = 0.02$) decreased formation of thiobarbituric acid-reactive substances (TBARS) over time in ferrous ion-induced peroxidation (Mayne and Parker 1989). Supplementation of rainbow trout with Canthaxanthin prior to processing into patties showed that the amount of Canthaxanthin deposited was critical if lipid stability was to be achieved (Clark et al. 1999). Likewise, a decreased susceptibility to lipid peroxidation and increased proportion of long chain omega-3 PUFAs, was observed in meat of broilers fed diets supplemented with or without Canthaxanthin at 10mg^{-1} Feed (Table 3-2) (Ajuyah et al. 1993).

Table 3-2 CXN effect on omega-3 PUFAs content of poultry meat

Ajuyah et al. 1993	Omega-3 PUFAs in Phosphatidyl Ethanolamine fraction from cooked dark broiler meat, %				
	CXN Dose in feed (mg^{-1})	20:5W3	22:5W3	22:6W3	Total W3
	0	2.5	4.6	4.1	13.7
	10	5.5	6.8	10.7	25.3
	P	<0.05	<0.05	<0.05	<0.05

Conclusions: CXN in the full fat seed supplemented chicken diet significantly increased proportion of easily oxidized long chain omega-3 PUFAs in chicken meat probably due to decreased oxidation, since MDA accumulation in the same samples was also reduced

Broiler breeders (ROSS 1) were fed a carotenoid combination, where the effect of tissue carotenoid content on the susceptibility of extracts of yolk and embryonic tissues to lipid peroxidation was evaluated (Surai and Speake 1998). In this study, the extent of lipid peroxidation during the incubation of tissue extracts, as estimated by the formation of TBARS, was significantly affected by the level of tissue-derived carotenoids in the mixtures. Thus, the rates of both spontaneous and Fe-stimulated peroxidation in extracts of yolk and of neonatal yolk sac membrane (YOLK SAC MEMBRANE) and liver were significantly reduced in samples from the high-carotenoid group compared with the control group (Table 3-3).

Table 3-3 Yolk and Embryo tissues susceptibility to lipid peroxidation in vitro

Surai and Speake, 1998	CXN Dose in feed* (mg/kg)	Antioxidant Effect					
		TBARS in tissues (ng/hour/mg lipid)					
		Initial yolk	YOLK SAC MEMBRANE	Liver of newly hatched chick	Initial yolk + Fe2+	YOLK SAC MEMBRANE + Fe2+	Liver of newly hatched chick + Fe2+
0	3.5	3.5	10.6	32.7	8.4	25.1	
3	2.5	2.7	9.3	25.5	6.6	20.9	
P	<0.001	<0.001	<0.01	<0.01	<0.01	<0.01	

*Canthaxanthin added in Feed as part of a carotenoid cocktail also including lutein, citranaxanthin and apoester.

Conclusions: CXN as a part of a carotenoid mixture is effectively transferred to the egg yolk and significantly contributes to antioxidant protection of the egg yolk and tissues of newly hatched chicks

Therefore, the efficiency of antioxidant protection afforded by carotenoids depends on their accumulation in the tissues, but an interaction of carotenoids with other antioxidants (e.g. alpha-tocopherol) could be considered as an additional factor regulating the efficiency of antioxidant defense in the tissue (Surai et al 2003). At the same time, a study by Galobart et al. (2001), has shown that CXN is effectively transferred to the egg yolk and numerically decreased lipid peroxidation and significantly increased proportion of long chain PUFA (presumably due to antioxidant effect), however, because of high result variability the difference in MDA levels did not reach significance (Table 3-4).

Table 3-4 CXN, MDA and PUFA (22:4n-6) in egg yolk from commercial layers supplemented with CXN.

Galobart et al., 2001	CXN Dose in feed (mg/kg)	Antioxidant Effect		
		CXN (µg/g), Malondialdehyde (mg/kg) and PUFAs (%) in egg yolk		
		CXN in egg yolk, µg/g	MDA in egg yolk, µg/g	PUFA (22:4n-6) in egg, %
0	Not determined	247.5	0.19	
5	8.9	121.9	0.28	
P	n/a	0.225 NS	0.0001	

Conclusions: CXN supplementation did not prevent lipid oxidation in PUFA-enriched eggs.

Canthaxanthin is effectively deposited and accumulates in egg yolk, with yolk color being the referent commercial indicator of such efficacy. In fact, egg yolk color directly reflects the concentration of pigments in the diets of laying hens (SCAN 2002), where CXN concentration in yolk can be estimated to 2.5-2.7 folds higher than the dietary CXN level (EFSA 2007). A study (Sidibé 2001) provided additional data on Canthaxanthin deposition in the egg yolk after feeding Canthaxanthin to laying hens at levels of 2-6 mg/kg feed, where the color intensity of the egg yolk reached a plateau after 10 days and the Canthaxanthin levels in the egg yolk measured between day 19 and 25 reflected a stable relationship between Canthaxanthin in feed and egg yolk (reported in SCAN 2002). As such, Canthaxanthin accumulation in egg yolk has been shown to be proportional to the dietary content, and associated with a decreased tissue susceptibility to lipid peroxidation (Surai et al. 2003).

3.2.3 Fate of dietary Canthaxanthin in poultry layers and offspring

3.2.3.1 Canthaxanthin deposition in poultry

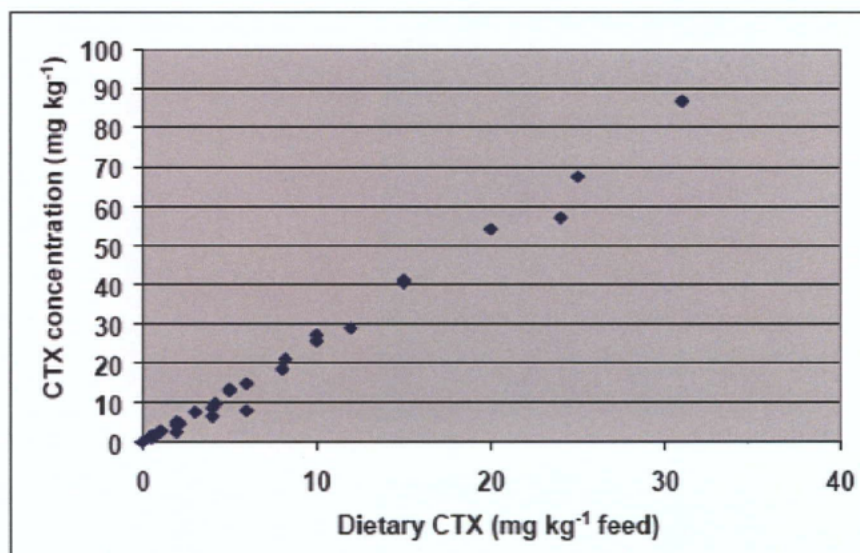
Oral administration of Canthaxanthin to poultry is followed by Canthaxanthin deposition in tissues. Target tissues include liver, as well as skin/fat and specially egg yolk. CXN (measured as the all-trans isomer) is considered as the marker residue (EFSA 2007). In poultry, Canthaxanthin is absorbed in the small intestine and carried via blood to the liver (SCAN 2002). After absorption, Canthaxanthin is packed in the portomicrons, then released from the enterocyte through the portal vein and transported to the liver. A portion of such portomicrons sustain hepatic metabolism, thereby forming VLDLg (Very Low Density Lipoproteins). Canthaxanthin is liposoluble and deposited in the yolk jointly with the lipids by VLDLg. As a result, Canthaxanthin remains in the VLDLg nucleus until the formation of the oocyte, coloring the yolk (Rocha et al. 2011).

CXN is metabolized in the liver to 4'-hydroxyechinenone, 4-oxoretinol and isozeaxanthin. Following repeated administration (four weeks) of 8 mg ³H-labelled CXN kg⁻¹ feed to hens, about 70% of the radioactivity was found in the ovaries, 5 to 6% in the liver, 3 to 8 % in the muscle, 1% in the fat and 1% in the skin. Unchanged CXN (essentially the all-trans isomer) represented about 40% and 80% of the total residues in the liver and skin, respectively. In the liver, 4-oxoretinol was the major metabolite (30%) and 4'-hydroxyechinenone and isozeaxanthin very minor metabolites. The major metabolite (30%) in the integuments (toe web) was 4'-hydroxyechinenone in an esterified form. After feeding chickens with 70 mg CXN kg⁻¹ feed, Canthaxanthin represented 96 and 81% of the total carotenoids measured in liver and skin, respectively, 4'-hydroxyechinenone amounted to 3 and 17% and isozeaxanthin was present at a very low level (EFSA 2007).

The deposition of CXN in egg yolks and broiler tissues is directly proportional to dietary levels (EFSA 2007). In laying hens, carotenoid deposition in egg yolk varies widely with Canthaxanthin being deposited at 30 to 45% efficiency (Hencken 1992; Schiedt 1998). According to EFSA

(EFSA 2007), up to 40 % of the Canthaxanthin intake is deposited in the egg yolk and up to 10 % in the tissues. The slope of the regression equation for dietary CXN vs. CXN in egg yolk is about 2.5-2.7 ((b) (4) 1987). This means that CXN concentration in yolk can be estimated to 2.5-2.7 folds higher than the dietary CXN concentration. A stable plateau is reached at the latest after 19 days of CXN administration (Grashorn and Steinberg 2002). A recent evaluation from EFSA of a number of studies (EFSA 2007), showed the linearity of the dose-response of dietary CXN to CXN in egg yolk (Figure 3-1).

Figure 3-3-1 Linearity of the dose response of dietary CXN to CXN in egg yolk*.

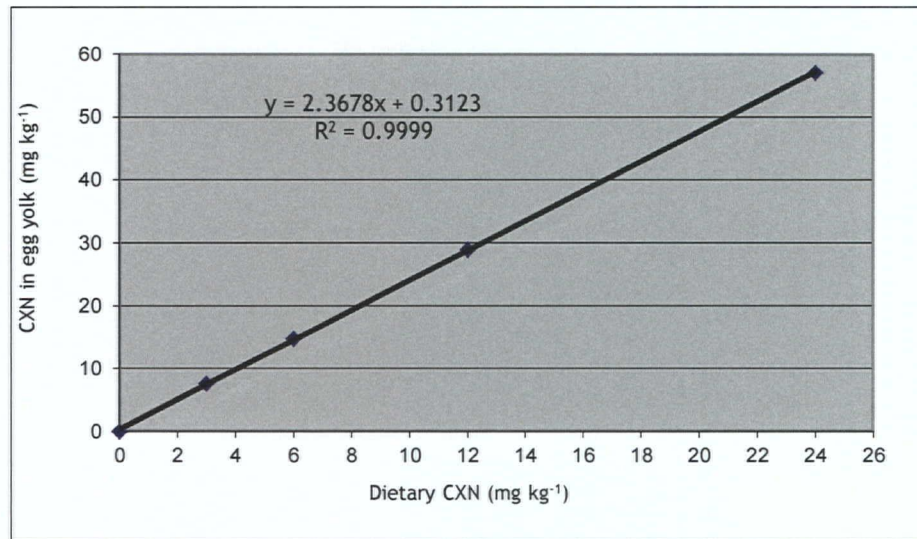


*Source: EFSA 2007, ($r^2 = 0.989$).

Canthaxanthin concentration in eggs from commercial layers fed at 8 mg CXN kg⁻¹ feed was measured to be 20.8 ± 2.3 mg kg⁻¹ in egg yolk (Grashorn and Steinberg 2002). After administration of the same CXN dose for 28 days, another study ((b) (4) 1987) reported values of CXN in egg yolk in the same range (20.5 ± 2.7 mg CXN kg⁻¹).

CXN deposition in egg yolk from "Commercial layers" as compared to deposition in "Breeding Layers" seems to follow a similar dose-response effect. In fact, broiler breeders and laying hens are fundamentally biologically similar (EFSA 2010a). When calculated via linear regression (Figure 3-3-2) based on analyzed contents from Surai et al. (2003), CXN concentration in egg yolks from "Breeding Layers" fed 8 mg CXN kg⁻¹ for five weeks, is of similar magnitude (19.3 mg kg⁻¹) as found in egg yolks from "Commercial Layers". At the recommended dose in feed of 6 mg / kg feed, the content of CXN in egg yolks from "Breeding Layers" has been found in the range of 14.75 mg kg⁻¹ (Surai et al. 2003) to 17.5 mg kg⁻¹ (Weber et al. 2013).

Figure 3-3-2 Linear regression: CXN content in egg yolks from “Breeding Layers” fed CXN



* Source: Calculated based on analyzed data reported by Surai et al 2003.

3.2.3.2 Maternal transfer of Canthaxanthin to poultry offspring

A good nutritional status of parent birds is crucial for the transfer to the egg of an adequate, balanced supply of nutrients required for normal development of the embryo (Wilson 1997). In the wild, Canthaxanthin is incorporated into gull egg yolk (Royle et al. 2001; Surai et al. 2001; Surai 2002). CXN was also found to be one of the most common carotenoids in liver, plasma and gut content of wild mallard (*Anas platyrhynchos*) ducklings (Butler and McGraw 2010).

Canthaxanthin concentration in tissues of adult wild gulls was reported to be 1.53; 0.59; 0.58; 0.38; 0.23; 0.16 and 0.11 µg/g in the liver, fat, kidney, breast muscle, heart, lung and pancreas respectively (Surai et al. 2000), while in newly hatched gulls CXN concentrations in the liver, heart and leg muscle were 21.2; 5.7 and 4.7 µg/g respectively (Surai et al. 2001). Dietary supplementation of Canthaxanthin in gulls increased the deposition of this carotenoid in their eggs (Blount et al. 2002a). In fact, Canthaxanthin was found in egg yolk of wild gulls at concentrations of about 5 µg/g and it was increased almost 5 times after Canthaxanthin supplementation, also linked to better antioxidant protection of egg yolk (Table 3-5) (Blount et al. 2002).

Table 3-5 CXN content and antioxidant status of Gull egg yolks supplemented with CXN in feed

Blount et al 2002	CXN Dose in feed (mg/nest)	Antioxidant Effect	
		CXN in egg yolk, µg/g	Malondialdehyde in gull egg yolk, µg/g
	0	4.5	31.1
	5	25.2	14.2
	P	<0.001	<0.05
Conclusions: CXN as a part of a carotenoid mixture is effectively transferred to the gull egg yolk and significantly contributes to antioxidant protection of the egg yolk			

Previously, it had been demonstrated that CXN was detectable in the plasma and liver of birds hatched from CXN-enriched eggs, while there was no CXN in plasma or liver of the control birds (Haq et al. 1995). In line with these findings, a study (Surai et al. 2003) reported that Canthaxanthin is effectively transferred from the egg yolk to the developing embryo and as a result, Canthaxanthin concentration in the liver, yolk sac membrane and plasma at d 16 of embryonic development and in 1-d-old and 7-d-old chicks, was significantly increased in proportion to its concentration in the egg yolk of breeders. Similar observations were reported earlier with gull embryos (Surai et al. 2001).

It was also reported that maternal diet, rich in carotenoids, influenced carotenoid concentration in up to 28-day-old chicks' tissues (Koutsos et al. 2003). Based on the important role played by carotenoids as antioxidant immediately after the hatching, the authors concluded that the consumption of carotenoids by the broiler breeders, in addition to affecting their incorporation by the progeny tissues, can be required to increase their viability (Rocha et al. 2011). According to Karadas et al. (2005), the enrichment of maternal diets with carotenoids is the main factor impacting the concentration of carotenoids on chicks' liver in their first week of life, with dietary sources impacting such levels thereafter (Rocha et al. 2011).

These data show the importance of the maternal diet on the composition of the chick during early postnatal development. It is also clear that after hatching, the Canthaxanthin that accumulates in the chicken's body during embryonic development is depleted. Thus, under these conditions, maternal effects predominate for at least the first week after hatching, whereas from 2 weeks onwards, the progeny's diet becomes the main determinant of its carotenoid status. Since the antioxidant and immunostimulatory roles of carotenoids are likely to be especially important during the immediate post-hatch period, maternal dietary intake of carotenoids may have important ramifications for the viability of the offspring (Karadas et al. 2005).

3.2.4 Canthaxanthin use in “Breeder Layers”

3.2.4.1 Needs of the poultry breeding industry

Poultry breeders play a key role within the overall poultry industry, being commercially reared for the production of fertile eggs and the ultimate objective of producing day-old chicks. Depending on the type of breeder strain reared, offspring are subsequently reared as broilers for meat production, as layers for production of commercial table eggs, or for the production of replacement breeders.

Because of its relevance for poultry production, there are three important goals that should be met in the poultry breeding industry for optimum performance. These are: 1) Good and uniform laying rate; 2) Maximum number of chicks per hen (Best fertility - Best hatchability) and 3) Uniform, good chick quality and optimum chick/broiler development. To achieve these goals, the poultry breeding industry faces the following challenges:

- To provide the highly valuable breeders with the best nutrition to guarantee optimum performance (laying rate, hatched chicks) and minimum stress.
- To favor chick embryo development, which is affected by an accumulation of polyunsaturated fatty acids in tissue lipids (Speake et al. 1998a) and that have a high risk of oxidation.
- To optimize quality of offspring and young chick development, which directly impact their best possible performance.

3.2.4.2 Susceptibility of egg yolk and embryo tissues to lipid oxidation

Chicken egg consists of egg yolk, egg white and egg shell. The yolk is the primary source of energy and nutrients for the embryo during its development (Speake et al. 1998b). Practically all lipids are located in the egg yolk, where a range of fat soluble compounds and antioxidants are also deposited (Surai 2002).

Yolk lipids are important for the nutrition of the evolving embryo (Cherian and Sim 1997), as they are the source of fatty acids and other components needed for the synthesis of membrane phospholipids in the growing tissues of the embryo (Noble et al. 1986). The energy needs of the embryo throughout the 21-d developmental period of incubation are obtained by the β -oxidation of the fatty acids derived from yolk lipids (Speake et al. 1998b). Overall, embryos secure more than 90% of their energy demand from the oxidation of fatty acids (Latour et al. 2000).

Yolk lipids are susceptible to attack by free radicals and oxidation via a chain reaction, where polyunsaturated fatty acids (PUFAs) are the most susceptible to oxidation, resulting in the formation of toxic compounds such as alkanes, aldehydes, alcohols and hydroperoxides, among other products (Rocha et al. 2010; Hogg and Kalyanaraman 1999). Free radicals are chemical substances with an odd number of electrons, and, as a result, they are highly unstable and energetic (Araújo 2006). To become stable, free radicals transfer the accumulated energy to adjacent molecules, especially PUFAs. During regular metabolism, the production of free

radical occurs, as byproducts of breathing and synthesis of complex structures. Therefore, the term free radical also incorporates oxygen and nitrogen reactive species generated in the metabolism of living systems, which cause oxidative stress whenever they exceed the biological antioxidant capacity (Rocha et al. 2011).

In living systems, this reaction between free radicals and PUFAs, triggers a chain-reaction process known as lipid peroxidation (Rocha et al. 2011), which changes the structure of cell membranes, causing modifications in the structure of amino acids, and resulting in changes in enzymatic activities and attacks on DNA.

In aerobic systems, the equilibrium between oxidation-reducing agents such as free radicals and the anti-oxidant defense system is essential. As free radicals are generated on an endogenous basis in the organism as the consequence of the metabolism, most living species have efficient protection system capable of neutralizing harmful effects from such reactive species. In this process, enzymes such as *glutathione peroxidase* (GSH-Px), superoxide dismutase (SOD) and catalase, are involved (Rocha et al. 2011).

Malondialdehyde (MDA) is generated during the PUFA oxidation due to the beta split of peroxidized PUFA, especially arachidonic acid. This aldehyde, with three carbon atoms ($C_3H_4O_2$) is widely used to assess lipid oxidation in foods and mainly the oxidative stress in biological samples through testing for substances that react with thiobarbituric acid known as TBARS. The TBARS principle is based on the reaction of one MDA molecule with two molecules of thiobarbituric acid (TBA), in an acid medium and at high temperatures, resulting in a red complex, which can be determined by absorption in the visible spectrum (532 nm) or through fluorescence (Rocha et al. 2011)

Research with table eggs has demonstrated that yolk lipids can also sustain oxidation during egg storage, with oxidation being influenced by time and temperature of storage and the degree of unsaturation of yolk fatty acids. Under consideration that storage is a standard procedure before transferring the eggs to the incubators, such oxidation may also occur in fertile eggs. The negative impact caused by a longer storage period of fertile eggs on incubation yield is well documented in the technical literature and explanations are based on physical changes taking place in the egg. In this case, the oxidation of yolk lipids of stored fertile eggs could result in reduced energy available for the embryos' development, in addition to the presence of toxic compounds that could cause the embryos' death (Rocha et al. 2010).

Experiments on commercial eggs demonstrated yolk lipids undergo oxidation during the storage period and such oxidation increases as the storage period increases, both under refrigeration at 4°C and room temperature at 25°C, and the higher temperature effect is noticeable (Franchini et al. 2002; Cherian et al. 1996, 2007).

While yolk lipids are primarily destined to fuel embryo development in fertile eggs, chick embryos also have a very high metabolic rate during development, which produces a relatively large amount of free radicals. The high PUFA concentration of embryonic tissues (Surai et al. 1997) combined with the increased consumption of oxygen in the second half of

the incubation period (Wilson et al. 1992), cause oxidative modifications to be accompanied by changes in the concentrations of antioxidant enzymes in the embryo tissues (Surai 1999b).

The presence of an antioxidant system within the egg is crucial for protecting the embryo against lipid peroxidation (Gaal et al. 1995; Surai and Sparks 2001). The embryo of the chicken develops within a closed system, the egg, which contains all the nutrients required for the 21-day developmental process (Noble and Cocchi 1990).

Therefore, it may be relevant that the antioxidant activity of carotenoids is mainly expressed at the low oxygen tensions that prevail in embryonic tissues (Burton 1989). Chick embryos also may be subjected to stress caused by excessive exposure to heat during the latter part of egg incubation (Tullett 1990). During the hatching period oxidative stress is high due to dramatic increases in the energy metabolism rate and exposure to atmospheric oxygen. This period is also marked by a high degree of unsaturation of tissue lipids (Noble and Cocchi 1990). Oxidative stress arising from the peroxidation of free radicals is likely to be most prominent in rapidly growing embryos because of their high levels of oxidative metabolism (Vleck and Bucher 1998).

3.2.4.3 Antioxidant protection of egg yolk and embryo tissues by Canthaxanthin

The ingestion of substances with antioxidant properties such as vitamin E, C and carotenoids helps the enzymatic defense mechanism to control the damages caused by free radicals in the cells. In the membranes, the main free radical neutralizer is alpha-tocopherol or vitamin E. Tocopherols remove peroxy radicals, donating its hydrogen atom to it, converting it into peroxide (Rocha et al. 2011).

Carotenoids also play an important antioxidant role; because they work as both a free radical remover and physical attenuator, absorbing and dissipating excess energy of highly reactive chemical species. It has been demonstrated that carotenoids can recycle vitamin E, as they are capable of donating electrons to alpha-tocopheroxyl radical ($TO\bullet + CAR + H^+ \rightarrow TOH + CAR\bullet^+$) (Böhm et al. 1997). Different authors have suggested there are different strategies for allocation of dietary antioxidants in nature, in particular carotenoids, in males and females fowl. Male birds allocate carotenoids to their plumage to indicate their health status (Blount et al. 2003) to be more successful in the mating process, while females deposit carotenoids mainly into the egg to help with oxidation defence of the developing embryo (Surai 2002, Surai 2012a,b; Monaghan et al. 2009).

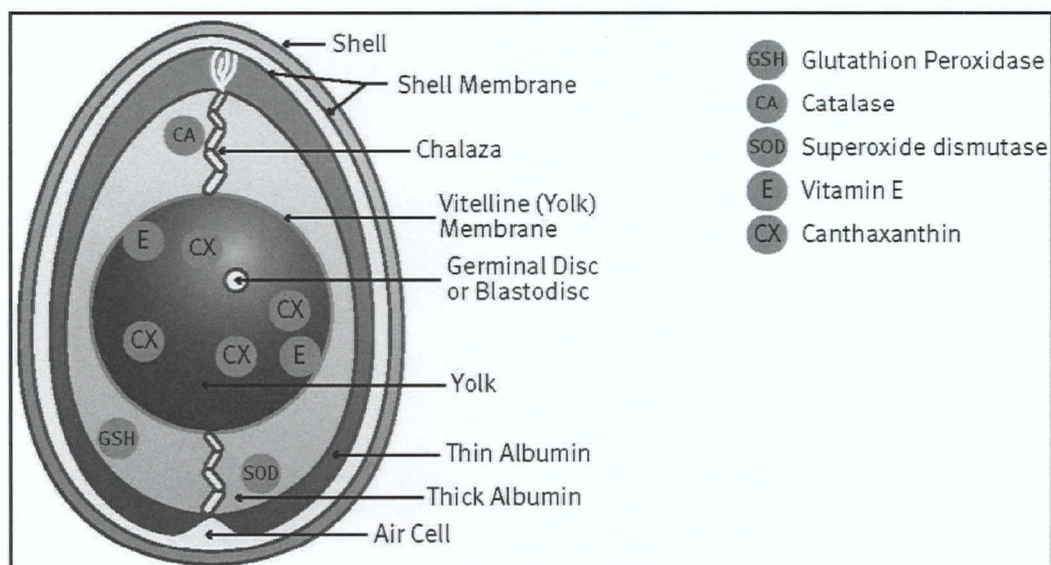
Hens allocate to the eggs diverse antioxidants that protect the embryo from oxidative stress. Furthermore, immunomodulating properties of carotenoids could be of great importance for the progeny (Constantini and Moller 2008). Analysis of yolk samples from clutches of wild birds that were subsequently partially cross-fostered revealed a positive effect of yolk antioxidant capacity on embryonic development and chick growth (Rubolini et al. 2006).

Likewise, pre-laying, wild Lesser Black-Backed Gulls (*Larus fuscus* L.) were given supplementary feed with a mixture of four carotenoids, or a carotenoid-free (control)

supplement. The yolk carotenoid profile and susceptibility to lipid peroxidation were then compared in eggs that they laid. Egg yolk produced by carotenoid-supplemented females was significantly less susceptible to lipid peroxidation in comparison with controls (Blount *et al.* 2002). These data support that antioxidants can be important mediators of maternal effects also in wild bird populations, especially during the critical early post-hatching phase.

Overall, antioxidant protection of the yolk is afforded mainly by vitamin E and carotenoids. Other compounds possessing antioxidant activities (e.g. coenzyme Q, glutathione, etc.) found in the egg are present only in very low concentrations and therefore play a minor role in the egg. It has been shown that increased vitamin E (Surai 1999a) or carotenoid concentrations (Surai 2002) in the egg yolk were associated with increased resistance to lipid peroxidation. Surai (2012b) has recently depicted the antioxidant system in the egg (Figure 3-3).

Figure 3-3 Antioxidant system in the egg



During egg incubation, vitamin E and carotenoids are effectively transferred from the egg yolk to the developing embryo and maximum concentrations of these compounds in the liver of newly hatched chicks are considered to be an adaptive mechanism to protect tissues from the oxidative stress of hatching (Surai 1999; Surai 2002; Surai *et al.* 1996, Surai and Fisinin 2012). During embryonic development other antioxidant compounds are synthesized in various tissues. These include glutathione, ascorbic and uric acids, as well as antioxidant enzymes such as glutathione peroxidase, catalase and superoxide dismutase (Surai 2006).

There is a tissue specificity of synthesis of such antioxidants (Surai 1999a) and in combination with vitamin E and carotenoids they build an integrated antioxidant system in the developing embryo and postnatal chicken (Surai 2006). This system is responsible for the protection of polyunsaturated fatty acids in biological membranes from the damaging effects of free radicals and toxic products of their metabolism. It has also been reported that feeding canthaxanthin to chicks results in an increased resistance to lipid peroxidation as a

result of increased alpha-tocopherol concentration in the liver (Mayne and Parker 1989).

A study (Surai and Sparks 2001) compared eggs, embryos and chicks produced by broiler breeders fed two diets: corn based, rich in carotenoids, specifically lutein and zeaxanthin - 11,8mg of carotenoids/kg and wheat based - 5,6mg of carotenoids/kg. The eggs from corn based diet fed hens resulted in greater concentrations ($P \leq 0,01$) of beta + gamma-tocopherol, total carotenoids, lutein and zeaxanthin, as well as in the tissues of such egg chicks. The authors concluded that the broiler breeders' diet play an important role in the formation of the antioxidant system during embryonic development and the corn-based diet increased the antioxidant potential of the egg yolk and the embryonic tissues in comparison with the wheat-based diet (Rocha et al. 2011)

Canthaxanthin has been shown to have good free radical-trapping properties at low partial pressures of oxygen, such as those found in healthy tissues (Frankel 1989). When added to poultry feed and tested for antioxidant protection of egg yolk and embryo tissues, CAROPHYLL[®] Red has been shown to be very effective (Surai et al. 2003, Robert et al. 2007, Rocha et al. 2013, Rosa et al. 2012, Zhang et al. 2011).

3.3 Studies with canthaxanthin (CAROPHYLL[®] Red) as a nutritive antioxidant that supports the development of chicks.

3.3.1 Introduction

As described in previous sections, Canthaxanthin is an important carotenoid that when fed in the diet is efficiently deposited in egg yolk and is further distributed in chick embryonic tissues (Surai and Speake 1998). Canthaxanthin is also one of the most powerful lipid-soluble antioxidants in nature, and identified as a potent free radical scavenger (Palozza and Krinsky 1992; Zhao et al. 1998; Rengel et al. 2000, Surai 2012a, Surai 2012b).

In addition to its more than 40 years of safe use as a Color Additive, researchers have increasingly focused on the antioxidant characteristics of Canthaxanthin in poultry, and their studies have shown that the presence of Canthaxanthin can effectively aid in reducing oxidation reactions in several tissues and in chick embryos (Surai et al. 2001a).

In the egg, Canthaxanthin is transferred from the yolk to the developing embryo and distributed to many organs and tissues (Llauradó et al. 1997; Surai et al. 2003; Karadas et al. 2005; Surai and Fisinin 2012; Surai 2012a, Surai 2012b) in which it might help protect the developing bird against oxidative damage, particularly during the sensitive periods of hatching and early post-hatch life (Robert et al. 2007).

Studies on the antioxidant activity of Canthaxanthin in poultry have been carried out with CAROPHYLL[®] Red as the Canthaxanthin source (Table 3-5) .

Table 3-5 Studies with poultry breeders on antioxidant activity of Canthaxanthin (CXN) after maternal feeding of CAROPHYLL® Red.

Study Canthaxanthin form used	Reference	Study Locati on	CXN Dose in feed (mg/kg)	Breeder type Birds age Study length	Antioxidant measure
<ul style="list-style-type: none"> (Surai et al. 2003) Effect of Canthaxanthin content of the maternal diet on the antioxidant system of the developing chick. CAROPHYLL® Red 	British Poultry Science 44 (4): 612–619	Scotla nd	0, 3, 6, 12, 24	<ul style="list-style-type: none"> Gallus gallus 21-25 wks old 4 wks 	<ul style="list-style-type: none"> MDA in Liver of 16day old embryos, 1day old chicks (hatchlings) and 7day old chicks
<ul style="list-style-type: none"> (Robert et al. 2007) Effects of Canthaxanthin supplementation in the ROSS breeder diet on oxidative stress of chicks. CAROPHYLL® Red 	Symposium proceeding	France (field trial)	0, 6	<ul style="list-style-type: none"> Gallus gallus ROSS PM3 Yellow 27-38 wks old 11 wks 	<ul style="list-style-type: none"> TBARS in Serum of 1-d-old chicks TROLOX in Serum of 1-d-old chicks
<ul style="list-style-type: none"> (Zhang et al. 2011) Influence of Canthaxanthin on broiler breeder reproduction, chick quality, and performance. CAROPHYLL® Red 15%. 	Poultry Science 90: 1516–1522	China	0, 6	<ul style="list-style-type: none"> Gallus gallus Chinese Three-Yellow 23-47 wks old 24 wks 	<ul style="list-style-type: none"> MDA and TAC of egg yolk MDA in Serum of 1-d-old and 7-d-old chicks SOD in Serum of 1-d-old and 7-d-old chicks TAC in Serum of 1-d-old and 7-d-old chicks
<ul style="list-style-type: none"> (Rosa et al. 2012) Effects of Canthaxanthin on the Productive and Reproductive Performance of Broiler Breeders. CAROPHYLL® Red 10% 	Poultry Science 91: 660–666	Brazil	0, 6	<ul style="list-style-type: none"> Gallus gallus COBB 500 45–66 wks old 21 wks 	<ul style="list-style-type: none"> MDA in egg yolk after 0, 4, 8 and 12 days of storage. MDA in egg yolk after 0, 7, 14 and 18 days of incubation MDA in Serum of 1-d-old chicks

MDA: Malondialdehyde
 TROLOX: Total Antioxidant Status (Randox kit - mMol Eq TROLOX/l)
 TAC: Total Antioxidant Content
 TBARS: thiobarbituric acid-reactive substances

Most studies have been conducted with commercial chicken (*Gallus gallus domesticus*) although information is also available for other bird species. Regardless of the species, these studies demonstrate that CAROPHYLL® Red has an impact on the antioxidant status of the developing chick via supplementation of the food of breeding hens. Its addition to poultry breeders diets is directly correlated with the level of Canthaxanthin in egg yolk and subsequent transfer to the tissues of the developing embryo (liver, vitelline membrane and plasma), which in turn supports the nutritional status of the offspring.

In these studies, the support of the nutritional status of the offspring by canthaxanthin has been demonstrated via various antioxidant measures, especially reduced TBARS values.

Overall, Canthaxanthin from CAROPHYLL® Red can provide antioxidant protection to nutrients that otherwise would have been subject to oxidative stress and lipid peroxidation. Supporting the antioxidant and nutritional status of the egg yolk-embryo-chick system has been considered to be of commercial interest to poultry producers.

3.3.2 Studies

3.3.2.1 Surai et al. 2003. Effect of Canthaxanthin content of the maternal diet on the antioxidant system of the developing chick.

Objective

Effects of Canthaxanthin supplementation of the maternal diet on the antioxidant system of the developing chick were investigated.

Methods

Three hundred and twenty female broiler breeder birds were housed in one of 4 controlled environment rooms (20 hens and 2 cocks per replicate) with 3 replicates for all treatments, with the exception of the control treatment of which there were 4 replicates. All birds received one of 5 diets: control low xanthophyll diet, or the same diet supplemented with 3, 6, 12 or 24 mg/kg Canthaxanthin in the form of CAROPHYLL® Red. The experiment started when the birds were 18 weeks of age. From 18 to 21 weeks of age all birds received a commercial pre-breeder ration. From 21 to 25 weeks of age all birds received treatment 1, control feed. Thereafter they received feed from one of the 5 treatments. At 30 weeks of age, 60 eggs from each of the 5 groups were incubated. At d 16 of the embryo development, and at d 1 and d 7 post-hatch, tissue samples were collected and analyzed by HPLC-based methods.

General Results

- Canthaxanthin accumulation in the egg yolk was proportional to dietary content.
- Furthermore, at 12 to 24 mg/kg Canthaxanthin was associated with an increase in γ -tocopherol concentration in the egg yolk. Canthaxanthin was transferred from the egg yolk to the developing embryo and, as a result, its concentration in the liver of the embryo at day 16 and in 1-d-old chicks was increased. Even at d 7 post-hatch Canthaxanthin concentrations in the chicken liver was elevated.

Specific results on antioxidant activity

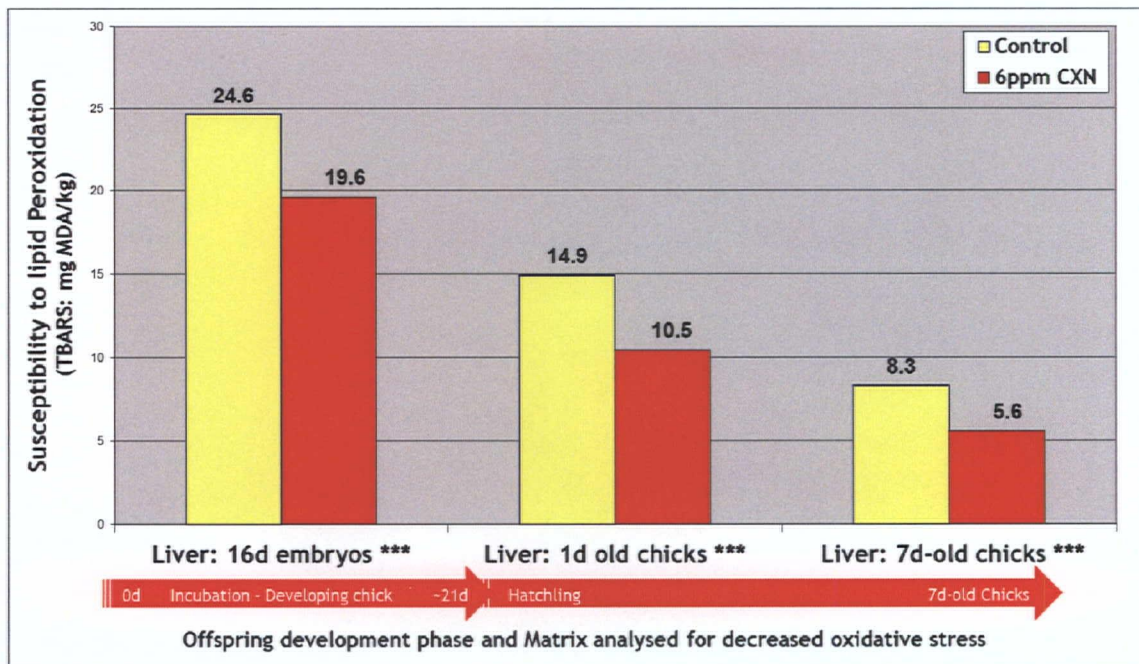
Embryo/Chicks

- Canthaxanthin supplementation of the maternal diet at 12 mg/kg was associated with an increased alpha-tocopherol concentration in the liver of 1-d-old chicks and resulted in decreased tissue susceptibility to lipid peroxidation.
- Canthaxanthin supplementation at 6 to 24 mg/kg was also associated with a delay in alpha-tocopherol depletion from the liver for 7-d post-hatch.

- As a result of the increased Canthaxanthin and vitamin E concentrations in the liver of 7-d-old chicks, tissue susceptibility to lipid peroxidation decreased.
- In practice, this means that Canthaxanthin concentrations in the liver and in the plasma significantly increased with chicks from one to seven days old. Moreover, with a Canthaxanthin dose of 12 mg/kg feed, there was a positive effect on the tocopherol concentration in the liver of the chicks after hatching and at 7 days after hatching, with the susceptibility of the liver to fat peroxidation being reduced (Figure 3-4) (Surai et al 2003).

CXN Dose in feed (mg/kg)	TBARS: MDA and CXN content in liver (mg/kg)					
	16-d-old embryos		1-d-old chicks		7-d-old chicks	
	MDA	CXN***	MDA	CXN***	MDA	CXN***
0	24.63	ND	14.92	ND	9.32	ND
3	19.88	1.96	13.51	25.19	6.69	1.11
6	19.61	4.76	10.47**	55.13	5.59**	3.73
12	18.90*	10.78	9.47***	145.85	5.47**	8.26
24	19.80	19.72	12.14	274.39	6.68	19.05
P	* <0.05 ** <0.01 *** <0.001 , differ from the other values in same column					

Figure 3-4 Surai et al. 2003***. Susceptibility to lipid peroxidation (TBARS) in livers from Offspring of hens fed with or without CAROPHYLL® Red - decreased oxidative stress.



Conclusion

The results support the hypothesis that dietary carotenoids can modulate the antioxidant systems of the developing chicken. Canthaxanthin is effectively transferred to the egg and chicken embryo and possesses a significant antioxidant activity.

3.3.2.2 Robert et al. 2007. Effects of Canthaxanthin supplementation in the ROSS breeder diet on oxidative stress of chicks

Objective

To determine the effect of 6 ppm Canthaxanthin in ROSS breeders feed (through supplementation with 60 ppm CAROPHYLL® Red) on the anti-oxidant status of their progeny (field trial).

Methods

Two experimental batches (2 X 9000 breeders) on the same site of breeding of a hatchery were integrated into the test. The experimental ROSS PM3 Yellow breeders received Canthaxanthin during 12 weeks, from 27 to 38 weeks of age. The feed was heat treated, wheat, corn and soy meal based. Blood samples were taken from 75 chicks from breeders from each batch. Serum Malondialdehyde (MDA) concentrations were evaluated by the TBARS technique (Satoh 1978). The antioxidant status of the chicks' sera was evaluated using the TAS (Total Antioxidant Status) Randox kit. This test evaluates the in vitro capacity of serum to scavenge free radicals.

General Results

Breeder performance:

- The production of eggs was higher within the Canthaxanthin supplemented breeders. Therefore the number of chicks was higher in this group.

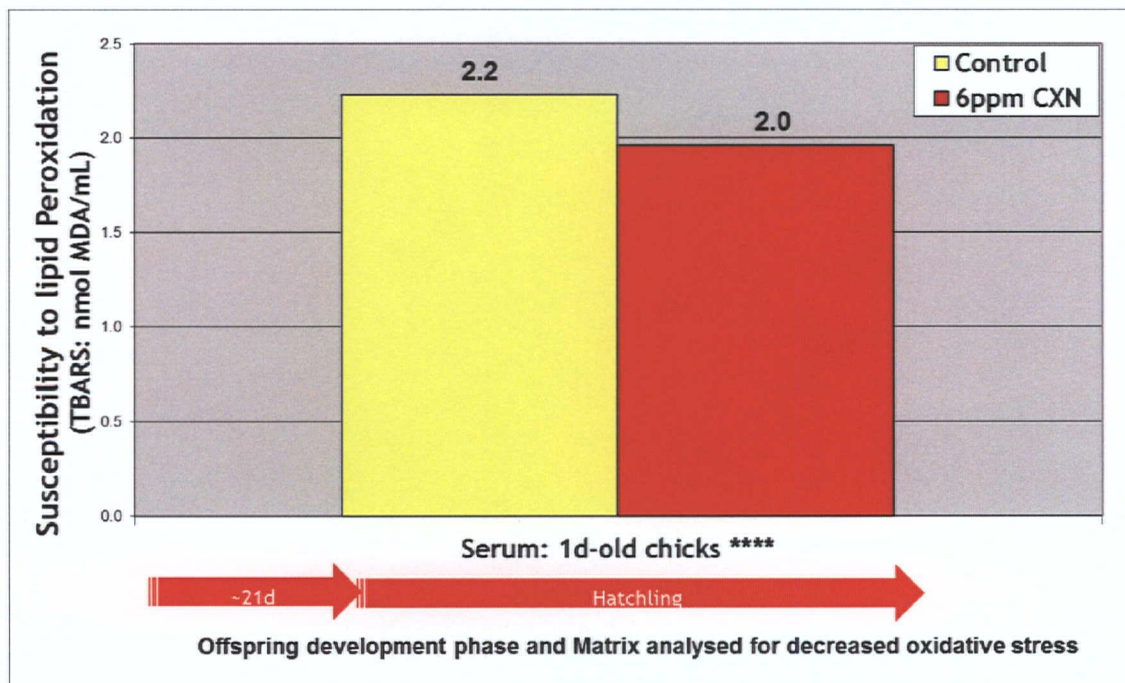
Specific results on antioxidant activity

Embryo/Chicks

- The antioxidant status of sera of 1-day chicks was significantly higher and the TBARS level significantly lower with 6 ppm Canthaxanthin in the breeder feed.
- These results indicated that maternal supplementation with Canthaxanthin (6ppm) enhances antioxidant capability and depresses oxidative stress in chicks.

CXN Dose in feed (mg/kg)	AO – effect in sera of 1-d-old chicks	
	TBARS: MDA (nmol/ml)	Total Antioxidant Status Randox (mMol Eq TROLOX/l)
0	2.23	0.56
6	1.96	0.89
P	<0.01	<0.01

Figure 3-4 Robert et al. 2007****. Susceptibility to lipid peroxidation (TBARS) in serum from 1d-old chicks of hens fed with or without CAROPHYLL® Red - decreased oxidative stress.



Conclusion

Positive effect of Canthaxanthin on the antioxidant status of the day-old chicks. This better antioxidative status decreases the detrimental effect of oxidative stress as shown by the TBARS results. These results indicated that maternal diet supplementation with Canthaxanthin at 6 mg/kg feed enhances antioxidant capability and depresses oxidative stress in chicks.

3.3.2.3 Zhang et al. 2011. Influence of Canthaxanthin on broiler breeder reproduction, chick quality, and performance

Objective

To investigate the effect of Canthaxanthin supplied via a maternal route on the production of both breeder hens and chickens.

Methods

270 Chinese Three-Yellow breeder hens were randomly divided into 2 groups consisting of 135 birds each (5 replicates of 27) for study. The breeder hens were fed either a basal diet or the basal diet supplemented with 6 mg of Canthaxanthin/kg for 24 wk. At the end of the 24-wk breeder experiment, all hatching eggs laid in 5 consecutive days of each group were collected and incubated. For each breeder group, 100 newly hatched chicks (5 replicates of 20) were reared under environmentally controlled conditions for 21 d.

General Results

- Canthaxanthin supplementation resulted in the following outcomes: an enhancement of the serum total antioxidant capacity (TAC) of breeder hens ($P < 0.029$), a significant increase in the yolk colorimetric score of Roche Yolk Color Fan (RYCF; $P < 0.001$), and a significant improvement of the antioxidant status of the egg yolk ($P < 0.05$).
- The chicks that hatched from eggs laid by breeder hens fed the Canthaxanthin supplementation diet demonstrated a higher pigmentation colorimetric RYCF for their shank skin ($P < 0.05$), and the antioxidant capacity of the newly hatched chicks was significantly increased ($P < 0.05$).
- Both of these positive effects on shank skin pigmentation colorimetric score of RYCF and antioxidant capacity were observed for at least 7 d post-hatching, and the chicks that hatched from Canthaxanthin-enriched eggs showed a lower mortality (0 vs. 4%) during the first 21d post-hatching.

Specific results on antioxidant activity

Hens

- Improvement of the serum TAC of breeder hens ($P < 0.029$).

CXN Dosed in feed (mg/kg)			
Antioxidant status of hens (serum)			
	TBARS: MDA(nmol/mL)	SOD (U/mL)	TAC (U/mL)
0	5.15	256.63	13.17
6	4.20	294.86	16.58
P	0.520	0.496	0.029

Egg yolk

- MDA content of the egg yolk decreased from 139.83 nmol/g down to 86.92 nmol/g ($P < 0.023$).

DSM1493-001



- Total antioxidant capacity (TAC) of the egg yolk increased from 1.87 U/g up to 3.16 U/g (P< 0.001).

CXN Dosed in feed (mg/kg)	Antioxidant status of egg yolk			
	TBARS:		SOD (U/g)	TAC (U/g)
	MDA (nmol/g)	mg MDA/kg egg* yolk		
0	139.83	(10.08mg/kg*)	110.92	1.87
6	86.92	(6.26mg/kg*)	131.53	3.16
P	0.023		0.155	<0.001

* original data in "nmol/g" converted to "mg MDA/kg egg yolk".

Embryo/Chicks

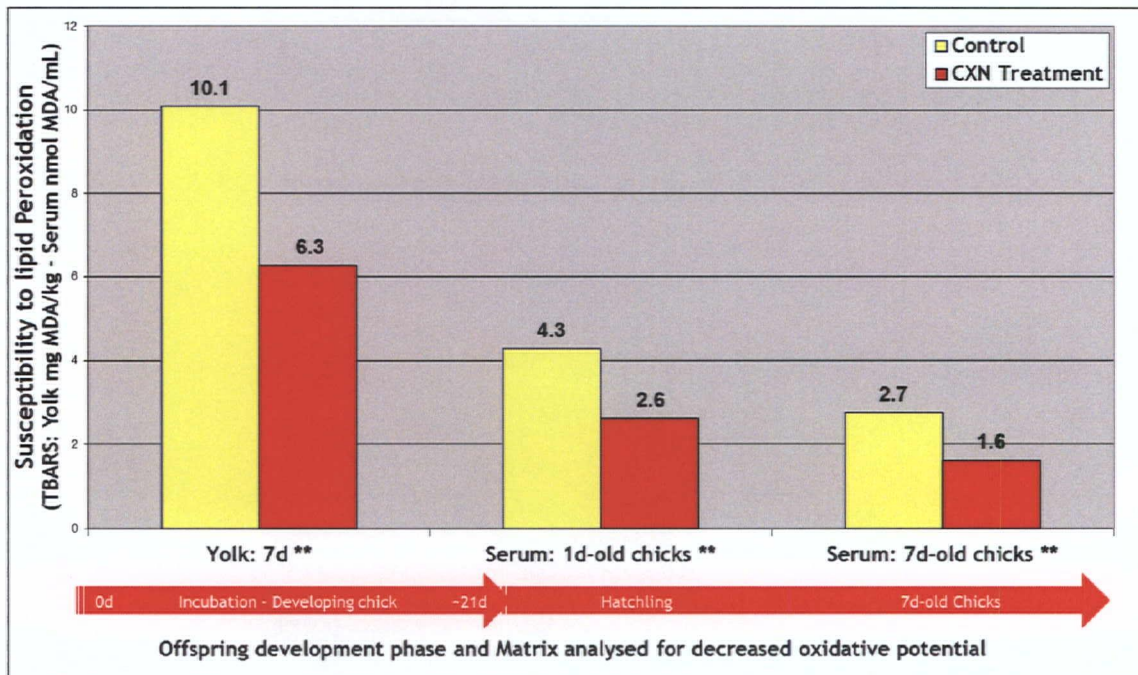
- The antioxidant capacity of the newly hatched chicks was significantly increased (P < 0.05).
- There was a significant decrease in MDA (from 4.28 down to 2.61 nmol/ml, P<0.001) in plasma of day-old chicks.
- Increased SOD activity in the chicken plasma (from 98.4 to 144.7 U/ml, P<=0.031) in agreement with previous observation indicating a stimulatory effect of CXN on SOD (Palozza et al. 2000).
- Positive effect on TAC of newly hatched chicken which increased by 33% (from 13.8 to 18.3 U/ml, P= 0.052).
- MDA in plasma was significantly lower in 7 day-old chicks hatched from the CX-enriched eggs.

CXN Dosed in feed (mg/kg)	TBARS: MDA in Chicks Serum (nmol/mL)	
	1-d-old chicks	7-d-old chicks
0	4.28	2.74
6	2.61	1.61
P	<0.001	<0.001

CXN Dosed in feed (mg/kg)	SOD in Chicks Serum (U/mL)	
	1-d-old chicks	7-d-old chicks
0	98.39	144.43
6	144.65	156.66
P	<0.05	NS

CXN Dosed in feed (mg/kg)	TAC in Chicks Serum (U/mL)	
	1-d-old chicks	7-d-old chicks
0	13.82	14.32
6	18.32	14.73
P Diet	NS	NS

Figure 3-5 Zhang et al. 2011**. Susceptibility to lipid peroxidation (TBARS) in yolk and serum from Offspring of hens fed with or without CAROPHYLL® Red - decreased oxidative stress.



Conclusion

Canthaxanthin supplementation of the maternal diet enhances the protective capacity of tissues against oxidative stress in vivo. CXN is effectively transferred to the egg and chicken embryo and possesses a strong antioxidant activity

3.3.2.4 Rosa et al. 2012. Effects of Canthaxanthin on the Productive and Reproductive Performance of Broiler Breeders

Objective

The effects of supplementing Canthaxanthin on productive and reproductive aspects of broiler breeders were examined in this study.

Methods

Three hundred and sixty female pullets and 36 roosters were placed in an open-sided house with 12 pens, each pen with 7.0 m². At the 42nd weeks of age, the breeder hens and roosters were distributed in two experimental groups with similar body weight and uniformity. From 46th to 66th weeks of age, one group received 6 ppm of Canthaxanthin, supplemented in the diet, and the other group received the diet without addition of Canthaxanthin (control diet). Body weight was measured every 28 days, the laying rate was calculated weekly and mortality was evaluated at the end of the study. Twenty one weekly incubations were performed to evaluate fertility and incubation parameters. To evaluate the antioxidant effect of Canthaxanthin at different storage time and during the incubation process eggs from each treatment were subject to Thiobarbituric Reactive Substances (TBARS) analysis.

General Results

- Body weight, mortality and laying rate were not affected by the inclusion of Canthaxanthin in the breeder's diets.
- No differences were observed on chick's body weight or quality.

Specific results on antioxidant activity

Yolk

- Reduction of TBARS was observed in yolks from stored eggs produced by breeders fed diets plus Canthaxanthin. This reduction was observed in eggs submitted to analysis on the same day they were produced ($P= 0.0214$) and in eggs stored for four ($P<0.0002$), eight ($P<0.0003$) and twelve days ($P<0.0001$).
- A reduction on TBARS levels was observed on eggs produced by hens fed with CXN for days 0 and 7 of incubation ($P<0.0001$). The difference was not significant for days 14 and 18 of incubation.

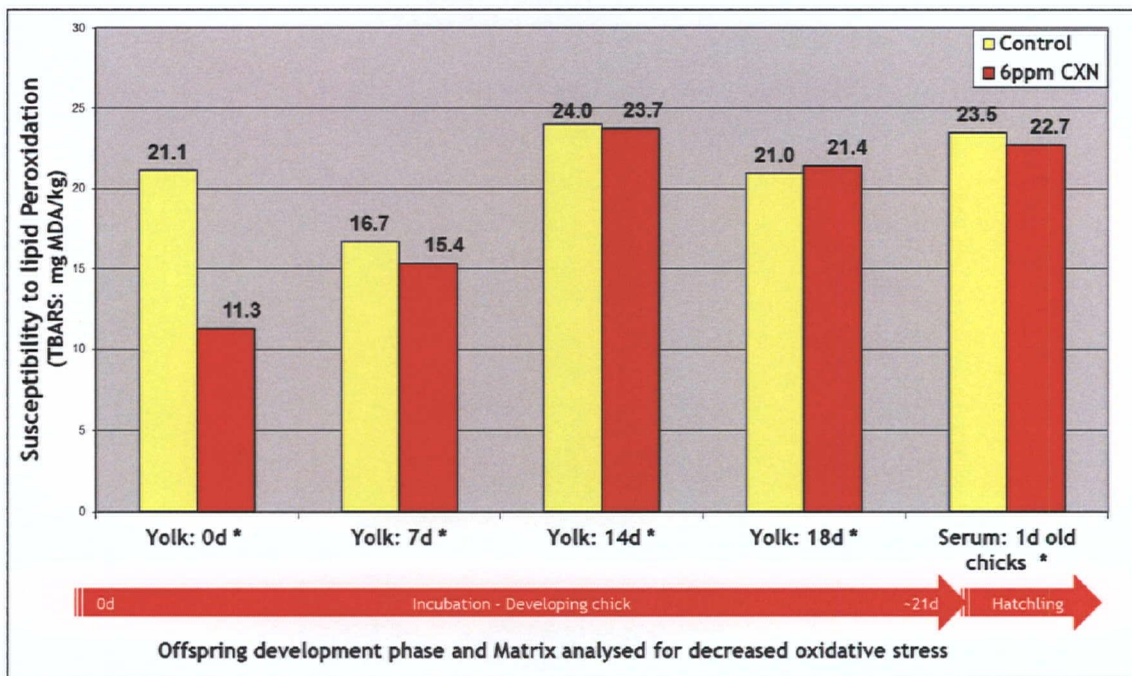
CXN Dose in feed (mg/kg)	TBARS: MDA in egg yolk (mg/kg)			
	0-d storage	4-d storage	8-d storage	12-d storage
0	13.53	20.87	20.73	28.97
6	10.7	15.54	14.61	16.86
P	0.0214	0.0002	0.0003	0.0001
CXN Dose in feed (mg/kg)	TBARS: MDA in egg yolk (mg/kg)			
	0-d incubation	7-d incubation	14-d incubation	18-d incubation
0	21.14	16.69	24.00	20.97
6	11.27	15.36	23.74	21.44
P	0.0001	0.0001	0.585 NS	0.680 NS

Chicks

- No differences in TBARS in blood sera samples collected from both groups after hatch.

CXN Dose in feed (mg/kg)	TBARS: MDA in 1-d-old chick blood sera (mg/kg)
0	23.50
6	22.74
P	0.3768

Figure 3-6 Rosa et al. 2012*. Susceptibility to lipid peroxidation (TBARS) in yolk and serum from Offspring of hens fed with or without CAROPHYLL® Red - decreased oxidative stress.



Conclusion

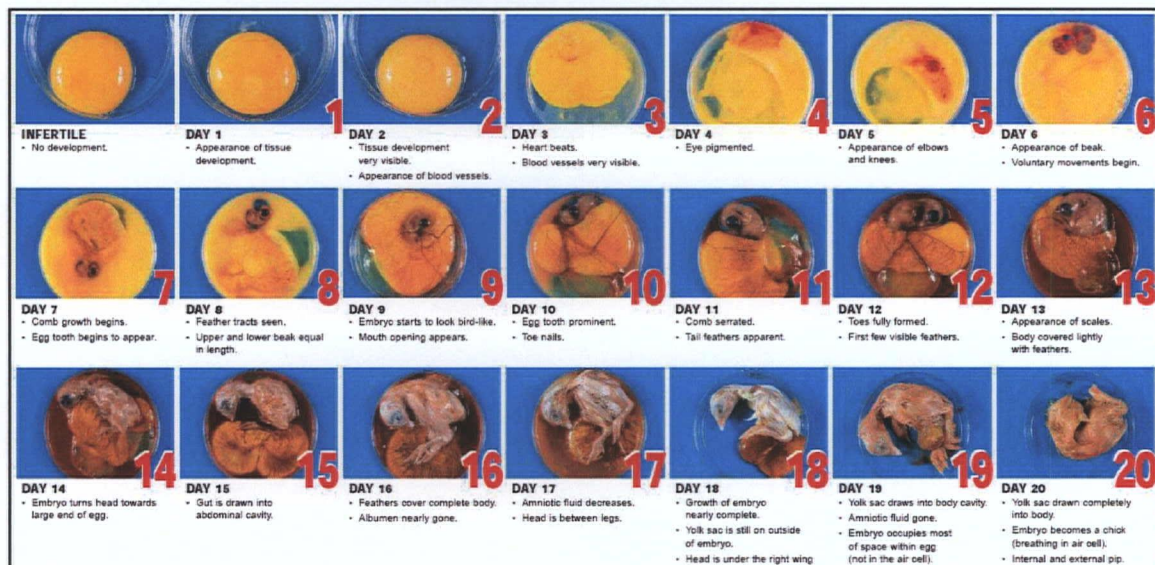
The supplementation of broiler breeder diets with Canthaxanthin improved the hatchability rate, fertility and reduced the presence of TBARS in eggs. CXN is effectively transferred to the egg yolk and possesses a significant antioxidant activity in fresh egg and during storage and incubation.

3.3.3 Discussion and Conclusions

In nature, high carotenoid levels in egg yolk have been reported to be of benefit for the developing embryo of fowl, particularly during the first part of incubation. The chick embryo, which will get most of its nutritional requirements from the yolk sac, has a high metabolic rate to support its development. This high metabolic rate produces a relatively large amount of free radicals, which in turn can attack healthy embryonic cells and tissues and potentially destroy them.

Yolk lipids are the source of fatty acids and other components needed for the synthesis of membrane phospholipids in the growing tissues of the embryo (Noble et al. 1986). Also, during the hatching period, oxidative stress may be high due to dramatic increases in the energy metabolism rate (Surai 2012a, Surai 2012b). This period is also marked by a high degree of unsaturation of tissue lipids (Noble and Cocchi 1990). Figure 3-7 illustrates the dramatic morphological changes seen in chick embryo development during the incubation period.

Figure 3-7 Chick Embryo Development (Source: Cobb 2012)

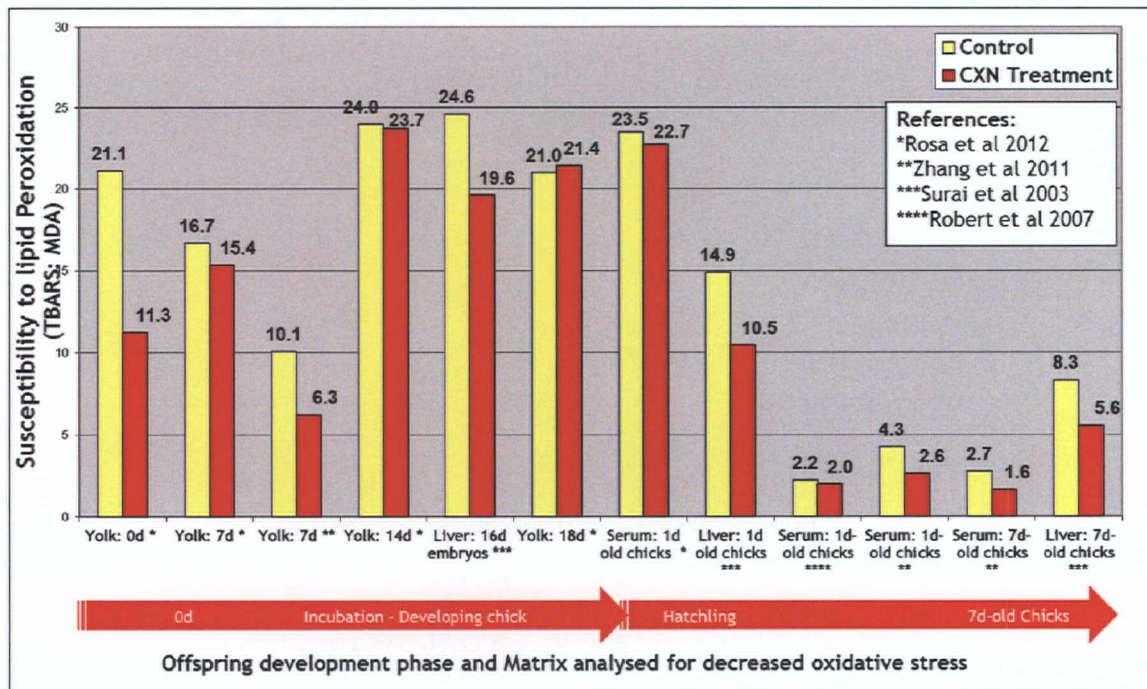


A number of studies have reported that dietary supplementation of hens with antioxidants, such as vitamin E, improved the activity of the embryo antioxidant system, thus decreasing the susceptibility of tissues to lipid peroxidation (Surai 2000; Surai and Sparks, 2001, Surai 2012a, Surai 2012b). Likewise, a protective effect against lipid peroxidation in embryonic tissues for using increased egg concentrations has been demonstrated for carotenoids (Surai and Speake 1998, Surai 2012a, Surai 2012b). Carotenoids also scavenge free radicals and protect lipids from oxidation. This antioxidant effect supports the integrity of the cellular membrane and help sparing other antioxidants, such as vitamins E and C, and selenium (Surai 2012b). In the case of Canthaxanthin, studies with chickens and gulls, have confirmed its antioxidant capacity alone or in combination with other carotenoids. Mayne and Parker (1989) also reported that by feeding Canthaxanthin to chicks there is an increased resistance to lipid peroxidation as a result of increased alpha-tocopherol concentration in the liver.

Among carotenoids, Canthaxanthin from CAROPHYLL® Red has one of the best deposition rates in egg yolk. This has been established for many decades from its use as a colour additive for poultry. This distinctive characteristic, in addition to Canthaxanthin's capacity to render free radicals harmless and decrease the adverse nutritional effects from lipid oxidation, makes of Canthaxanthin an effective dietary antioxidant to protect yolk nutrients required for the development of poultry offspring. In order to demonstrate the utility of Canthaxanthin from CAROPHYLL® Red as a nutritional antioxidant, one field trial (Robert et al. 2007) and three research studies (Surai et al. 2003; Zhang et al. 2011 and Rosa et al. 2012) are discussed in this GRAS Notice.

Results from these trials confirm that, when compared to unsupplemented treatments, Canthaxanthin dosed to breeder hens at 6mg/kg feed, result in decreased oxidative stress (TBARS-TAC-SOD) at different times and in different matrixes (yolk, liver, plasma) during offspring development. In these studies Malondialdehyde (MDA) was used as the common marker to evaluate the degree of oxidative stress through the testing of TBARS. A comparative overview of results on the susceptibility to lipid peroxidation based on TBARS is provided in (Figure 3-8).

Figure 3-8 Overview of four studies: Susceptibility to lipid peroxidation (TBARS) in offspring from hens fed with or without CAROPHYLL® Red and decreased oxidative stress.



Results consistently show that maternal supplementation with CAROPHYLL® Red reduces the susceptibility to lipid peroxidation during embryo and chick development. These findings support the premise that Canthaxanthin as a nutritional antioxidant, has a positive effect on protecting valuable nutrients in yolk sac, liver and serum of embryos and chicks. With TBARS measured at

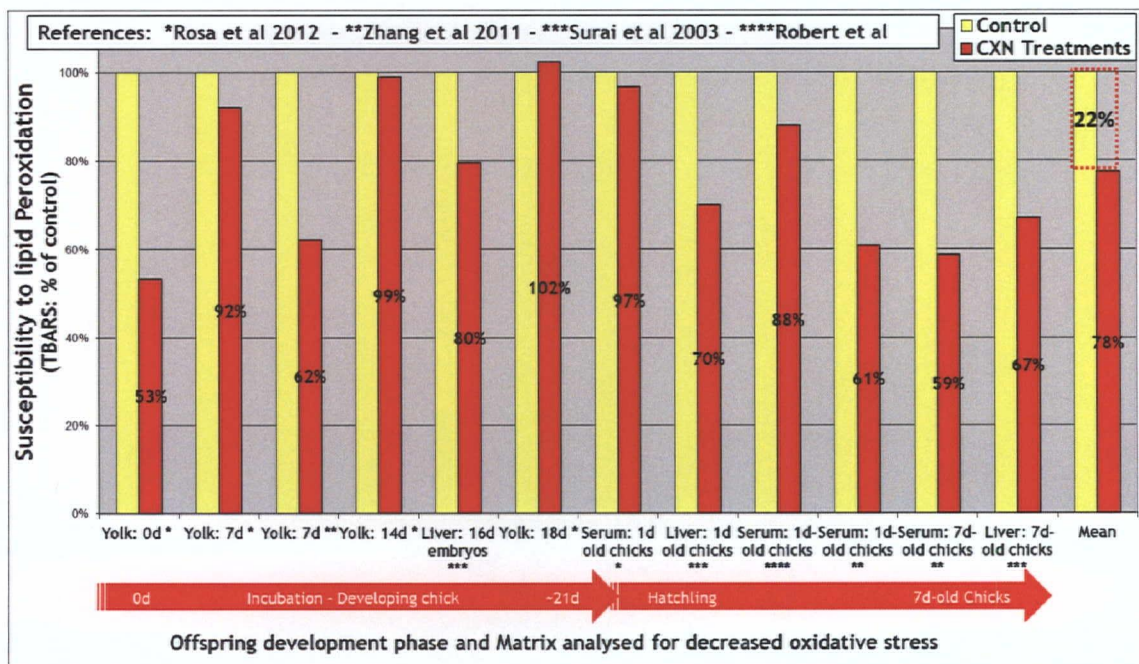
different times and in different matrixes during incubation and after hatch, it is also possible to recognize the biological shifts on the antioxidant effect as the embryo develops (Figure 3-7).

Overall, susceptibility to lipid peroxidation was first significant in yolk sac at day 0 (after egg laying) and 7th of incubation, but not at days 14th and 18th. The loss of significance in yolk sac after day 14th, however, seem to parallel the mobilization of Canthaxanthin from the yolk to deposit in the embryo liver. Similar findings were reported by Rocha et al. 2013 who tested TBARS in the viteline sac of day old chicks and didn't find a better antioxidant staus in canthaxanthin treatments compared to control treatments.

In terms of antioxidant capacity, this mobilization from yolk to liver, is evidenced by the numerically lower TBARS values in embryo livers at day 16th of incubation, which further reached significance in livers from hatchlings (1 day-old chick) and 7 day-old chicks.

In the case of serum of day old chicks, the antioxidant effect was significant in two out of three studies where serum was tested, and also significant in the 7 day-old chicks. The better antioxidant status after maternal dietary supplementation with CAROPHYLL® Red can then play a significant role in the development of the chick, by favoring optimum development during the critical phases of incubation, hatching and post hatch. In summary, when TBARS results from the four studies were compared in terms percentage, CAROPHYLL® Red treatments had in average 22% less susceptibility to lipid peroxidation. as compared to unsupplemented treatments (Figure 3-9)

Figure 3-9 Overview of four studies: Susceptibility to lipid peroxidation (TBARS) in Offspring from hens fed with or without CAROPHYLL® Red - Oxidative stress of CXN Treatments respect to Control (%).



4. SAFETY

4.1 Introduction

The characterization of the safety risk of CAROPHYLL® Red is directly related to Canthaxanthin, its active substance.

With more than 40 years of practical use in poultry nutrition and as a food additive, the safety of Canthaxanthin for Consumers and Target Species (e.g. Poultry), has been the subject of numerous public evaluations by different scientific bodies in the US (FDA, ILSI), the EU (SCAN, SCF, EFSA) and at FAO/WHO (Joint Expert Committee on Food Additives- JECFA). An overview of key safety evaluations and assessments for Canthaxanthin is presented in (Table 4-1).

In the Federal Register of Tuesday November 19, 1985 FDA published the final rule for the use of Canthaxanthin as a Color Additive exempt from certification. Within the supplementary information of the rule, FDA noted that the Acceptable Daily Intake (ADI) from all sources for Canthaxanthin was 150 mg/p/d based upon the No Observable Effect Level (NOEL) in rats after a 2 year feeding study. In its most recent assessment in the US, the FDA maintained the ADI of 150 mg/person/day ($2.5 \text{ mg kg bw}^{-1} \text{ day}^{-1}$) (63 Fed. Reg. 1998, 14814).

A number of evaluations outside the US have assigned Canthaxanthin with an ADI of 0.03 mg/kg bw, based upon the reduction in b-waves in the human electroretinography (ERG) at doses of 0.25 mg/kg bw/d and above. At the same time, crystal deposition in the retina of individuals consuming large amounts of Canthaxanthin in the form of tanning pills (unapproved use in the United States), is only observed at doses of 0.5 mg/kg bw/d and above (Köpcke et al. 1995). It has been demonstrated that the crystal deposition is reversible and does not result in morphological changes (Hueber et al. 2011)

The ADI of 0.025 mg/kg bw, rounded up to 0.03 mg/kg bw was first established by JECFA in 1995. Two years later an independent assessment by the European Scientific Committee on Food agreed upon the same ADI. The Scientific Committee for Animal Nutrition (SCAN) endorsed this ADI in 2002, which was also taken by the European Food Safety Authority (FEEDAP Panel) for their 2007 assessment for Canthaxanthin use in animal nutrition. The most recent assessment for Canthaxanthin uses as Food and Feed Additive by (EFSA 2010, EFSA 2014), further confirmed this ADI.

Canthaxanthin is likely to be the most widely evaluated carotenoid for which safety has been studied and relevant end points determined. DSM Nutritional Products (previously Hoffman-LaRoche, Vitamins and Fine Chemicals Division) has provided evaluators with internal study reports for their independent assessment. Information is also available from other sources. A cross-check of available literature, listing the references used in some most recent evaluations of JECFA (1995), SCF (1997), ILSI (1999) and EFSA (2010) is provided (DSM 2011).

Table 4-1 Safety Evaluations for Canthaxanthin in the US and the EU

Evaluator	Year	Title of Evaluation	Relevance	Link
European Food Safety Authority. (EFSA 2014)	2014	Scientific opinion on the safety and efficacy of canthaxanthin as a feed additive for poultry and for ornamental birds and ornamental fish	Canthaxanthin is safe for the target species (including poultry) and for the human consumer. ADI re-confirmed at 0.03 mg/kg bw/day.	http://www.efsa.europa.eu/en/efsajournal/doc/3527.pdf
European Food Safety Authority. (EFSA 2013)	2013	Scientific opinion on the safety and efficacy of CAROPHYLL® Red 10% (preparation of canthaxanthin) for all poultry for breeding purposes (chickens, turkeys and other poultry)	Safe use of canthaxanthin for poultry breeders at 6ppm Feed has been determined. Confirmed potential of canthaxanthin to stabilise the reproductive performance of breeder hens. Proposed use safe for consumers.	http://www.efsa.europa.eu/en/efsajournal/pub/3047.htm
European Food Safety Authority. (EFSA 2010)	2010	Scientific Opinion on the re-evaluation of Canthaxanthin (E 161 g) as a food additive	ADI re-confirmed at 0.03 mg/kg bw/day, by the EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)	http://www.efsa.europa.eu/en/efsajournal/doc/1852.pdf
European Food Safety Authority. (EFSA 2007)	2007	Opinion of the Scientific Panel on Additives and Products or substances used in Animal Feed on the Maximum Residue Limits of Canthaxanthin coming from animals fed with Canthaxanthin used as a feed additive in accordance with Council Directive 70/524/EEC	Proposal of Maximum Residue Limits (MRL's) in tissues for control within the food chain and based on the maximum permitted limits in feed set in 2003. (implemented in 2008 by EC)	http://www.efsa.europa.eu/en/efsajournal/doc/507.pdf
European Commission. (SCAN 2002)	2002	Opinion of the Scientific Committee on Animal Nutrition on the use of Canthaxanthin in feedingstuffs for salmon and trout, laying hens, and other poultry.	Review of maximum permitted levels of Canthaxanthin in complete feed (implemented in 2002 by EC) Trigger for the setting of MRL's	http://ec.europa.eu/food/fs/sc/scan/out81_en.pdf
ILSI North America Technical Committee on Food Components for Health Promotion. (ILSI 1999)	1999	Safety Assessment and potential health benefits of food components based on selected scientific criteria. "Canthaxanthin"	Comprehensive review of data of Canthaxanthin, where the committee found that Canthaxanthin has been marketed in the U.S. and the EU as a direct and indirect food additive for more than 30 years and during that time, there have been no problems of safety with regard to the use of Canthaxanthin for either purpose.	Crit Rev Food Sci Nutri 39(3): 203-316
FDA	1998	Color Additive Petition listing Canthaxanthin as a color additive in the feed of salmonid fish	Review of specifications, manufacturing, use and labelling of Canthaxanthin to color fish feed with a limit of 80 mg/kg of feed	63 Fed. Reg. 14814 1998
European Commission. Scientific Committee on Food (SCF 1997)	1997	SCF Opinion on Canthaxanthin.	Setting an Acceptable Daily Intake (ADI) based on laboratory animals and data on humans – the ADI was set by SCF at 0.03 mg /kg bw/d (i.e. for a 60 kg person: 1.8 mg per day)	http://ec.europa.eu/food/fs/sc/oldcomm7/out10_en.html
Joint Expert Committee on Food Additives (JECFA 1996)	1996	Toxicological evaluation of certain food additives and contaminants. Canthaxanthin.	The Committee allocated an ADI of 0-0.03 mg/kg bw to Canthaxanthin, based on a NOEL of 0.25 mg/kg bw/day in humans and a safety factor of 10.	http://www.inchem.org/documents/jecfa/jecmono/v35je08.htm WHO food additives Series No. 35. 839.
FDA	1985	Color Additive Petition listing Canthaxanthin as a color additive in broiler chicken feed	Review of specifications, manufacturing, use and labelling of Canthaxanthin to color broiler chicken feed with a limit of 4.41 mg/kg of feed	50 Fed. Reg. 47532 1985
FDA	1969	Color Additive Petition listing Canthaxanthin for Food Use Exempt From Certification	Review of specifications, manufacturing, use and labelling of Canthaxanthin to color food. Limit of 30 mg/lb of food	34 Fed. Reg. 250 1969

For this GRAS Notice, an up to date review on the safety of canthaxanthin for consumers (Chapter 4.3) has been prepared by DSM. This included public scientific safety and toxicological data. Public literature from January 1997 through November 2013 was searched in the following databases: NCBI Pubmed, SciFinder, Scopus, TOXNET, OECD eChem Portal, RTECS, IPCS INCHEM, NTP (National Toxicology Program), ILSI (International Life Sciences Institute) and BIBRA toxicity profiles. Results of these searches can be found as annex "DSM 2013". For completeness, EFSA and JECFA assessments, which in addition to public literature also report internal/unpublished DSM/Roche data, are also provided.

Results from the literature search were evaluated in the light of the relevance of their toxicological information (e.g.: studies addressing the different fields of toxicological risk assessment such as acute and repeated dose toxicity, local effects on skin & eyes, sensitization, genotoxicity, carcinogenicity, developmental & reproductive toxicity, effects on the immune and endocrine system, and toxicokinetics, etc). Articles were screened to retrieve the original references. Studies from the literature search were incorporated into the safety review (Chapter 4.3) if they delivered new insights or additional data supporting the presence or absence of a toxicological effect. Furthermore, an exposure calculation for humans from the proposed and approved uses of canthaxanthin is provided, see section 4.3.12.

For public literature on Canthaxanthin prior to 1997, the ILSI report from 1999 provides a comprehensive compilation. Recent Scientific Opinions from EFSA (2010, 2013, 2014) also provide a good indication of the current status of safety and toxicological data available for Canthaxanthin use in animal and human nutrition. In relation to target animal safety and consumer exposure, besides the demonstrated safe use of Canthaxanthin for over 40 years as a food Color Additive in the US, target animal safety studies with poultry are discussed in section 4.2.

These safety data will be discussed in detail in the following sections, where the following is established:

- Target species: Canthaxanthin at a dietary supplementation of up to 10 times (10x) the recommended dose of 6 mg/kg feed, is found to be safe for poultry breeders. Furthermore, Canthaxanthin has already been confirmed as safe and is an approved color additive for broilers in the US.
- Humans: Canthaxanthin fed to poultry breeders as a GRAS substance does not represent a safety concern for humans. On one hand, tissues from spent hens, same as discarded eggs from poultry breeders, are not aimed for human consumption. Furthermore, assuming a worst case scenario, where eggs and tissues would reach the final consumer, human exposure to canthaxanthin from the GRAS use and all US approved color uses, is always below the ADI.

4.2 Target Animal Safety

4.2.1 History of safe use

It is important to emphasize from a perspective that FDA's approval of Canthaxanthin's safety for multiple food uses has been in place for more than 40 years. The FDA on January 8, 1969 published in the Federal Register, Vol. 34, 250-251, the final rule for Canthaxanthin with its listing as a Color Additive that is safe for general use in foods and drugs.

The only restrictions specified were that (a) the quantity of Canthaxanthin not to exceed 30 milligrams per pound of solid or semisolid food or per pint of liquid food and (b) it may not be used to color foods for which standards of identity have been promulgated under section 401 of the FD&C act unless added color is authorized by such standards. In the Federal Register Vol 42, March 22, 1977, 15643-15645, Part 73 Listing of colors exempt from certification, 21 CFR §73.75, the identity, specifications and approved uses of Canthaxanthin are specified.

In 1985, in the Federal Register Vol. 50, No. 223, November 19, 1985 pp. 47532-47534, the FDA amended the Color Additive regulation to provide for the safe use of Canthaxanthin as a Color Additive in broiler chicken feed to enhance the yellow color of broiler chicken skin in accordance with the following conditions: The quantity of Canthaxanthin incorporated in the feed shall not exceed 4.41 milligrams per kilogram (4 grams per ton) of complete feed to supplement other known sources of xanthophyll and associated carotenoids accomplish the intended effect.

In the Federal Register Vol. 63 No. 59 March 27, 1998 pp 14814-14817; the FDA amended the Color Additive regulations to provide for the safe use of Canthaxanthin as a Color Additive in the feed of salmonids fish to enhance the color of their flesh. FDA determined that a use level of Canthaxanthin in fish feed of 80 mg/kg (72 grams per ton) is safe. As part of this determination, FDA conducted a reevaluation of the cumulative exposure to Canthaxanthin and determined that their original calculations were unreasonably exaggerated in that they assumed Canthaxanthin was added to all foods and determined that the proposed amended use was safe.

For the specific use in poultry breeders, EFSA has recently confirmed canthaxanthin safety at the proposed dose of 6ppm feed (EFSA 2013). For all other poultry (layers, broilers, ornamental birds), the use of canthaxanthin as a color additive has also been confirmed (EFSA 2014)

4.2.2 Absorption, Distribution, Metabolism, Excretion in poultry

Canthaxanthin metabolism in avian species is different than that of mammals and is illustrated in the figure below for chickens. Canthaxanthin is absorbed in the small intestine and transported via the blood to the liver. There, a part of the absorbed Canthaxanthin undergoes metabolic change and is transformed into 4-hydroxyechinenone and isozeaxanthin but also 4-oxoretinol, a vitamin A precursor, in both laying hens and broilers (Tyczkowski et. al., 1988; Schiedt, 1998). The remaining unchanged Canthaxanthin is transported by lipoproteins via blood to the target deposition sites. Less than 40% of the dietary Canthaxanthin is deposited in

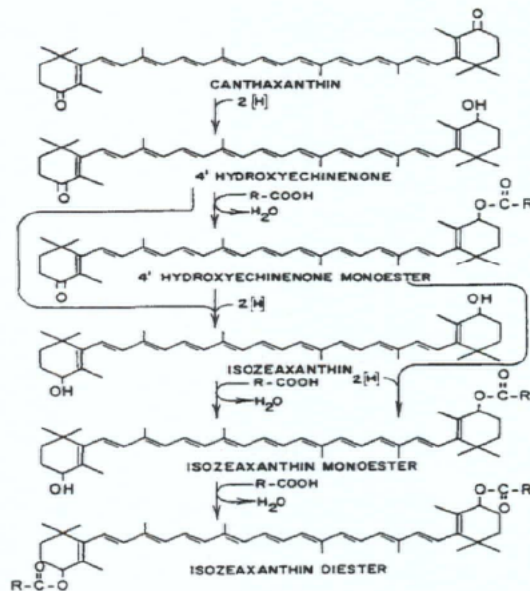
egg yolk, whereas the deposition in the body tissues is lower than 10% ((b) (4) 1987; EFSA 2007).

The distribution of the total radioactivity in the different tissues and organs following the repeated administration (8 mg/kg feed) of radioactive Canthaxanthin to the hen is the following: ovaries (68-69%), liver (5.2-6.3%), muscle (3.2-7.5%), fat (1.0-1.2%), skin (1.1-1.1%) ((b) (4) 1987).

Further studies carried out using radiolabelled Canthaxanthin have allowed the isolation of metabolites from the liver of both laying hens and broiler chicks (Schiedt, 1998) as well as from egg yolk, spleen, kidney and perineal fat of layers ((b) (4) 1987). Non-metabolized (unchanged) Canthaxanthin represented 40% of the total residues in the liver while the 4-oxoretinol was the major metabolite (30%).

The relatively low content of the reduction products 4-hydroxy-echinenone and isozeaxanthin is noteworthy, whereas these metabolites are present at a much higher concentration (30% for 4-hydroxyechinenone) and in esterified form in the toe-web and integumentary tissues (Tyczkowski et al., 1988). No radioactivity could be recovered in β,β -carotene, which was therefore not an intermediate in the conversion of Canthaxanthin into vitamin A. Even if these biotransformations are limited, they may reduce the pigmenting properties of Canthaxanthin in the hen (Hencken, 1992).

Figure 4-1 Pathway for the metabolism of Canthaxanthin in chickens (Tyczkowski et al. 1988)



Analysis of tissue samples from forty, 3-week old chicks fed a Canthaxanthin fortified corn-soy diet revealed that most of the substance was deposited intact with the liver being the primary site (Table 4-2).

Overall EFSA concluded in 2007, that “Canthaxanthin is by far the major component of residues in target tissues of poultry and fish”. The European FEEDAP Panel considers Canthaxanthin as the only residue of concern and therefore retains Canthaxanthin (measured as the all-trans isomer) as the marker residue.

Table 4-2 Distribution of Canthaxanthin and its metabolites in chicken tissues (Tyczkowski et al. 1988)

Tissue	Canthaxanthin	Hydroxy-echinonone		Isozeaxanthin		
		Free	Ester	Free	Mono-ester	Diester
		(µg/g)				
Intestinal contents	70	.63	tr. ²	tr.	tr.	tr.
Intestinal mucosa	90	4.5	tr.	.18	tr.	tr.
Serum	37	3.2	.04	.44	tr.	.04
Liver	144	5.0	.14	.28	tr.	.14
Skin	29	3.5	2.4	.55	tr.	.20
Toe web	38	2.9	6.8	.34	.05	.48
Toe nails	68	6.0	30	.95	2.0	46
Feathers	10	1.6	.49	.23	.01	.08
Whole body homogenate	22	1.9	.62	.29	tr	.13

¹ Values are means of 4 groups of 10 birds.

² tr. = Trace.

4.2.3 Tolerance Studies

Target animal safety of Canthaxanthin with poultry (*Gallus gallus*) has been previously evaluated by FDA with the Color Additive Petition submitted by Hoffman-La Roche (now DSM Nutritional Products) in 1971. These original studies demonstrated the safe use of Canthaxanthin with broilers, roosters and breeder hens (Table 4-3), and FDA conclusions on the suitability of the results (FDA 1972) were a key element for the current approval of Canthaxanthin as a Color Additive in the US for Broilers.

Table 4-3 Studies on target animal safety from Roche’s 1971 Color Additive Petition for Canthaxanthin.

Author	CXN Dose (mg/kg feed)	Description	Reference
(Roche 1971a)	0, 5, 50, 500	Three reports presented <ul style="list-style-type: none"> • Marusich et al. 1963: Toxicity studies on Canthaxanthin in Poultry. • Schwartz 1963: Histopathologic Study of tissues from hens and roosters fed Canthaxanthin • Marusich 1967: 24-week Hen study with graded levels of Canthaxanthin fed in colloidal type beadlets. 	Canthaxanthin 1971 Color Additive Petition CAP 1C0101, approved in 1985 <ul style="list-style-type: none"> • Report 1: pp 298-318 • Report 2: pp 319-321 • Report 3: pp 322-332

The safe use of Canthaxanthin as a Color Additive in poultry has been further confirmed in the European Union (EFSA 2013, EFSA 2014). Approved level for canthaxanthin use in broilers in the EU is 25 mg/kg. Furthermore, Canthaxanthin safe use has been also confirmed in the EU as a

Color Additive to pigment egg yolk from laying hens, where approved dose is 8 mg/kg, higher level as compared to the 6 mg/kg proposed in this GRAS Notice. EFSA has also confirmed cantaxanthin safe use when fed to breeder hens at the recommended dose of 6 mg/kg Feed (EFSA 2013).

As summarized in (Table 4-4), a number of studies have evaluated the performance of poultry breeders at the proposed dose of 6mg Canthaxanthin per kg of feed. While these studies were not specifically designed to evaluate target animal safety, provide consistent evidence that no adverse effects are expected from Canthaxanthin when used as proposed with breeder hens. A dedicated study with overdoses of canthaxanthin in poultry breeder diets (Weber et al. 2013) is presented in chapter 4.2.4.

Table 4-4 Studies with poultry breeders fed Canthaxanthin at different doses in feed.

Author	Dose (mg/kg feed)	Description	Study Length (weeks)	Findings	Reference
Llaurado et al. 1997	0, 2, 4, 6	Effect of Canthaxanthin supplementation on the hatchability of eggs of broiler breeders.	13	Poultry breeders were not adversely affected by CXN added to the feed, at any of the doses tested.	Proceedings of the 11th European Symposium on Poultry Nutrition, Denmark, 1997. pp. 280—282.
Surai et al. 2003	0, 3, 6, 12, 24	Effect of Canthaxanthin content of the maternal diet on the antioxidant system of the developing chick	4		British Poultry Science Volume 44, Number 4 (September 2003), pp. 612—619
Robert et al. 2007	0, 6	Effects of Canthaxanthin supplementation in the ROSS breeder diet on oxidative stress of chicks	12		World Poultry Science Association, Proceedings of the 16th European Symposium on Poultry Nutrition. August 26 - 30, 2007 Strasbourg, France
Rocha et al. 2013	0, 6	The effect of storage and dietary canthaxanthin on fertile egg quality and embryonic development	13		Arq. Bras. Med. Vet. Zootec., v.65, n.3, p.792-800
Rosa et al. 2012	0, 6	Effects of Canthaxanthin on the Productive and Reproductive Performance of Broiler Breeders	21		Poultry Science 2012 91: 660–666
Souza et al. 2008	0, 6	Effect of the use of CAROPHYLL [®] Red on reproductive indices in broiler breeders	10		APINCO – Poultry Science and Technology Conference Proceedings. 27-29 of May 2008. Santos. SP
Zhang et al. 2011	0, 6	Influence of canthaxanthin on broiler breeder reproduction, chick quality, and performance.	24		Poultry Science 2011 90: 1516-1522

4.2.4 Target Animal Safety study with Poultry breeders

Study	Weber et al. 2013: Tolerance of poultry against an overdose of canthaxanthin as measured by performance, different blood variables and post-mortem evaluation
GLP Status	Not GLP. Comparison of the breeders study to GLP standard is provided (Annex (b) (4) 2012).
Objective	To investigate the safety of canthaxanthin under conditions of an accidental oversupply in all poultry categories (broilers, laying hens and poultry breeders)
Methods	<ul style="list-style-type: none"> • Studies in all poultry categories were performed, comparing a non-supplemented control to treatments with the respective recommended dietary supplementation of CXN and a ten times overdose of the recommended CXN level. • Breeders experiment was performed with 162 breeder hens at the IRTA Animal Research Center Reus (Constantí, Tarragona, Spain). • From week 18 to 22 the birds were acclimatized in the cages and fed the basal diet without the test product. On week 22 the hens were allocated to 3 dietary treatments, differing in the supplementation level of CXN (Carophyll Red 10%) in feed. Each treatment was replicated 9 times with 6 hens each (3 cages, housing 2 hens, per replicate): <ul style="list-style-type: none"> ○ 0 mg CXN/kg (control); ○ 6 mgCXN/kg (recommended level) or ○ 60 mg CXN/kg (overdose). • Mash Feed - ad libitum: Based on maize and soybean meal (dietary nutrient composition as fed; ME: 11.5 MJ/kg, CP: 170 g/kg; Lys: 9.2 g/kg; Met: 5.2 g/kg, Met + Cys: 8.2 g/kg; Ca: 40 g/kg, P: 6.1 g/kg; calculated content). • Hens were inseminated artificially with semen from roosters that were fed a diet without CXN supplementation. All eggs, produced during the week before the evaluation of hatchability, were collected and stored at 17°C before being placed in the incubator at once. Candling to evaluate fertility and embryo development was performed on day 12 of incubation. • Parameters recorded: growth performance, egg production, egg weight, feed consumption, laying rate and FCR, deposition of CXN in eggs on week 30, reproductive parameters, deposition of CXN in different tissues (liver, kidney, adipose tissue, muscle and skin) at week 52 of the study (2 hens per replicate of the control and the treatment with the recommended dose), post-mortem evaluation and blood analyses (hematology and biochemistry).
Results	<ul style="list-style-type: none"> • Body weight and productive performance were not different among treatments. • Egg weight with 6 mg CXN/kg feed was higher (P=0.045) than in control group. • CXN content in eggs increased proportionally to CXN supplementation (P<0.001). • No differences in fertility were observed among the treatments, but hatchability of fertile eggs was depressed by overdosing CXN (P<0.001). • Certain hematology and blood chemistry traits differed between the treatments, but the values remained within the normal range. • Final post-mortem examination did not reveal any health problems related to the dietary treatments or attributable to the high CXN contents. • There were no outward indications that any of the treatment group of animals suffered from impaired vision during the course of the study. There were no signs of aggression, weight loss or dehydration due to an inability which would have been indicated that the animals were experiencing an impaired or loss of vision (Annex Perez-Vendrell 2014).
Conclusions	CXN at dietary supplementation doses of up to ten times the recommended levels is safe for broilers, laying hens and poultry breeders.

4.2.5 Conclusions on Target Animal Safety

Besides a history of safe use in poultry nutrition, a dietary supplementation of poultry breeders with canthaxanthin at 6mg/kg feed does not negatively affect the performance, health or product quality of hens.

4.3 Safety of Canthaxanthin for consumers

4.3.1 Introduction

The hazard profile of Canthaxanthin has been thoroughly reviewed by various competent authorities over the more than forty years this carotenoid has been available in the marketplace. Of key relevance, the safety profile of Canthaxanthin was evaluated several times by the US Food and Drug Administration (FDA), the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the European Commission Scientific Committee on Food (SCF) and the European Food Safety authority (EFSA). In addition to these reviews, a comprehensive evaluation of public toxicity and ADME studies were summarized and reviewed by the US International Life Sciences Institute (ILSI 1999).

After (b) (4) the FDA determined an ADI for Canthaxanthin of 150 mg/person/day (2.5 mg/kg bw/day) (FDA 1968). With follow up submissions and approvals for broilers (CAP 101) and salmonids, this ADI remained. In 1998, the FDA (63 Fed Reg 1998) evaluated the safety for the use as Color Additive for salmon and broilers and concluded that Canthaxanthin when used as regulated is safe with a cumulated exposure of 0.36 mg/person/day (0.006 mg/kg bw/day for a 60 kg person) (FDA 1998). The current exposure to Canthaxanthin from food uses in the US is 0.018 mg/person/d (FDA 2006).

JECFA (1996) as well as SCF (1997) came to the conclusion that an Acceptable Daily Intake (ADI) of 0.03 mg Canthaxanthin/kg bw/day should be set based on the observation of reduction of the b-wave in the electroretinography (ERG) of human (exact value 0.025 mg/kg bw/day). Recent re-evaluations by EFSA have confirmed the ADI of 0.03 mg Canthaxanthin/kg bw/day (EFSA 2010, EFSA 2014).

The European Scientific Committee on Medicinal Products and Medical Devices has published in its opinion in 1998 that the use of Canthaxanthin as coloring agent in medicinal products constitutes no risk for public health (SCMPMD 1998). This opinion is based on the basis that compared with the quantities of colorants that can be consumed without limitation in certain foodstuffs, the quantities consumed with medical products are absolutely negligible.

The International Agency for Research on Cancer (IARC) has evaluated the cancer-preventive potential of carotenoids (Vainio and Rautalahti 1998). No human data regarding associations with cancer risk have been published for Canthaxanthin. In animal models, however, there is sufficient evidence of cancer-preventive activity for Canthaxanthin (Vainio and Rautalahti 1998, EFSA 2010). Pending further research into their cancer-preventive activity, supplemental Canthaxanthin and other carotenoids should not be recommended for cancer prevention in the general population.

With consideration that the safety and toxicological properties of Canthaxanthin have been subject of multiple investigations over the past four decades, this makes a relatively large safety dataset to be summarized in this GRAS Notice. In order to streamline the dataset provided, only the key conclusions from FDA on the toxicological data submitted by Hoffman-La Roche with

(b) (4) are included in this GRAS notice (see: FDA 1968 and FDA 1972, respectively).

Because a large amount of safety studies and reviews have been generated from the time of those early submissions in the 1960' and 1970', this chapter provides a summary of internal and public safety and toxicological data. Regarding the public data considered, JECFA (1996), SCF (1997), ILSI (1999) and EFSA (2010, 2013, 2014) provide thorough reviews.

With consideration to the availability of these reviews, a comprehensive search of scientific literature was then conducted for data from January 1997 through November 2013. The following databases were searched: NCBI Pubmed, SciFinder, Scopus, TOXNET, OECD eChem Portal, RTECS, IPCS INCHEM, US NTP (National Toxicology Program), ILSI (International Life Sciences Institute) and BIBRA toxicity profiles. Three searches were carried out and the result of these literature searches compiled in Annexes Literature Search 1: Jan 1997-Aug 2010; Literature Search 2: Aug 2010-Aug 2011; and DSM 2013: Aug 2011-Nov 2013.

4.3.2 Absorption, Distribution, Metabolism, and Excretion

4.3.2.1 Summary of the ADME data

Overall, toxicokinetic studies in animals indicated that only 3 to 9% of orally administered radiolabelled Canthaxanthin is absorbed and faecal excretion is the major route of elimination (85 to 89% of the dose in monkeys). Canthaxanthin is further distributed to liver, spleen, adipose tissue and adrenals. In monkeys, the concentration of Canthaxanthin in the retina is dose-related, but nonlinear, suggesting that saturation could occur. In animals, elimination from adipose tissue is slow, while Canthaxanthin is eliminated from other tissues soon after withdrawal (EFSA 2010).

In humans, a part of the orally ingested Canthaxanthin is absorbed (8 to 34% of the dose) (EFSA 2010). About sixty percent of the absorbed dose was transferred to fat tissue as estimated by measuring the concentration of Canthaxanthin in the chylomicrons after fractionation of serum lipids. In plasma, Canthaxanthin was associated with lipoprotein fractions, and 52% was transferred from chylomicrons to fat tissues. Remobilization from fat was described as slow and the half-life was estimated to be approximately 5 days. No data on Canthaxanthin metabolism in humans were published.

4.3.2.2 Animal Studies

Besides public studies, several major studies describing the absorption, distribution, metabolism & excretion of Canthaxanthin in animals were reported by JECFA (1990) and EFSA (2010).

Reference	EFSA (2010) Reported as: Glatzle D and Bausch J, 1988a. Canthaxanthin balance studies - first results. Research report No. B-106'707. Unpublished report submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland. Glatzle D and Bausch J, 1989. Canthaxanthin Balance Studies. Unpublished research report No. B-106714 submitted to WHO by F. Hoffmann-La Roche & Co., Basel, Switzerland (as referred to by JECFA, 1995).
Type	Balance Study
Guideline	Not applicable
GLP	No
Test substance	Unlabelled for pre-treatment Canthaxanthin 10% WS Radioactive material for single dose experiment 6,7,6',7'- ¹⁴ C-Canthaxanthin in liposomal preparation 6,7,6',7'- ¹⁴ C-Canthaxanthin in beadlet preparation
Species / sex	Rat (RORO strain)/ male
Number animals	52 animals in total (1 to 4 animals per experiment)
Doses males	Pre-feeding 0, 0.001, or 0.01% Canthaxanthin 10% WS in diet (0, ca. 350 nmol, and ca. 3500 nmol Canthaxanthin per animal) ¹⁴ C-radioactivity 34 and 350 nmol/animal
Doses females	Not applicable
Route of administration	Oral

In rats given a single oral administration of Canthaxanthin (0.08 and 0.8 mg/kg bw) the absorption accounted for only 9% of the dose, based on the amounts recovered in tissues and excreted in urine. In these studies, 6,7,6',7'-¹⁴C-canthaxanthin was administered either as a liposomal preparation (using soya lecithin) in phosphate buffered saline (PBS) using a probe applied into the stomach or as beadlets (containing 3.3% ¹⁴C-Canthaxanthin) administered in the feed (2 g feed mixed with Canthaxanthin followed by another 2 g of feed). There was no significant difference between the results for animals pre-fed with Canthaxanthin (0.001% or 0.01% in the diet), done in order to achieve steady state conditions, and those receiving no Canthaxanthin before the application of the radioactive pulse label. Canthaxanthin added to the feed was in the form of beadlets containing Canthaxanthin (as a water dispersible formulation). For a lower dosage (34 nmoles) of radioactive Canthaxanthin applied in liposomes to rats on a Canthaxanthin-free diet, the percentage of the applied radioactivity which was absorbed, and also the percentage of the applied radioactivity which was excreted in urine within the first 24 hours, was higher as compared to the values obtained at the higher dosage (337 nmoles) (20% versus 9% of the dose). The pattern of distribution in the tissues and of excretion was rather similar for all preparations and applications.

DSM1493-001



The distribution of Canthaxanthin was studied in several species including rats, guinea pigs, dogs and cynomolgus monkeys using single doses of 6,7,6',7'-¹⁴C-Canthaxanthin. After oral administration (as beadlets added in the feed or by oral gavage of liposomes using ¹⁴C-Canthaxanthin), the radiolabel was identified mainly in liver and spleen. Canthaxanthin was also found in small intestine, eye, skin, bone marrow and brain. In several of the radiotracer studies described above, the tissue distribution data for ¹⁴C-Canthaxanthin were obtained in animals pre-dosed with (cold) Canthaxanthin in the diet (5-52 weeks) (EFSA 2010, reported as Hoffmann-La Roche, 1986. Canthaxanthin. Unpublished report submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland).

In a 52-week study in dogs (7 animals/dose) receiving Canthaxanthin (unlabelled) at 0, 50 or 250 µg/kg bw/day, the highest Canthaxanthin levels were recorded (after 250 µg/kg bw/day) in fat (24 µg/g tissue), adrenals (15.1 µg/g tissue), the skin (9.6 µg/g tissue) and the liver (5.2 µg/g tissue). Background levels were < 0.1 µg/g tissue in untreated animals (EFSA 2010, reported as Hoffmann-La Roche, 1986. Canthaxanthin. Unpublished report submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland).

Reference	EFSA (2010) Reported as: Glatzle D and Bausch J, 1988b. Accumulation and depletion of canthaxanthin and astaxanthin in kidney fat of rats. Research report No. B-106'690. Unpublished report submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland.
Type	Accumulation and Depletion Study
Guideline	Not applicable
GLP	No
Test substance	Canthaxanthin 10% WS
Species / sex	Rat (RORO strain)/ male
Number animals	4 to 5 per dietary concentration
Doses males	1, 10, 100, 1000 ppm Canthaxanthin in diet
Doses females	Not applicable
Route of administration	Feed admix

In rats (RORO strain), elimination from adipose tissue was reported to be very slow, while Canthaxanthin was eliminated from other tissues soon after withdrawal. In this strain of rats, approximately 50% of the absorbed (radiolabelled) Canthaxanthin dose was excreted in the urine in 24 hours, and 98-99.7% in 7 days. In contrast, measurements of Canthaxanthin levels in kidney fat after 31-week supplementation of the diet with 100 mg/kg diet showed a total Canthaxanthin content of 4.3 µg/g tissue (n=3). After cessation of the supplementation and feeding the animals a Canthaxanthin-depleted diet for 14 and 31 weeks, respectively, kidney fat Canthaxanthin content had decreased by only 35% (n=1) and 49% (n=4)

Reference	EFSA (2010) Reported as: Bausch J, 1992a. (14C)-Canthaxanthin: Absorption, Distribution and Excretion Following Oral Administration at Steady State in the Cynomolgus Monkey. Unpublished research report No. 106 798, submitted to WHO by F. Hoffmann-La Roche & Co., Basel, Switzerland. (as referred to by JECFA, 1995 and SCF, 1997)
Type	Balance Study
Guideline	Not applicable
GLP	Yes
Test substance	¹⁴ C-labelled Canthaxanthin (6,7,6',7'- ¹⁴ C-Canthaxanthin) Non-labelled Canthaxanthin 10% formulation
Species / sex	Cynomolgus Monkey (<i>Macaca fascicularis</i>) / both sexes
Number animals	1 per sex per dose
Doses males	0.2 and 0.6 mg/kg bw/day
Doses females	0.2 and 0.6 mg/kg bw/day
Route of administration	Oral by gavage

In cynomolgus monkeys (2 males and 2 females), a single oral dose of radiolabelled 6,7,6',7'-¹⁴C-Canthaxanthin was administered at dose levels of 0.2 and 0.6 mg/kg bw. Steady-state conditions were maintained after the radiolabelled dose by daily administration of non-radiolabelled Canthaxanthin. The radiolabelled Canthaxanthin was administered orally using a dosing apparatus whereas the non-radiolabelled Canthaxanthin formulation was prepared as a solution in distilled water, and was administered by gavage. Following the administration of radiolabelled compound, blood and plasma radioactivity profiles were similar over the 96 hours post-dose period, but plasma concentrations were twice those of blood. Concentrations in female animals tended to be higher than those in males with peak blood concentration occurring within 4 and 6 hours, in male and female respectively. Faecal excretion was the major route of elimination (85 to 89 %), most recovered within 48 hours of dosing. Urinary excretion (1.6 to 3.6%) and radioactivity retained in tissues (1.6 to 4.6% (excluding gastro intestinal tract)) indicates that 3 to 8% of the dose was absorbed. By 96 hours post dose, radioactivity was distributed into most of the tissues sampled. Highest concentrations were observed in the adrenal gland, with moderate levels in the spleen, liver, bone marrow, skin, fat and ovaries. Noticeably, low levels were found in parts of the brain and eye. The half-life of (radiolabelled) Canthaxanthin in the systemic circulation of monkeys ranged from 36 to 92 hours, irrespective of maintenance dose or sex.

In other studies in monkeys using unlabelled Canthaxanthin, the concentration in the retina was found to correlate with concentration in plasma and was dose related, but nonlinear, suggesting that saturation can occur. Distribution was dependent on the time after dosing.

Reference	EFSA (2010) Reported as: Bausch J, 1992b. Canthaxanthin Metabolic Studies: Comparison of Rat Results with Monkey Results. Unpublished research report No. B-106 799, submitted to WHO by F. Hoffmann-La Roche & Co., Basel, Switzerland. (as referred to by JECFA, 1995 and SCF, 1997)
Type	Expert Report
Remark	This expert evaluation compares the results of two balance studies with Canthaxanthin in rats and in monkey. In addition, further information on metabolites in urine and faeces is presented.

Canthaxanthin metabolism in monkeys and rats was compared using the same radiolabelled 6,7,6',7'-¹⁴C-Canthaxanthin administered orally at single dose levels of 0.2 or 0.6 mg/kg bw. Canthaxanthin was metabolized and excreted more slowly in monkeys than in rats. The levels of the total remaining radioactivity in monkey tissues were 7.4% after 96 hours, compared to less than 1% of the administered dose in rat tissues. The adrenals were the target organ for retention of radioactivity in the monkey, accumulating levels per g tissue that were 20-50 times higher than in any other tissue. In the rat, the levels of radioactivity measured in the adrenals were lower than the levels detected in liver and spleen. In both species, noticeably low levels were found in the eye. Urinary metabolites in the rat contained some very polar compounds, which were only present in trace amounts in monkey, while their urine contained some less polar compounds that were absent in rat urine. In the monkey retina, 4-hydroxyechinenone (4HE) and isozeaxanthin (IZX) were identified as metabolites. No similar investigation was carried out in rats.

Astorg et al. (1997b) have evaluated the effect of different carotenoids on the initiation stage of carcinogenesis in a feeding experiment in rats using 10% cold-water dispersible Canthaxanthin provided by Hoffmann-La Roche (300 ppm Canthaxanthin in the diet). Additionally, liver lobes were removed 4 weeks after a 3-4 week feeding period and analyzed for carotenoid content by HPLC. Results showed that Canthaxanthin content in the rat liver was 26.2±4.7 µg/g liver of which 66.2% were the all-trans and the remaining 33.8% were cis isomers. This distribution of both isomers reflects the proportion of the respective isomers in the powder fed to the rats. After Canthaxanthin feeding, the content of retinyl esters in the liver samples was lowered to 88.8% of control.

A similar experiment was conducted by Gradelet et al. (1998), feeding 300 ppm Canthaxanthin (10% cold-water dispersible powder, Hoffmann-La Roche) to a group of 10 male Wistar rats for a time span of 3 weeks. Canthaxanthin content in hepatectomy samples (obtained 4 weeks after cessation of Canthaxanthin treatment) was 20.0 ±4.4 µg/g liver. The content of retinyl esters in the liver samples was slightly lowered after Canthaxanthin feeding, compared to the control group.

A similar experiment was conducted by Astorg et al. (1996), feeding 300 ppm Canthaxanthin (10% cold-water dispersible powder, Hoffmann-La Roche) to a group of 10 male Wistar rats for a time span of 3 weeks. Canthaxanthin content in hepatectomy samples (obtained either at day 15 of Canthaxanthin administration or at 28 and 49 days after cessation of treatment) was

DSM1493-001



477.8 ± 147.9, 82.2 ± 13.5, and 13.5 ± 2.0 µg/g liver. The content of retinol and retinyl esters in the liver samples after Canthaxanthin feeding was not significantly different from control group.

Using mesenteric lymph duct cannulated rats; Clark et al. (1998) have assessed the absorption of the carotenoids Lycopene (purified from tomato) and Canthaxanthin (from Sigma Chemicals) in the GIT of rats. Lipid emulsions containing the respective carotenoid at different concentrations were continuously infused into the duodenum. The time course for absorption of both carotenoids and triglycerides was similar, reaching steady-state conditions in the lymph by 6 h. Uptake of carotenoids from the intestine was found to happen simultaneously with applied lipids. Canthaxanthin concentration in the lymph increased in a dose dependent manner. Average efficiency of Canthaxanthin absorption was 16% (range: 10-20%) and was not dependent on the concentration of Canthaxanthin administered. Administration of both carotenoids in parallel revealed that they do not significantly affect each other's absorption from the GIT.

In the same model, Clark et al. (2001) could show that absorption of Canthaxanthin by the rat is influenced by the total amount of lipids in the intestinal lumen: The recovery of Canthaxanthin in the lymph when infused with 10 mg/h olive oil was 14.2 ± 1.2% and with 90 mg/h was 26.9 ± 5.7%, with a correlation between lipid load and Canthaxanthin absorption of $r = 0.85$.

Grolier et al (1997) have noticed that in rats dietary Canthaxanthin at 2.5-20 µM (obtained from Hoffmann-La Roche, Basel) competes with beta-Carotene for intestinal absorption. It inhibits the enzyme beta-carotene-dioxygenase (the enzyme that converts beta-Carotene to Vitamin A) at >1 µM but is not itself converted to Vitamin A by this enzyme.

Hageman et al. (1999) have conducted an uptake study in rats. Canthaxanthin (5-20 µmol/L in corn oil) was infused into the duodenum of rats with/without concomitant administration of alpha-Tocopherol (300 µmol/L); the amount of Canthaxanthin found in the lymph was measured. Results show a significant linear dependency of Canthaxanthin concentration applied and the amount found in the lymph. However, Canthaxanthin absorption was impeded by co-administration of large doses of alpha-Tocopherol: absorption was approximately 12% without alpha-Tocopherol but only 5% with 300 µmol/L alpha-Tocopherol, independent of Canthaxanthin concentration.

Bendich & Shapiro (1986) fed Canthaxanthin (0.2%) to groups of 8 male Wistar Kyoto rats for 20 weeks. Canthaxanthin concentrations were determined in plasma (68 ± 0.1 µg/dL), liver (14.1 ± 2 µg/g), and spleen (175 ± 17 µg/g).

4.3.2.3 Human Studies

Besides anecdotal reports describing the distribution of Canthaxanthin in necropsied tissues, two major studies were reported by JECFA (1990) and EFSA (2010):

Reference	JECFA (1990) Kübler W, von Reinersdorf D (2002) EFSA (2010) Reported as: Kubler W, 1986. Biokinetic evaluation of canthaxanthin plasma levels after multiple doses of 1 mg and 8 mg Canthaxanthin. Unpublished report submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland. Schalch W, 1988. Biokinetic evaluation of canthaxanthin in humans. Unpublished Research Report No. B-107'134 submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland.
Type	Plasmakinetics in human volunteers after single and multiple doses
Guideline	Not applicable
GLP	No
Test substance	Canthaxanthin 10% WS
Species / sex	Healthy human volunteer / both sexes (between 23 and 37 years of age)
Number animals	5 to 10 volunteers per sex per dosage group
Doses males	30 x 1 mg / 5 volunteers 12 x 8 mg / 5 volunteers 1 x 75 mg / 5 volunteers 1 x 150 mg / 6 volunteers
Doses females	30 x 1 mg / 5 volunteers 12 x 8 mg / 5 volunteers 1 x 75 mg / 5 volunteers 1 x 150 mg / 10 volunteers
Route of administration	Oral

In the first study, ten volunteers (5 females and 5 males, age not indicated) and 16 volunteers (10 females and 6 males) received respectively a single oral dose of 75 and 150 mg Canthaxanthin, mixed with full milk cream. From this study, the elimination half-life was calculated as 4.5 days in each group and the proportion of the dose absorbed was estimated to be 12% and 9% in each group respectively. In the second study, ten volunteers (5 males and 5 females) were each given either 1 mg or 8 mg Canthaxanthin (presumably water dispersible Canthaxanthin) (in capsule together with full milk cream) 6 times a day for 5 days or 2 days, respectively, corresponding to a total dose of 30 mg or 96 mg, respectively. From this study, the calculated steady state plasma concentrations of Canthaxanthin after daily ingestion of 6 mg (6 times 1 mg) or 48 mg (6 times 8 mg) were 1.8 or 10.3 mg/l, respectively. The authors concluded that Canthaxanthin was cleared from serum with a half-life of 5.3 days, and after administration in multiple oral doses, the maximum serum concentrations were achieved after approximately 48 hours. The absorption of Canthaxanthin was no more than 34% of a 1 mg oral daily dose, and about 60% of the absorbed dose was transferred to fat tissue as assumed by measuring the concentration of Canthaxanthin in the chylomicrons after fractionation of serum lipids.

In a further study in male and female human volunteers single doses of 150 mg or 75 mg Canthaxanthin (as water dispersible Canthaxanthin) were administered orally to fasted volunteers (10 per group) as a Canthaxanthin/milk/cream mixture in a total volume of 660 ml. The authors of this study reported that the absorption of Canthaxanthin ranged from 8 to 15% of

the administered dose. Pharmacokinetic analysis of the experimental data by the authors showed an upper limit for Canthaxanthin maximum absorption of around 20% of the initial dose after intake of very low doses of Canthaxanthin. The same authors report that following uptake by the liver, Canthaxanthin is released into the circulation where approximately 48% is found in the plasma, associated with VLDL, LDL and HDL fractions, and 52% is transferred from chylomicrons to fat tissues. Remobilization from fat was described as very slow and the half-life estimated to be approximately 5 days (EFSA 2010, reported as Cohn W and Schalch W, 1990. The biokinetic properties of canthaxanthin. Unpublished Research Report No. B-156'905. F. Hoffmann-La Roche & Co., Basle, Switzerland).

Reference	Pateau et al. (1997)
Type	Plasmakinetics in human volunteers after single dosing
Guideline	Not applicable
GLP	No
Test substance	Canthaxanthin 10% water dispersible beadlets (Hoffmann-La Roche)
Species / sex	Healthy premenopausal women (age 20-36 years) / female
Number animals	9
Doses males	Not applicable
Doses females	25 mg Canthaxanthin 25 mg Canthaxanthin + 25 mg beta-Carotene
Route of administration	Oral

Plasma kinetic of Canthaxanthin was assessed in 9 healthy, non-smoking, premenopausal women (age: 20-36 years), each of which ingested 25 mg beta-Carotene, 25 mg Canthaxanthin, and beta-Carotene plus Canthaxanthin (25 mg each) in a random order; doses were separated by 10 week washout periods. One day before until four days after administration, subjects were instructed to consume a carotenoid-low diet. Blood samples were obtained in hourly intervals for 12 h and at 1, 2, 3, 4, 8, 15, and 22 days after administration. Blood samples from five subjects were used for lipoprotein fractionation into chylomicrons, 3 VLDL subfractions, IDL, and LDL. Carotenoids were quantified in plasma and plasma lipoprotein fractions by HPLC-UV/Vis.

The plasma appearance of Canthaxanthin was monophasic, with a rapid increase to the 12h measurement ($1.41 \pm 0.11 \mu\text{mol/L}$) followed by a steady decrease thereafter to concentrations near baseline at 15 days postdosing ($0.03 \pm 0.01 \mu\text{mol/L}$). Mean Canthaxanthin content in plasma lipoproteins peaked 6h postdosing in the chylomicron and VLDL_A fractions (0.17 ± 0.03 and $0.21 \pm 0.04 \mu\text{mol/L}$, respectively) and 8h postdosing in the VLDL_B and VLDL_C fractions (0.09 ± 0.02 and $0.10 \pm 0.03 \mu\text{mol/L}$, respectively). Canthaxanthin content in LDL was detected 2h postdosing and increased steadily; at 10h postdosing, Canthaxanthin content in LDL was $0.45 \pm 0.08 \mu\text{mol/L}$. Ingestion of a combined dose of beta-Carotene and Canthaxanthin significantly reduced the plasma Canthaxanthin AUC as well as the appearance of Canthaxanthin in chylomicrons and the VLDL subfractions; incorporation into IDL and LDL was not affected.

Canthaxanthin plasma concentrations increased in the first 12h after administration; 24h postdosing Canthaxanthin elimination was already ongoing. Canthaxanthin was found in all lipoprotein subfractions but is mainly incorporated into LDL.

Elmadfa and Majchrzak (1999) have assessed absorption and excretion of Canthaxanthin and Astaxanthin in human volunteers after consumption of a single portion of salmon. A significant increase of plasma Canthaxanthin level was observed already 6 h after consumption. Maximum plasma concentration was reached 10 h after uptake. Excretion of Canthaxanthin in faeces was approximately 25% of the administered dose over a period of 3 days.

In a study with human female volunteers, Riedl et al (1999) showed that bioavailability of Canthaxanthin (determined as plasma concentration) is not significantly affected by concomitant ingestion of dietary fiber.

4.3.3 Acute Toxicity

4.3.3.1 Acute Oral Toxicity

JECFA (1990), as well as ILSI (1999) and EFSA (2010) report that acute oral toxicity of Canthaxanthin is very low: the LD₅₀ in mice exceeds 10'000 mg/kg bw (JECFA 1990, EFSA 2010, reported as Hoffmann-La Roche, 1966. Canthaxanthin. Unpublished report submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland).

4.3.3.2 Acute Dermal Toxicity

The conduct of acute dermal toxicity studies for Canthaxanthin or Canthaxanthin-containing preparations was not considered necessary in the light of the high oral LD₅₀ in the mouse being greater than 10'000 mg/kg bw (EFSA 2010, ILSI 1999).

4.3.3.3 Acute Inhalation Toxicity

The conduct of acute inhalation toxicity studies for Canthaxanthin or Canthaxanthin-containing preparations was not considered necessary. Particle size distribution measurement of a Canthaxanthin preparation (CAROPHYLL® Red 10%) revealed that all particles have a size of 100 µm or greater and are therefore not deemed to be respirable (EFSA 2013).

4.3.4 Irritation

4.3.4.1 Summary

Canthaxanthin is neither a skin nor an eye irritant and showed no skin sensitizing potential.

4.3.4.2 Eye Irritation

Based on a skin irritation study submitted to EFSA, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) concluded that Canthaxanthin is considered to be not irritating to the eye (EFSA 2013):

Reference	EFSA (2013)
Type	Eye Irritation Study in Rabbits
Guideline	OECD 405
GLP	Yes
Test substance	Canthaxanthin 93.2% all-trans Canthaxanthin
Species / sex	NZW Rabbits / both sexes

The applicant submitted one study that conformed to OECD Test Guideline 405 and to GLP. Canthaxanthin caused no irritation when instilled into the conjunctival sac of rabbit eyes.

4.3.4.3 Skin Irritation

Based on a skin irritation study which has been submitted to EFSA, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) concluded that Canthaxanthin is considered to be no skin irritant (EFSA 2013):

Reference	EFSA (2013)
Type	Skin irritation study in rabbits
Guideline	OECD 404
GLP	Yes
Test substance	Canthaxanthin 93.2% all-trans Canthaxanthin
Species / sex	NZW Rabbits / both sexes

The applicant submitted one study that that conformed to OECD Test Guideline 404 and to good laboratory practice (GLP). Canthaxanthin caused no primary irritation of rabbit skin when applied for 4 hours under a semioclusive dressing.

4.3.4.4 Skin Sensitisation

Reference	EFSA (2013), JECFA (1996), reported as: Geleick, H, & Klecak, G. (1983). Determination of the sensitizing potential of canthaxanthin crystalline In guinea pigs by the Optimization Test (Maurer). Unpublished research report no. 104'987 submitted to WHO by F. Hoffmann-La Roche & Co., Basel, Switzerland.
Type	Skin sensitization
Guideline	Comparable to OECD 406 (GMPT)
GLP	No, pre-GLP study
Test substance	Canthaxanthin crystalline
Species / sex	Guinea pig / female

Canthaxanthin did not show sensitizing effects in the guinea pig optimization test.

Additionally, EFSA (2013) mentions two reports of observations in workers from two different plants: no cases of irritation or hypersensitivity following exposure to canthaxanthin during production were observed.

A recent EFSA evaluation found Canthaxanthin unlikely to be a skin sensitizer (EFSA 2013): Considering (i) the absence of adverse findings in the Maurer optimization test, (ii) the absence of observations of adverse effects in workers and (iii) the conclusion of the ANS Panel (EFSA 2010) that Canthaxanthin had caused no biologically significant adverse effects in studies on its potential for allergenicity, hypersensitivity and intolerance, the EFSA FEEDAP Panel considers it unlikely that exposure to Canthaxanthin as a result of the use of CAROPHYLL® Red 10 % (a beadlet formulation containing 10% Canthaxanthin) would cause skin sensitisation.

4.3.5 Repeated Dose Toxicity

4.3.5.1 Summary

Toxicity of Canthaxanthin was studied in different laboratory animal species: dog, rat, mouse, and monkey.

In all species, consistently red discoloration of faeces was noted in most cases accompanied by discoloration of fur and skin. Macroscopically, yellow / orange / red staining of adipose tissue was observed and in almost all species also liver (rat, mouse, monkey), pancreas (rat, mouse) as well as gastrointestinal tract were discolored.

In rats, the major target organ was the liver. Liver toxicity was manifested by clinical chemistry parameters (increased cholesterol and liver enzyme levels) as well as increased liver weights. Histologically, hepatocyte vacuolation, centrilobular hypertrophy, reduced centrilobular glycogen, increased periportal lipid content, iron-positive content of parenchymal and interstitial cells, and pigment deposition in hepatocytes and periportal macrophages were noted. Liver findings increased in incidence and / or severity with increasing duration of exposure, and / or were observed also at lower dose levels with increasing duration i.e. increasing age of the animals. Females were more affected from mild hepatotoxicity when compared to males. Liver toxicity was reversible in low to mid doses showed evidence of reversibility at high doses. Reversibility of observed liver toxicity gives evidence that the dose-related hepatocellular findings are more an adaptive response to non-physiological conditions and not a toxic manifestation. Furthermore, older animals exhibit diminished capacity of metabolism with age. It is therefore not unexpected that a lipophilic overload may affect overload of biological elimination pathways leading to the observed accumulation in hepatocytes and macrophages and fatty change/vacuolation. Overall, the NOAEL in female rats based on liver toxicity was 5 mg/kg bw/day; for males 25 mg/kg bw/day.

In dogs, histologically, no adverse findings were noted up to and including a dose level of 500 mg/kg bw/day. Indications of Canthaxanthin particulate matter in the reticuloendothelial system in the spleen and to lesser extent in liver were noted. In mice and monkey, also non-adverse pigment inclusions were noted in sinusoidal liver cells, liver macrophages, and hepatocytes.

Conventional ophthalmoscopy did not show any effects on eyes in standard laboratory animal (rats, mice, dogs, and monkey). However, in monkey, birefringent inclusions in the retina and

macula were noted using special investigative methods. These findings were, however, without any adverse effect on visual performance. A threshold of 0.6 mg Canthaxanthin/kg bw/day could be established for Canthaxanthin crystalline deposits in the retina of monkey. The NOEL was 0.2 mg/kg bw/day.

4.3.5.2 Studies in mice

Reference	EFSA (2010) Reported as: Steiger A and Hummler H, 1981. Tolerance study with Ro 01-9915 (Canthaxanthene, beadlets 10% water soluble) administered orally to mice over 13 weeks. Unpublished Report No. RCR B-87 284 submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland.
Type	Dose-range finder
Guideline	Comparable to OECD 408, clinical chemistry and hematology were not performed.
GLP	Yes
Test substance	Canthaxanthin, 10% water-soluble beadlets Placebo powder without Canthaxanthin
Species / sex	Mouse / both sexes
Strain	Albino-SPF, outbred
Route of administration	Feed admix
Period of administration	90 days
Frequency of administration	Daily
Post-exposure period	No
Doses males	10 animals / dose Target: 0 (negative control), 0 (placebo control 1), 0 (placebo control 2), 125, 250, 500, 1000, and 2000 mg Canthaxanthin/kg bw/day
Doses females	10 animals / dose Target: 0 (negative control), 0 (placebo control 1), 0 (placebo control 2), 125, 250, 500, 1000, and 2000 mg Canthaxanthin/kg bw/day
Control group	Yes (negative and placebo)
NOAEL	2000 mg/kg bw/day
LOAEL	> 2000 mg/kg bw/day

Canthaxanthin was fed to albino outbred mice (10 animals/sex/group) at doses of 0, 125, 250, 500, or 1000 mg/kg bw/day in the first experimental series and 0 or 2000 mg/kg bw/day in the second experimental series for 13 weeks. Water dispersible Canthaxanthin was incorporated in beadlets containing 11.2% pure substance (food grade) that were added to a control diet. Control animals received either a control diet added placebo beadlets at the same proportion as treated animals (placebo controls) or a control diet.

In the first experimental series significantly lower body weights as compared to the placebo controls were recorded for males receiving doses of 125 mg/kg bw/day from week 2-12 ($p < 0.05$) and 13 ($p < 0.01$) and of 1000 mg/kg bw/day in weeks 3, 6, 7, 9, ($p < 0.05$) and 2, 8, 10, 12 and 13 ($p < 0.01$), and for females receiving 1000 mg/kg bw/day only in week 10 ($p < 0.05$). In the second series the significantly lower body weights as compared to the placebo controls were recorded for 2000 mg/kg bw/day group males in weeks 2, 3 and 13 ($p < 0.05$), and for females in weeks 1, 4, 7-12 ($p < 0.05$) and 2, 3, 5 ($p < 0.01$). In all dosage groups a red discoloration of the faeces was reported. At necropsy the only difference to the placebo controls was the orange to yellow discoloration of livers, fat and pancreas in the Canthaxanthin-treated groups. Lower absolute

weights were noted for some organs in the two highest dose groups compared to the concurrent placebo controls. The observed lower absolute organ weights are considered secondary due to lower body weights. Microscopic examination of the organs from the two highest dose groups did not reveal any compound-related pathological alterations.

Based on the observations, the maximum dose level for a long-term study in mice was considered to be 1000 mg/kg bw/day because the feed inclusion level of in long-term studies should not exceed 10%.

4.3.5.3 Studies in rats

Reference	EFSA (2010) Reported as: Steiger A and Buser S, 1982. Tolerance study with Ro 01-9915 (Canthaxanthene, beadlets 10% water soluble) administered orally as food admixture to rats over 13 weeks. Unpublished Report No. B-87 283 submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland.
Type	Dose-range finder
Guideline	Comparable to OECD 408
GLP	Yes
Test substance	Canthaxanthin, 10% water-soluble Placebo powder Canthaxanthin
Species / sex	Rats, both sexes
Strain	Albino-SPF, outbred
Route of administration	Feed admix
Period of administration	90 days
Frequency of administration	Once per day
Post-exposure period	No
Doses males	10 animals / dose Target: 0 (negative control), 0 (placebo control 1), 0 (placebo control 2), 125, 250, 500, 1000, and 2000 mg Canthaxanthin/kg bw/day
Doses females	10 animals / dose Target: 0 (negative control), 0 (placebo control 1), 0 (placebo control 2), 125, 250, 500, 1000, and 2000 mg Canthaxanthin/kg bw/day
Control group	Yes (negative and placebo control)
NOAEL	2000 mg/kg bw/day
LOAEL	> 2000 mg/kg bw/day

Canthaxanthin was fed to albino outbred rats (10 animals/sex/group) at doses of 0, 125, 250, 500, 1000 mg/kg bw/day in the first experimental series and 0 or 2000 mg/kg bw/day in the second experimental series for 13 weeks. Water dispersible Canthaxanthin was incorporated in beadlets containing about 11% pure substance (food grade) that were added to the control diet. Control animals received either a control diet added placebo beadlets at the same proportion as treated animals (placebo controls) or a control diet.

In all dosage groups a red discoloration of the faeces was observed. The body weight of males and females from all treated groups in the first series and of males in the second series was not statistically significantly different from the placebo controls. The body weight of females in the second series was significantly lower than the placebo controls in weeks 1 ($p < 0.05$), 3 ($p < 0.01$)

and 13 ($p < 0.05$). The plasma cholesterol in all treated groups of both sexes in both series was significantly higher than in the placebo controls, except for 500 mg/kg bw/day female group, but the values were within the physiological ranges for the species. All other clinical chemistry parameters (plasma aspartate transaminase, alanine aminotransferase, glutamate dehydrogenase, urea, bilirubin, glucose) and hematological parameters were unaffected by the treatment. The only finding at necropsy was a red or orange discoloration of liver, fat, pancreas, intestines and faeces. In males, a statistically significantly lower absolute kidney weight in the 2000 mg/kg bw/day group and a statistically significantly lower adrenal weight in the 500 mg/kg bw/day group compared to the placebo controls were recorded. In female groups, absolute adrenal weights at dose level from 250 to 2000 mg/kg bw/day were statistically significantly reduced in a dose-dependent manner compared to the placebo controls. The absolute liver weight of 2000 mg/kg bw/day females was significantly lower compared to the placebo controls (6.9 g versus 7.4 g, $p < 0.05$) but the absolute liver weights in all other treated groups of both sexes in the first series and of males in the second series were not statistically significantly different from the placebo controls. Microscopic examination did not reveal any treatment related lesions.

Based on the observations, the maximum dose level for a long-term study in rats was considered to be 1000 mg/kg bw/day because the feed inclusion level of in long-term studies should not exceed 10%.

Supporting studies

Kumar et al. (2011 and 2012) have conducted 28 day studies in rats, feeding them partially saturated Canthaxanthin (PSC, 11,11',12,12',13,13',14,14',15,15'-decahydro-Canthaxanthin) purified from *Aspergillus carbonarius*.

In the first study (Kumar et al. 2011) PSC was mixed into rat feed at concentrations of approximately 5, 10, and 25 mg/kg bw/d. Feed was offered ad libitum. None of the animals showed adverse effects throughout the study except reduced food uptake and consequently no body weight gain of the treatment groups. Liver enzymes (ALT, AST, AP, CK) remained unchanged.

In the second study (Kumar et al. 2012), doses of approximately 50, 100, and 250 mg/kg bw/d in feed were administered; a vehicle control group was treated in parallel with the vehicle (ground nut oil). Administration of PSC to rats over a period of 28 days resulted in no deaths. Neither signs of toxicity nor treatment-related changes in feed consumption, body weight, body weight gain, hematological parameters or clinical biochemistry were noted. Gross necropsy and histopathology revealed no differences between treatment groups and the control animals. In a concurrent pharmacokinetic study, 5, 10, and 25 mg/kg bw of PSC were administered to rats by gavage followed by blood collection every three hours over a period of 48 h. PSC was found in blood samples already at 3 h after dosing; T_{max} was found at the 3h dosing point for the 5 mg/kg bw dose and at the 6h sampling point for the other doses (10 and 25 mg/kg bw). Taken together, partially saturated Canthaxanthin, when administered to rats in feed over a period of 28 days at a dose of up to 250 mg/kg bw/d, resulted in no adverse effects.

4.3.5.4 Studies in dogs

Reference	EFSA (2010) Reported as: Hoffmann-La Roche, 1966. Canthaxanthin. Unpublished report submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland
Type	15-Week study
Guidelines	Pre OECD-Guideline study
GLP	No, Pre-GLP study
Test substance	Canthaxanthin crystalline
Species / sex	Dog / both sexes
Strain	Beagle
Route of administration	Oral, gelatine capsule
Period of administration	15 weeks
Frequency of administration	Daily
Post-exposure period	No
Doses males	0, 100, 400 mg Canthaxanthin/kg bw/day / 3 animals per group
Doses females	0, 100, 400 mg Canthaxanthin/kg bw/day / 3 animals per group
Control group	Yes
NOAEL	400 mg/kg bw/day
LOAEL	> 400 mg/kg bw/day

In a further study on dogs eighteen dogs (3 male and 3 female animals/group) were exposed to daily oral doses of 0, 100, or 400 mg/kg bw Canthaxanthin in gelatine capsules for 15 weeks. All parameters recorded in the treated animals (growth, pathology, haematology, hepatic and renal function, histopathology) were within normal limits or comparable to those of the control group.

Reference	EFSA (2010) Reported as: Chesterman H, Fox PA, Heywood R, Street AE and Prentice DE, 1979. Canthaxanthin preliminary toxicity study in beagle dogs. Unpublished Report No. HLR76/79686 of Huntingdon Research Centre submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland. Buser S and Hummler H, 1980. Tolerance study with Ro 01-9915 (Canthaxanthine) beadlets, 10% water soluble) administered orally as a food admixture to dogs over 13 weeks. Unpublished report.
Type	Dose-range finder
Guidelines	Dose-range finder; description of materials and methods see below
GLP	Pre-GLP study
Test substance	Canthaxanthin powder Placebo powder without Canthaxanthin
Species / sex	Dog, both sexes
Strain	Beagle dog
Route of administration	Feed admix
Period of administration	90 days
Frequency of administration	Once per day
Post-exposure period	No
Doses males	1 animal / dose 0 (placebo control), 250, and 500 mg Canthaxanthin /kg bw/day
Doses females	1 animal / dose 0 (placebo control), 250, and 500 mg Canthaxanthin /kg bw/day
Control group	Yes (placebo control)
NOAEL	500 mg/kg bw/day
LOAEL	> 500 mg/kg bw/day

In a further study, Canthaxanthin (incorporated as a water dispersible formulation in beadlets) fed to Beagle dogs (3 animals/sex/group) at dose levels of 0, 250 or 500 mg/kg bw/day for 13 weeks, showed no treatment-related effects on food and water intake, body weight gain or organ weights. Apart from red/orange staining of the feet, muzzle, abdominal fat and faeces, there were no abnormalities related to the test compound on clinical, ophthalmoscopic, and histological parameters.

The No-Observed-Adverse-Effect-Level (NOAEL) was considered to be 500 mg/kg bw/day, the highest dose tested.

Reference	EFSA (2010), reported as: Harling RJ, Burford P, Heywood R, Street AE, Chanter DO, Read R and Gopintah C, 1987. Canthaxanthin toxicity to dogs by repeated dietary administration for 52 weeks. Unpublished Report No. HLR137/85682 of Huntingdon Research Centre Ltd., submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland.
Type	One-year study
Guidelines	Not indicated, materials and methods are comparable to OECD 409
GLP	Yes
Test substance	Canthaxanthin 10% water-soluble beadlets Placebo beadlets containing 0% Canthaxanthin
Species / sex	Dog, both sexes
Strain	Beagle dog
Route of administration	Feed admix
Period of administration	1-year
Frequency of administration	Once per day
Post-exposure period	No
Doses males	4 animals / dose 0 (negative control), 0 (placebo control), 50, 100, and 250 mg Canthaxanthin /kg bw/day
Doses females	4 animals / dose 0 (negative control), 0 (placebo control), 50, 100, and 250 mg Canthaxanthin /kg bw/day
Control group	Yes (negative and placebo control)
NOAEL	250 mg/kg bw/day
LOAEL	>250 mg/kg bw/day

In another study, beagle dogs (4 animals/sex/group) were given Canthaxanthin (in beadlets containing water dispersible Canthaxanthin in the diet at doses of 0, 0 (placebo), 50, 100 or 250 mg/kg bw/day for 52 weeks.

No deaths occurred during the study period. No signs of systemic toxicity and no adverse effects of treatment on food intake and body weights were observed. No dose-related adverse effects were found by ophthalmoscopy, in hematological and urinalysis parameters. Mean values for the clinical chemistry parameters in the treated groups showed occasional statistically significant differences from placebo controls but these were not systemic or dose-related and were generally within physiological limits. At necropsy reddish discoloration was observed in fur and hair, and in some other tissues. Absolute and relative organ weights were not statistically significantly different between the groups. Histopathological examination did not reveal any treatment related lesions. Moderate amounts of dark pigment were seen in midzonal hepatocytes and in some Kupffer cells of one female from the 50 mg/kg bw/day group and one

male from the 100 mg/kg bw/day group but no such pigmentation was seen in all other dogs receiving 50, 100 or 250 mg/kg bw/day Canthaxanthin or in any control animal.

It can be concluded that prolonged feeding of a diet containing Canthaxanthin up to a dose of 250 mg/kg bw/day to Beagle dogs was well tolerated and there were no signs related to an adverse effect of the test substance.

4.3.5.5 Studies in monkeys

Reference	Goralczyk et al. (2000) and Goralczyk et al. (1997) EFSA (2010), reported as: Buser S, Goralczyk R, Bausch J and Schüep W, 1993. Canthaxanthin (Ro 01-9915) in a long-term study with Cynomolgus monkeys (oral gavage); 3-year interim report. Unpublished research report No. B-161'152 submitted to WHO by F. Hoffmann-La Roche & Co., Basel, Switzerland. Buser S, Goralczyk R, Bausch J and Schüep W, 1994. Canthaxanthin (RO 01-9915) in a long-term study with Cynomolgus monkeys (oral gavage); 3-year final report. Unpublished research report no. B-161'152 submitted to WHO by F. Hoffmann-La Roche & Co., Basel, Switzerland.
Type	Long-term study with interim kills
Guideline	Not indicated, Materials and methods are described below
GLP	Yes
Test substance	Test article 1: Canthaxanthin 10% WS Control article 1: Canthaxanthin placebo beadlets Test article 2: Canthaxanthin 30% suspension in rape seed oil Control article 2 (vehicle control group): Rape seed oil Test article 1 and control article 1 were suspended in distilled water and used for application of the following doses: 0 (placebo), 0.2, 0.6, 1.8, 5.4, 16.2, and 48.6 mg/kg bw/day. Test article 2 was used for application of 0, 200 and 500 mg/kg bw/day
Species / sex	Monkey / both sexes
Strain	Cynomolgus monkey
Route of administration	Gavage
Period of administration	0 - 48.6 mg/kg bw/d 155-156 weeks with interim kills at weeks 35-36, 54, 83, 136-137. Doses of 200 and 500 mg/kg bw/d were administered for 4.5 years as a 30% oily suspension. Thereafter the formulation was changed to 48.6 mg/kg bw/d beadlets for the remaining 6 months.
Frequency of administration	Once daily
Post-exposure period	No
Doses males	0, 0.2, 0.6, 1.8, 5.4, 16.2, 48.6, 200, 500 mg Canthaxanthin/kg bw/day
Doses females	0, 0.2, 0.6, 1.8, 5.4, 16.2, 48.6, 200, 500mg Canthaxanthin/kg bw/day
Control group	Yes
NOAEL	Systemic: 500 mg/kg bw/day Birefringent inclusions in the eye: 0.2 mg/kg bw/day
LOAEL	Systemic: > 500 mg/kg bw/day Birefringent inclusions in the eye: 0.6 mg/kg bw/day

Groups of four cynomolgus monkeys (*Macaca fascicularis*) per gender and dose were administered 0.2, 0.6, 1.8, 5.4, 16.2, and 48.6 mg Canthaxanthin /kg bw/day for 2.5 to 3 years. A second group of monkeys were administered 200 and 500 mg/kg bw/d for 5 years. Control

animals receiving placebo were treated in parallel. In vivo ophthalmoscopy was performed at intervals of 3 months along with electroretinography after 12 and 24 months and retinal biomicroscopy just before the monkeys were killed. Retinal wholemounts or frozen sections were investigated postmortem by polarization, bright field, and differential interference contrast microscopy. Retinal and preterminal plasma Canthaxanthin concentrations were determined by HPLC.

By ophthalmoscopy and retinal biomicroscopy in vivo, crystals or other light-reflecting particles were observed in the central paramacular retina only in the extreme high doses of 200 to 500 mg/kg bw/d but not at 48.6 mg/kg bw/d and lower. By postmortem polarization microscopy, crystals in the peripheral retina and/or in the macula were detected in all Canthaxanthin treated groups except at the lowest dose of 0.2 mg/kg bw/d. The grade of crystals increased up to a dose of 16.2 mg/kg bw/d. The inclusions were located mainly in the inner retinal layers, that is, the nerve fiber layer and ganglion cell layer, inner plexiform layer, and inner nuclear layer. Canthaxanthin inclusions in the retina of monkey eye are clearly different from those reported in humans regarding the variability of crystal density and size as a function of retinal location. Crystal sizes were between 4 - 25 µm in humans compared to 2.5 - 10 µm in monkey which might explain the invisibility of the inclusions by standard ophthalmoscopy. Dose-dependent increases in Canthaxanthin levels also were noted in the liver and in plasma. Retinas of placebo-treated monkeys were free of birefringent, crystal-like inclusions. The HPLC confirmed the presence of all-trans-Canthaxanthin, 4-OH-echinenone and isozeaxanthin in the retinas of all Canthaxanthin-treated animals. In the peripheral and paracentral retina of very highly dosed animals all-trans Canthaxanthin was the major compound, where its concentration correlated largely with the grade of inclusions. In the macula, 4'-OH-echinenone was the dominant Canthaxanthin metabolite. Neither electroretinography (ERG) nor histopathology indicated any adverse effects on visual functions.

It is concluded that prolonged treatment with Canthaxanthin for up to 2.5 or 5 years was well tolerated by Cynomolgus monkeys, even at very high doses which exceed intakes from food in humans. On the basis of results obtained from this study, Canthaxanthin did not induce any adverse effects at dose levels from 0.2 - 500 mg/kg bw/d. Clinical and post-mortem observations represent expected effects with a carotenoid such as the discoloration of the faeces, or the dose-related coloration of the digestive tract and mainly organs and tissues containing lipids.

A high intake of Canthaxanthin for up to 5 years led to the deposition of crystal-like birefringent inclusions in the inner layers of the peripheral retina and, to some extent, the central retina of cynomolgus monkeys. The grade of crystals in monkey retinas was dose dependent with a threshold level at 0.6 mg Canthaxanthin/kg bw/d. It correlated in the retinal periphery with the concentrations of all-trans-Canthaxanthin and in the macula with its metabolites. The presence of these deposits did neither interfere with morphology nor with retinal function.

The NOAEL for systemic toxicity was considered to be the highest dose of 500 mg/kg bw/day. The NOEL for eye deposits is 0.2 mg/kg bw/day. However, it is important to note that the observed deposits had no influence on visual performance.

4.3.6 Genotoxicity

4.3.6.1 Summary

There was no evidence of genotoxicity or mutagenicity in a comprehensive battery of studies with or without metabolic activation.

4.3.6.2 Studies

EFSA (2010) and JECFA (1990) concluded that the available data do not raise concern with respect to the genotoxic potential of Canthaxanthin. Also ILSI (1999) judged that Canthaxanthin was found not to be genotoxic.

Reference	JECFA (1990) and EFSA (2010) reported as: Chételat A, 1981. Mutagenicity evaluation of Ro 01-9915 (Canthaxanthin) in the Ames Salmonella/mammalian microsome plate test. Unpublished Report No. B-95 575 submitted to WHO by F. Hoffmann La-Roche & Co., Basle, Switzerland.
Type	Reverse mutation in bacteria (<i>Salmonella typhimurium</i> TA 1535, TA 1537, TA 1538, TA 98 and TA 100)
Guideline	Materials and methods described are comparable to OECD 471, except that no concurrent positive control for the experiments without metabolic activation was described.
GLP	Yes, QUA statement is included
Test substance	Canthaxanthin 10% in water-soluble beadlets
Concentration	0, 0.25, 0.625, 1.25, 2.5 mg/plate
Metabolic activation	Yes, rat liver S9 mix (phenobarbitone induced)

Water soluble beadlets containing nominal 10% Canthaxanthin did not induce mutations in *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537, TA 1538.

Reference	EFSA (2014)
Type	Reverse mutation in bacteria (<i>Salmonella typhimurium</i> TA 1535, TA 1537, TA 98, TA 100 and <i>Escherichia Coli</i> WP2uvrA)
Guideline	Compliant to OECD 471
GLP	Yes
Test substance	Canthaxanthin Crystalline
Concentration	3, 10, 33, 100, 333, 1000, 3333, 5000 µg/plate
Metabolic activation	Yes, 5 and 10% rat liver S9 mix (phenobarbital / β-naphthoflavone induced)

Crystalline Canthaxanthin (purity 97.1 %) was tested in the reverse mutation assay in *Salmonella typhimurium* (strains TA1535, TA1537, TA98 and TA100) and in *Escherichia coli* (strain WP2uvrA). The test was performed in two independent experiments in the presence and absence of S9-mix (rat liver S9-mix induced by a combination of phenobarbital and β-naphthoflavone), in compliance with OECD guideline 471 (rev 1997). Results at 5000 µg/plate were not reported due to precipitation. The test substance did not induce a significant dose-

DSM1493-001



related increase in the number of revertants at a concentration range of 100 to 3330 µg/plate both in the absence and presence of metabolic activation. The negative and strain-specific positive controls performed as expected.

Reference	JECFA (1990) and EFSA (2010) Reported as: Strobel R, 1986. Gene mutation assay in mammalian cells with dry canthaxanthin Type 10 CWS, Ro 01-9915/063 (V79/HGPRT Test). Unpublished Research Report No. B-153'076 submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland.
Type	Forward Mutation in V 79 cells, HPRT Assay
Guideline	Materials and Methods described are comparable to OECD 476
GLP	Yes, QUA statement is included
Test substance	Canthaxanthin water soluble beadlets, containing 10% Canthaxanthin
Concentration	Without metabolic activation: 1 st experiment: 50, 250, 400, 500 µg beadlets /mL 2 nd experiment: 350, 400, 500 µg beadlets /mL In both experiments placebo control was used With metabolic activation: 1 st experiment: 50, 250, 400, 500 µg beadlets /mL 2 nd experiment: 50, 250, 400, 500 µg beadlets /mL In both experiments placebo control was used
Metabolic activation	With and without rat liver S9 (Arocolor 1254 induced)

Canthaxanthin did not induce mutations to 6-thioguanine-resistance in V79 Chinese hamster lung cells in the presence of metabolic activation.

Reference	JECFA (1990) and EFSA (2010) reported as: Chetelat, A. (1986). Mutagenicity evaluation of Ro 01-9915 (canthaxanthin) with <i>Saccharomyces cerevisiae</i> D7. Unpublished Research Report No. B-105'448 submitted to WHO by F. Hoffmann-La Roche, & Co., Basle, Switzerland.
Type	Mutagenicity in Yeast cells
Guideline	Materials and methods described are comparable to OECD 480
GLP	Yes, QUA statement is included
Test substance	Canthaxanthin
Concentration	1 st experiment (stationary phase): 1.6, 8, 40, 200, 1000 µg test substance / mL 2 nd and 3 rd experiment (logarithmic growth phase): 0, 10, 33.33, 100, 333.33, 1000 µg test substance / mL 4 th experiment (stationary phase): 200, 333.33, 500, 1000, 2000 µg test substance / mL
Metabolic activation	Yes, rat liver S9 mix (phenobarbitone / beta-naphthoflavone)

Canthaxanthin did not induce mutations in *Saccharomyces cerevisiae*.

DSM1493-001



Reference	EFSA (2010) and JECFA (1990) Reported as: Strobel, R. (1986). Gene mutation assay in mammalian cells with dry canthaxanthin Type 10 CWS, Ro 01-9915/063 (V79/HGPRT Test). Unpublished Research Report No. B-153'076 submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland.
Type	UDS in vitro
Guideline	Materials and methods are comparable to OECD 486
GLP	Yes, QUA statement is included
Test substance	Canthaxanthin water soluble beadlets containing 10% Canthaxanthin
Concentration	1 st experiment: 5, 25, 125, 200, 250 µg beadlets / mL 2 nd experiment: 2.5, 12.5, 62.5, 100, 125 µg beadlets /mL 3 rd and 4 th experiment: 375 and 500 µg beadlets / mL In both experiments placebo control was used. Concentration of placebo corresponded to the highest concentration of beadlets tested in each experiment.
Metabolic activation	Not applicable, primary hepatocytes

Canthaxanthin did not induce DNA damage resulting in unscheduled DNA synthesis in primary cultures of rat hepatocytes.

Reference	EFSA (2014)
Type	in vitro Micronucleus Assay in cultured peripheral human lymphocytes
Guideline	Compliant to OECD 487
GLP	Yes
Test substance / Batch	Canthaxanthin Crystalline
Concentration	3, 10, 33 µg/mL (3h exposure ±S9 mix) 1, 3, 10 µg/mL (24h exposure -S9 mix)
Metabolic activation	Yes, rat liver S9 mix (phenobarbital / β-naphthoflavone induced)

The possible clastogenicity and aneugenicity of crystalline canthaxanthin (purity 97.1 %) was tested in an in vitro micronucleus assay in cultured peripheral human lymphocytes. The substance was tested in the presence and absence of a metabolic activation system (phenobarbital and β-naphthoflavone induced rat liver S9-mix) in two independent experiments, according to OECD guideline 487. In the first experiment, canthaxanthin was tested up to 33 µg/mL for a 3 hours exposure time with a 27 hours harvest time in the absence and presence of S9-fraction. In the second experiment, the substance tested up to 10 µg/mL for a 24 hours exposure time with a 24 hours harvest time in the absence of S9-mix. A precipitate was observed in the culture medium at 33 and (slightly) at 10 µg/mL. The test item did not induce a statistically significant or biologically relevant increase in the number of mono- and binucleated cells with micronuclei in the absence and presence of S9-mix, in either of the two independently repeated experiments, while the positive control chemicals induced micronuclei as expected.

Reference	EFSA (2010) and JECFA (1990) Reported as: Gallandre F, 1980. Mutagenicity studies with canthaxanthin in mammalian systems. Unpublished report submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland.
Type	In vivo MNT in the bone marrow of the mouse
Method	Materials and Methods described are comparable to OECD 474, except the group size consists of 3 instead of 5 animals.
GLP	Pre-GLP study, QUA statement is included
Test substance	Canthaxanthin 10% in beadlet formulation
Species / sex	Mouse / both sexes
Strain	Füllinsdorf Albino, SPF quality
Route of administration	Oral by gavage
Period of administration	Two doses, 30 and 6 hours prior to sacrifice
Doses male	0, 500, 1000, 2000 mg test substance (beadlets) /kg bw Equivalent to 0, 55.5, 111, 222 mg Canthaxanthin /kg bw
Doses female	0, 500, 1000, 2000 mg test substance (beadlets)/kg bw Equivalent to 0, 55.5, 111, 222 mg Canthaxanthin /kg bw

Canthaxanthin was negative in a mouse micronucleus assay in which it was administered orally 30 and 6 hours before sacrifice at doses of 0, 55.5, 111 and 222 mg/kg bw.

De Flora et al. (1999) have reviewed several hundred results of different genotoxicity assays (including Ames test, umu assay, *in vivo* micronucleus assay, *in vivo* detection of DNA strand breaks) testing either the carotenoids themselves or the modulation of genotoxic effects induced by known mutagens. The available data support the view that carotenoids, including Canthaxanthin, do not induce genotoxic effects.

Azuine et al. (1992) have studied both, mutagenic and anti-mutagenic effects of Canthaxanthin. They tested the mutagenicity of Canthaxanthin in *S. typhimurium* tester strains TA 98 and TA 100 with and without metabolic activation (Aroclor 1254-induced rat liver S9-mix). No significant increase in revertants was observed in any of the strains, be it with or without metabolic activation. After treatment of TA100 with the known mutagen N-Methyl-N9-nitro-N-nitrosoguanidine (MNNG), revertant numbers significantly decreased after co-incubation with Canthaxanthin in a dose-dependent manner. The same effect was observed when Benzo[a]pyrene (BaP) + S9 mix was used instead of MNNG.

Santamaria et al. (1998) have studied the prevention of photomutagenicity by co-incubation of 8-methoxypsoralen (MOP) and UV light by carotenoids. Both, Canthaxanthin and β -Carotene prevented photomutagenicity in *S. typhimurium* TA102. Co-incubating Canthaxanthin with the mutagen MNNG did neither increase nor decrease mutation frequency in TA 100 and TA 1535 compared to MNNG incubation without Canthaxanthin.

Also other studies showed that carotenoids such as Canthaxanthin seem to protect cells from genotoxic agents such as X-rays (Pung et al. 1988), Aflatoxin B1 (Gradelet 1997a, He & Campbell 1990), 3-Methylcholanthrene (Bertram et al. 1991), or heterocyclic amines (Okai et al. 1996).

Other studies showed that carotenoids seem to inhibit in most cases those genotoxicants which require metabolic activation to electrophilic derivatives, suggesting that carotenoids are able to modulate phase I- and phase II-metabolism of xenobiotics.

As reported by Stich et al. (1984) and reviewed by Thomas et al. (2011) oral administration of 180 mg/week Canthaxanthin over a period of 9 weeks to members of a local Philippinean tribe chewing betel nuts did not significantly increase the micronucleus frequency in buccal mucosa cells.

4.3.7 Carcinogenicity

4.3.7.1 Summary

Carcinogenic potential of Canthaxanthin was studied in rats and mice. In both species, no test item-related carcinogenicity was observed.

Dietary treatment of male and female mice up to the limit dose of 1000 mg/kg bw/day for up to 98 weeks resulted in no toxicity or treatment-related changes in tumor incidences. Treatment-related findings were discoloration of tissues and pigment deposition in the liver (sinusoidal cells, macrophages, and hepatocytes). However, these were not considered to be adverse in the absence of tissue damage. Canthaxanthin was neither tumorigenic nor carcinogenic in mice. The NOAEL was considered to be 1000 mg/kg bw/day.

Dietary two-year studies in male and female rats resulted in a NOAEL for female rats of 5 mg/kg bw/day; for male rats a NOAEL of 25 mg/kg bw/day was obtained. The NOAELs are based on observed non-neoplastic liver toxicity (see repeated toxicity). In female rats, a slightly increased incidence in benign liver tumours being non-dose related was noted at dose levels equivalent or greater than 250 mg/kg bw/day (lowest dose level tested). However, in a second combined long-term carcinogenicity study no increased incidence in benign liver tumours was noted in female rats up to and including a dose level of 250 mg/kg bw/day. There was no indication of increased incidences in malignant tumours, neither in males nor in females up to the limit dose of 1000 mg/kg bw/day.

In contrast, Canthaxanthin was found to exert anticarcinogenic activity in several animal studies.

4.3.7.2 Studies

EFSA (2010) considered that the studies in mice and rats are negative with respect to carcinogenicity. Canthaxanthin safety has also been reviewed by Hallagan et al. (1995) and by ILSI North America (ILSI 1999). Both stated that in different studies no carcinogenic potential of Canthaxanthin could be detected.

Furthermore, Canthaxanthin was found to act anticarcinogenic in different models of carcinogenesis in laboratory animals. Cancer-preventive activity of Canthaxanthin has been reviewed by IARC (1998), Chew et al. (1999a) and Tanaka et al. (2012).

Reference	EFSA (2010) Reported as: Buser S, 1987a. Canthaxanthin potential tumourigenic and toxic effects in prolonged dietary administration to mice. Unpublished Report No. HLR135/861058 of Huntingdon Research Centre Ltd. Submitted to WHO by F. Hoffmann-La Roche & Co., Basel, Switzerland.
Type	Combined long-term / carcinogenicity study with interim kill after 12 months
Guideline	This study was in accordance with OECD "Short Term and Long Term Toxicology Group Guidelines" published in Chemical Regulations Reporter, 14 August 1981.
GLP	Yes
Test substance	Canthaxanthin 10% water soluble beadlets Placebo beadlets containing 0% Canthaxanthin
Species / sex	Mice / both sexes
Strain	CD-1
Route of administration	Feed admix ad libitum
Period of administration	Main groups (males 90 weeks, females 98 weeks) Interim sacrifice (52 weeks)
Frequency of administration	Once per day
Post-exposure period	No
Doses males	0 (negative control), 0 (placebo control), 250, 500, and 1000 mg Canthaxanthin/kg bw/day 60 animals / dose level
Doses females	0 (negative control), 0 (placebo control), 250, 500, and 1000 mg Canthaxanthin/kg bw/day 60 animals / dose level
Control group	Yes
NOAEL	1000 mg/kg bw/day
LOAEL	> 1000 mg/kg bw/day

CD-1 mice (50 animals/sex/group and 10 animals/sex in satellite groups for interim sacrifice in week 52) were given water dispersible Canthaxanthin in beadlets in the diet at doses of 0 (negative control), 0 (placebo control beadlets), 250, 500 or 1000 mg/kg bw/day for 90 weeks (males) or 98 weeks (females).

In most of animals from all groups receiving the test compound a reddish discoloration of faeces and orange staining of fur and skin were observed. The body weight gain was slightly decreased in dose related manner in all treatment groups during the study, reaching statistically significant difference to the placebo controls at the dose of 1000 mg/kg bw/day in males in the second part of the study (weeks 52-91) and in females during the first 52 weeks only. No treatment-related hematological and ophthalmoscopic abnormalities were observed. The only clinical chemistry finding was a statistically significantly higher blood cholesterol level in all treated groups of both sexes in week 52 and in all treated female groups in weeks 98 but no clear dose-relationship was apparent. The plasma cholesterol levels in treated males of all groups in week 91 were not significantly higher than in the placebo control group.

At termination, no differences apart from a generalized orange discoloration of fur/skin, subcutis, adipose tissue and gastrointestinal tract content were recorded macroscopically. There were no statistically significant differences in absolute organ weights between the treated groups and the controls. The incidence of any tumor type or the total number of tumours per group was not

statistically significantly different between the treated and the control groups. Histopathological examination revealed a dose-related higher incidence of lipid positive granules in the sinusoidal cells in the livers of all treated groups. There was also orange/brown pigment seen in sinusoidal cells and, to a lesser degree, in macrophages and some hepatocytes. Other microscopic findings in mice that had been treated with Canthaxanthin were similar to the findings in placebo controls or in untreated controls.

It was concluded that continuous feeding for the life-time of the CD-1 mice on a diet containing Canthaxanthin in doses amounting up to 1000 mg/kg bw/day showed no carcinogenic effect. Furthermore, no effect on the state of health or survival of mice was apparent, except for an increase in plasma cholesterol levels and alteration of histological picture of the liver. which were considered not adverse. The NOAEL was 1000 mg/kg bw/day.

Reference	EFSA (2010) Reported as: Hoffmann-La Roche, 1966. Canthaxanthin. Unpublished report submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland
Type	Chronic study
Guideline	Pre-OECD-guideline study
GLP	No, pre-GLP study
Test substance	Canthaxanthin
Species / sex	Rats, both sexes
Strain	Wistar
Route of administration	Feed admix
Period of administration	Almost 2 years (93 and 98 weeks)
Frequency of administration	Once per day
Post-exposure period	No
Doses males	Growth phase (first 20 weeks): 0, 325 – 700, 1250 – 2500, 3200 – 7000 mg Canthaxanthin/kg bw/day From 20 weeks onwards: 0, 325, 1250, and 3200 mg/kg bw/day 25 animals for control, low, and mid dose group 15 animals for high dose
Doses females	Growth phase (first 20 weeks): 0, 380 – 700, 1600 – 2800, 4000 – 7800 mg Canthaxanthin/kg bw/day From 20 weeks onwards: 0, 380, 1600, and 4000 mg/kg bw/day 25 animals for control, low, and mid dose group 15 animals for high dose
Control group	Yes
NOAEL	3200 and 4000 mg/kg bw/day for males and females, respectively
LOAEL	> 3200 mg/kg bw/day

Wistar rats (25 male animals and 25 female animals per group) were exposed to 0, 0.5, 2 or 5% Canthaxanthin in semi-synthetic diet for 93-98 weeks. Based on food consumption data, the exposure corresponded to 0, 325, 1250 and 3200 mg Canthaxanthin/kg bw/day.

Except for the yellow-to-orange pigmentation of the body fat and a darker color of the livers, no treatment-related effect on clinical chemistry, haematology or histopathology were reported.

The administration of up to 5% Canthaxanthin inclusion level in the diet administered over almost 2 years had no negative influence on the test animals. The NOAEL in this study was considered to be 5% (3200 and 4000 mg/kg bw/day for males and females, respectively).

Reference	EFSA (2010) Reported as: Rose PH, Crook D, Gopinath C, Gibson WA and Majeed SK, 1988. Canthaxanthin potential tumorigenic and toxic effects in prolonged dietary administration to rats. Unpublished Report No. HLR134/86980 of the Huntingdon Research Centre Ltd. Submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland. Buser S and Banken L, 1988. Canthaxanthin (Ro 01-9915) in a combined tumourigenicity/toxicity study in rats. Histopathological re-evaluation and statistical analysis. Unpublished Research Report No. B-119'973 submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland.
Type	Combined long-term / carcinogenicity study with interim kill after 12 months
Guideline	This study was in accordance with OECD "Short Term and Long Term Toxicology Group Guidelines" published in Chemical Regulations Reporter, 14 August 1981.
GLP	Yes
Test substance	Canthaxanthin 10% water soluble beadlets Placebo: water soluble beadlets containing 0% Canthaxanthin
Species / sex	Rats / both sexes
Strain	CD of Sprague-Dawley origin
Route of administration	Feed admix ad libitum
Period of administration	Main groups (104 weeks) Interim sacrifices (52 weeks and 78 weeks) Recovery groups (79 weeks)
Frequency of administration	Once per day
Post-exposure period	Yes, from week 79 on for 16 weeks (males) and 20 weeks (females)
Doses males	0 (negative control), 0 (placebo control), 250, 500, and 1000 mg Canthaxanthin/kg bw/day 70 animals / dose level
Doses females	0 (negative control), 0 (placebo control), 250, 500, and 1000 mg Canthaxanthin/kg bw/day 70 animals / dose level
Control group	Yes
NOAEL	< 250 mg/kg bw/day based on observed liver toxicity in both sexes
LOAEL	250 mg/kg bw/day

CD Sprague-Dawley rats (70 animals/sex/group) were given Canthaxanthin in the diet at target doses of 0, 0 (placebo), 250, 500 or 1000 mg/kg bw/day for up to 104 weeks. The Canthaxanthin was microencapsulated in water-soluble beadlets containing water dispersible Canthaxanthin. The control beadlets devoid of Canthaxanthin were prepared for the control placebo group. Interim sacrifices (10 animals/sex/group) were performed in week 52 and in week 72 (3-4 animals/sex/group). Furthermore 10 rats of each sex per dose group were placed from week 78 on a normal control diet up to 16 weeks (males) or 20 weeks (females) as a recovery group.

Food and water intake, and body weight gain of placebo control and Canthaxanthin-treated groups were statistically significantly lower than those in the untreated controls. Body weight gain of Canthaxanthin-treated groups was also statistically significantly lower than that of the placebo control group, especially for females, but did not vary in dose related way between dose groups. Body weight gain of treated rats and of placebo controls increased to similar extent during the recovery period. The reduced food intake and body weight gain were considered to be not due to Canthaxanthin but to the presence of the beadlets in the diet. No clinical abnormalities other than reddish discoloration of faeces, fur and skin were observed. Ophthalmoscopic examination did not reveal any differences between the treated and control

groups. The mortality of the treated groups and the placebo controls was lower than of the untreated controls.

The clinical biochemistry examinations revealed treatment-related increases in the sera of female rats only in alkaline phosphatase (AP), aspartate transaminase (AST), alanine transaminase (ALT), cholesterol, gamma-glutamyl transpeptidase (gamma-GT) and bilirubin on several occasions. No treatment-related effects were seen in the hematological or urinalysis parameters.

Gross pathological examination at interim and terminal sacrifices revealed orange discoloration of liver, intestinal contents, skin, fur, subcutis, adipose tissue and extremities in treated animals in all dose groups. Liver weights of treated females were increased compared to the placebo or untreated control groups at the interim and terminal sacrifices. Histopathological examination at the interim and terminal sacrifices revealed treatment-related changes only in the liver. In the treated groups of both sexes hepatocellular hypertrophy and deposition of brown pigment was seen. Eosinophilic hepatocellular foci, hepatocyte vacuolization, bile duct hyperplasia and cystic bile ducts were observed in all treated female groups.

At the terminal sacrifice, a higher incidence of benign hepatocellular adenomas in all treated female groups but not in a dose-related manner was recorded. Re-evaluation of liver histopathology led to the conclusion that hepatocellular changes were seen at all dose levels in females only (i.e. from 250 mg/kg bw/day onwards), and that there was no indication of an increase in malignant tumours in the treated groups of both sexes compared to the controls

Canthaxanthin was not carcinogenic up to and including the limit dose of 1000 mg/kg bw/day. Regarding systemic toxicity, the lowest dose tested (250 mg Canthaxanthin/kg bw/day) was considered the LOAEL based on a dose-related increase in cholangiofibrosis observed in females only.

Reference	EFSA (2010) Reported as: Buser S, 1992a. Canthaxanthin (Ro 01-9915) in a long-term study with male rats (feed admixture). Unpublished research report B-157'342, submitted to WHO by F. Hoffmann-La Roche & Co., Basel, Switzerland (as referred to by JECFA, 1995 and SCF, 1997). Buser S, 1992b. Canthaxanthin (Ro 01-9915) in a long-term study with female rats (feed admixture). Unpublished research report B-157'343 submitted to WHO by F. Hoffmann-La Roche & Co., Basel, Switzerland (as referred to by JECFA, 1995 and SCF, 1997).
Type	Long-term study with interim kills after 52 and 78 weeks and recovery period (females only)
Guideline	Comparable to OECD 453, with the following exceptions: histopathology was limited to liver, male group size was 30 animals scheduled for 2-year treatment.
GLP	Yes
Test substance	Canthaxanthin 10% water soluble beadlets Placebo beadlets containing 0% Canthaxanthin
Species / sex	Rats / both sexes
Strain	CrI: CD (SD) BR
Route of administration	Feed admix ad libitum
Period of administration (males)	Main groups (104 weeks): 30 animals Interim sacrifices (52 weeks and 78 weeks): 10 animals

Period of administration (females)	Main groups (104 weeks): 50 animals Interim sacrifices (52 weeks and 78 weeks): 10 animals Recovery groups (52 weeks of treatment + 26 weeks of recovery): 10 animals Recovery groups (78 weeks of treatment + 26 weeks of recovery): 15 animals
Frequency of administration	Once per day
Post-exposure period	Males: No Females: Yes: 26 weeks
Doses males	0 (negative control), 0 (placebo control), 5, 25, 75, and 250 mg Canthaxanthin/kg bw/day 50 animals / dose level: 30 animals for the 2-year treatment and 10 animals for each of the 2 interim sacrifices
Doses females	0 (negative control), 0 (placebo control), 5, 25, 75, and 250 mg Canthaxanthin/kg bw/day 80 – 105 animals / dose level
Control group	Yes
NOAEL (males)	25 mg Canthaxanthin/kg bw/day
LOAEL (males)	75 mg Canthaxanthin/kg bw/day
NOAEL (females)	5 mg Canthaxanthin/kg bw/day
LOAEL (females)	25 mg Canthaxanthin/kg bw/day

In a further study, CD Sprague-Dawley rats (50 males and 80-105 females/group) were given Canthaxanthin in the diet at doses of 0, 0 (placebo), 5, 25, 75 or 250 mg/kg bw/day for up to 104 weeks. Canthaxanthin was supplied to the animals as beadlets containing water dispersible Canthaxanthin. Interim sacrifices were performed after 52 and 78 weeks (10 animals/sex/group). A 26-week recovery period was scheduled after 52 weeks (10 additional females) and 78 weeks (15 additional females) from groups receiving placebo and the two highest doses. No recovery group was scheduled for male animals.

The survival was not affected by the treatment. Red staining of the fur and tail was observed in rats of both sexes given 25 mg Canthaxanthin/kg bw/day or higher doses. Eye examinations showed no abnormalities related to treatment. Mean body weight gain of females receiving placebo or 250 mg/kg bw/day were statistically significantly lower than the untreated controls. No effects on food consumption were observed. No effects on hematological parameters or urine parameters were observed, except for a light to dark orange/brown discoloration of urine samples collected from a few animals, predominantly at dose levels of 75 and 250 mg/kg bw/day at week 51 in females. Plasma cholesterol was statistically significantly increased in males (250 mg/kg bw/day) and females (75 and 250 mg/kg bw/day). This increase was reversible during the recovery periods. In males treated with 75 and 250 mg/kg bw/day, slightly higher activity of AP was found after 104 weeks. In males, no effects on organ weight were found. In females, a statistically significant increase of relative liver weight in animals receiving Canthaxanthin at doses higher than 5 mg/kg bw/day was found, but not after the recovery period.

Gross pathology showed an orange/red discoloration of the digestive tract and orange discoloration of the subcutis and adipose tissue at all dose levels in both sexes and in some female rats previously treated with 75 and 250 mg/kg bw/day after recovery. Discoloration of the liver was seen in a number of animals after treatment at a dose level of 25 mg/kg bw/day and above, and in a few animals treated with 5 mg/kg bw/day.

Histopathological examination revealed treatment-related increases in the incidence or severity of non-neoplastic lesions in the liver (hepatocellular hypertrophy) in males receiving doses of 75 mg/kg bw/day and 250 mg/kg bw/day, and in females receiving doses from 25 mg/kg bw and higher from week 52 and onwards, higher incidence or severity of centrilobular hepatocyte vacuolization in males in the two highest doses after weeks 52 and 104 and higher severity of periportal fat in males in the two highest dose groups after 78 and 104 weeks, higher severity of periportal vacuolization in the high-dose group females after 52 weeks and higher incidence or severity of general vacuolization from a dose of 25 mg/kg bw/day after 104 weeks, focal or zonal fine orange/brown pigment in hepatocytes in the two highest dose male groups and from 25 mg/kg bw/day female group), which decreased or disappeared after the recovery period.

There was no increased incidence of liver cell tumours in Canthaxanthin-treated rats in comparison with controls. The NOAEL was considered to be 25 and 5 mg Canthaxanthin/kg bw/day for male and female animals, respectively; female rats being more susceptible to the effects of Canthaxanthin than males.

Cancer-preventive activity of Canthaxanthin

Bertram et al. (1991) have shown that Canthaxanthin inhibited methylcholanthrene-induced neoplastic transformation of pluripotent C3H/10T1/2 stem cells. When continuously administered to methylcholanthrene-treated cultures 7 days after removal of the carcinogen, Canthaxanthin (0.3-10 μ M final concentration, from Hoffmann-La Roche, Basel, Switzerland) inhibited the production of transformed foci in a dose-dependent manner.

Astorg et al. (1997b) have tested the effect of different carotenoids on the initiation stage of carcinogenesis in a feeding experiment in male Wistar rats. Two weeks before until one week after initiation of liver tumours (using either diethylnitrosamine or 2-nitropropane i.p. injections), Canthaxanthin at a concentration of 300 ppm (10% cold-water dispersible powder, Hoffmann-La Roche) and other carotenoids were applied in feed to groups of 10 rats each. Four weeks after cessation of Canthaxanthin administration, the tumor promoter 2-acetylaminofluorene was administered in the animals' diet for 2 weeks. In the middle of this period, a partial hepatectomy was conducted; liver specimens were analyzed for carotenoid content by HPLC. At the end of the experiments animals were sacrificed, liver slices were obtained and stained for gGT and GST-P, two markers of liver preneoplasia. The number and volume of stained foci were determined and compared to the placebo control group. Results showed that four weeks after cessation of Canthaxanthin administration, its content in the rat liver was still quite high (26.2 \pm 4.7 μ g/g liver). The content of retinyl esters in the liver samples was lowered to 88.8% of control group value. No data on development or food consumption are given. In the rats that were initiated with 2-nitropropane, feeding of Canthaxanthin had no statistically significant effect on the number of foci. Overall, results revealed that oral administration of Canthaxanthin before and during initiation of hepatocarcinogenesis had no significant influence on number and size of liver preneoplasia.

Gradelet et al. (1998) have evaluated the anticarcinogenic effect of Canthaxanthin and other carotenoids (beta-Carotene, beta-apo-8'-carotenal, Astaxanthin, and Lycopene) on liver carcinogenesis induced by Aflatoxin B1 in male weanling Wistar rats (10 rats/group). Two weeks before until one week after initiation of liver tumours by Aflatoxin B1 i.p. injections, Canthaxanthin (10% cold-water dispersible powder, Hoffmann-La Roche) was applied in feed at a concentration of 300 ppm. Three weeks after cessation of Canthaxanthin administration the promoter 2-acetylaminofluorene was administered in the animals' diet for 2 weeks. In the middle of this period, a partial hepatectomy was conducted. At the end of the experiments animals were sacrificed, liver slices were obtained and stained for GST-P, a marker of liver preneoplasia. The number and volume of stained foci were determined and compared to a control group which received a placebo. Liver DNA single-strand breaks induced by Aflatoxin B1 and in vivo binding of [3H]-Aflatoxin B1 to liver DNA and plasma albumin were measured. Additionally, the modulation of Aflatoxin B1 metabolism by carotenoids was investigated in vitro using liver microsomes of carotenoid-fed rats. For the treatment interval, no information on body weight gain, development or food consumption is given. Canthaxanthin content in hepatectomy samples (obtained 4 weeks after cessation of Canthaxanthin treatment) was still high: 20.0 ± 4.4 $\mu\text{g/g}$ liver. The content of retinyl esters in the liver samples was lowered after Canthaxanthin feeding to 92% compared to control group. Canthaxanthin significantly decreased the initiating effect of Aflatoxin B1: the number and size of foci were significantly decreased. Aflatoxin B1-induced DNA single strand breaks and liver DNA-adducts were also significantly reduced. In the presence of microsomes from rats fed Canthaxanthin, the in vitro metabolism of Aflatoxin B1 was enhanced, principally towards less genotoxic metabolites. In conclusion, oral administration of Canthaxanthin reduced the number of preneoplastic lesions induced by Aflatoxin B1, presumably due to alterations in liver xenobiotic metabolism.

Astorg et al. (1996) have evaluated the anticarcinogenic effect of Canthaxanthin and other carotenoids (beta-Carotene, vitamin A) on liver carcinogenesis induced by diethylnitrosamine in male weanling Wistar rats (10 rats/group). Two weeks before until one week after initiation of liver tumours by diethylnitrosamine i.p. injections, Canthaxanthin (10% cold-water dispersible powder, Hoffmann-La Roche) was applied in feed at a concentration of 300 ppm. Three weeks after cessation of Canthaxanthin administration the tumor promoter 2-acetylaminofluorene was administered in the animals' diet for 2 weeks. In the middle of this period, a partial hepatectomy was conducted. At the end of the experiments animals were sacrificed, liver slices were obtained and stained for gGT and GST-P, two markers of liver preneoplasia. The number and volume of stained foci were determined and compared to a control group which received control diet. The rats reduced food intake and stopped gaining weight during the week following the diethylnitrosamine injection. This effect was significantly less marked in rats fed Canthaxanthin compared to the other groups. Canthaxanthin content in liver was 477.8, 82.2, and 13.5 $\mu\text{g/g}$ liver measured during Canthaxanthin feeding, at 28 and 49 days after cessation of Canthaxanthin administration, respectively. The content of retinol and retinyl esters in the liver samples were not significantly influenced by Canthaxanthin feeding compared to the control group. No significant effect of Canthaxanthin administration on number and size of preneoplastic foci was observed.

Schwartz and Shklar (1997) have tested the anticarcinogenic effect of Canthaxanthin, beta-Carotene (both obtained from Sigma Chemical), and 13-cis-retinoic acid on dimethylbenz[a]anthracene (DMBA) induced tumours in hamster cheek pouch. To this end, right buccal pouches of male Syrian Golden hamsters (10 per group) were painted thrice weekly with a 0.5% DMBA solution in heavy mineral oil. On alternate days, animals received the retinoid/carotenoids (10 mg/kg bw) orally. Additionally, control groups receiving either DMBA or the retinoid/carotenoids were treated in parallel. After the 14-week treatment period, animals were sacrificed and the number and size of tumours of the right buccal pouch were determined. No statistical evaluation was performed for any parameter. Results showed that at the end of the 14-week treatment period, Canthaxanthin-treated animals demonstrated a significant inhibition of tumor development compared with the tumor control animals. In the Canthaxanthin/DMBA and the DMBA group, the number of animals with tumours was 8 and 10, respectively; the overall number of tumours was 30 and 130, respectively and the tumor burden (No. of tumours x volume) was 116.1×10^3 vs. 162.5×10^3 mm³. Tumours found were well to moderately differentiated epidermoid carcinomas. No tumours were observed in animals treated only with Canthaxanthin but not with DMBA. In the experimental group receiving Canthaxanthin, the underlying connective tissue demonstrated considerably fewer capillaries with lower intensity than the tumor control group. Mean body weight of the animals receiving Canthaxanthin (140 g) was higher compared to the tumor control (103 g) and the untreated group (121 g). Taken together, the tumor-inducing effects of DMBA were significantly reduced by concomitant treatment with Canthaxanthin.

Chew et al. (1999a) have assessed anticarcinogenic activity of Canthaxanthin in a mouse model: BALB/c mice were fed a synthetic diet containing 0, 0.1 or 0.4% Canthaxanthin, Astaxanthin or β -Carotene. After 3 weeks, all mice were inoculated with 1×10^6 WAZ-2T tumor cells into the mammary fat pad. All animals were killed 45 days after inoculation with the tumor cells. In general, all three carotenoids decreased mammary tumor volume. Mammary tumor growth inhibition by Astaxanthin was dose-dependent and was higher than that of Canthaxanthin and β -Carotene. Mice fed 0.4% β -Carotene or Canthaxanthin did not show further increases in tumor growth inhibition compared to those fed 0.1% of each carotenoid. Therefore, β -Carotene, Canthaxanthin and especially Astaxanthin inhibit the growth of mammary tumors in mice; their anti-tumor activity is also influenced by the supplemented dose.

Tanaka et al. (1995) tested the chemopreventive effects of Canthaxanthin and Astaxanthin on oral carcinogenesis induced by 4-nitroquinoline-1-oxide (4-NQO) in male F344 rats. Rats received 20 ppm of 4-NQO for 8 weeks in their drinking water to induce oral neoplasms or preneoplasms. Animals were fed diets containing 100 ppm of the respective xanthophyll for 10 weeks during the initiation phase or for 22 weeks thereafter. In week 32 the incidences of preneoplastic lesions and neoplasms in the oral cavity of rats treated with 4-NQO and Canthaxanthin were significantly smaller than those of rats given 4-NQO alone. In animals fed Astaxanthin or Canthaxanthin but no 4-NQO no (pre-)neoplastic lesions were observed in the oral cavity.

Azuine et al. (1992) tested the chemopreventive effect of Canthaxanthin and other carotenoids against BaP-induced forestomach carcinogenesis in mice. Mice were administered

Canthaxanthin (4.7 μM /animal/day) by gavage over a period of 8 weeks. Starting in week three, eight 1 mg-doses of BaP were administered to the animals over a period of 4 weeks. After a total duration of 180 days animals were sacrificed and forestomach papillomas were counted. All 20 animals receiving only BaP developed forestomach cancer with a multiplicity of 7.0 ± 0.3 tumors/animal. In the animals fed Canthaxanthin in parallel, tumor incidence was only 20% with a multiplicity of 1.0 ± 0.0 tumors/animal. No tumors were found in the control animals receiving only Canthaxanthin.

Canthaxanthin was also effectively preventing or reducing cancers of the skin after treatment with UV-B irradiation alone or in the presence of benzo[a]pyrene:

Mathews-Roth (1982) has administered Canthaxanthin in feed (33000 ppm, equivalent to 6680 mg/kg bw/day) to SKH/hr hairless mice. After receiving the carotenoid or a placebo over 10 weeks skin tumors were induced using one of the following methods: 1) UV-B irradiation, 2) painting with DMBA and Croton oil, or 3) painting with DMBA followed by UV-B irradiation. In animals receiving treatment 1 (UV-B irradiation) appearance of the first tumor was noted in week 11 in Canthaxanthin-fed animals vs. week 9 in the placebo group. At study end, tumor multiplicity was lower in the Canthaxanthin-fed animals when compared to the placebo group (1.75 vs. 15.05 tumors/animal). Also the animals treated with DMBA / UV-B irradiation (method 3) showed delayed formation of skin tumors when fed with Canthaxanthin (first tumor in week 13, placebo: week 6). At termination of the study in week 20, tumor multiplicity was 0.75 in Canthaxanthin-fed animals vs. 2.75 in the placebo group. No difference in tumor incidence and multiplicity was observed in animals treated with DMBA / Croton oil (method 2) with or without Canthaxanthin.

In a similar study, Mathews-Roth & Krinsky (1987) have treated hairless mice a single high dose of UV-B irradiation. Animals in group 1 received Canthaxanthin (1000 ppm, equivalent to about 150 mg/kg bw/d) for 6 weeks before irradiation and placebo for 24 weeks thereafter. Group 2 received placebo first and Canthaxanthin after irradiation. Group 3 animals received Canthaxanthin throughout the whole experiment. A control group receiving only placebo was run in parallel. Tumor incidence 24 weeks post UV-B treatment was 14/24 in the control group, 3/24 in group 1, and 2/24 in groups 2 and 3, indicating that Canthaxanthin administration can significantly prevent the development of UV-B induced skin tumors.

Santamaria et al. (1983) have administered Canthaxanthin (500 ppm, equivalent to about 75 mg/kg bw/d) to mice for a period of 4 weeks. Thereafter, animals were painted with benzo[a]pyrene solution and exposed to UV-A light or kept in the dark. Canthaxanthin, when perorally administered to mice, markedly prevented benzo[a]pyrene and UV-A induced carcinogenesis.

4.3.8 Reprotoxicity and Developmental Toxicity

4.3.8.1 Summary

In a 3-generation reprotoxicity study in rats with 2 matings per generation, there were no adverse effects on mating performance, gestation duration, parturition, or ability of the dams to lactate and rear their progeny successfully up to and including the highest dose level administered (1000 mg/kg bw/day). Orange / red coloration of viscera and adipose tissue was noted in adults and weanlings. There was indication of liver toxicity in adult females as indicated by clinical biochemistry and histopathology (foamy macrophages in liver sinusoids in F₀ and F₁ females and slightly increased hepatocyte vacuolation in F₂ adults at 500 and 1000 mg/kg bw/day). In addition, adult females showed decreased adrenal weight in all dose groups and increased spleen weight at 1000 mg/kg bw/day. Culled weanlings had significantly increased liver weights, a finding which showed considerable tendency for recovery. The NOAEL for reprotoxicity and developmental toxicity was 1000 mg/kg bw/day. The NOAEL for parental toxicity was 250 mg/kg bw/day.

In a combined developmental toxicity and post-natal toxicity study in rats with Canthaxanthin exposure during organogenesis, no adverse effects were seen on dams. There was no indication of embryotoxicity, developmental toxicity or teratogenicity up to and including the limit dose of 1000 mg/kg bw/day. The rearing study showed no evidence of adverse effects on lactation or functional abnormality in the offspring. The NOAELs for maternal and developmental toxicity were 1000 mg/kg bw/day, the highest dose.

In a developmental toxicity study in rabbits, no adverse effects were noted on does. There were no indications of embryotoxicity, developmental toxicity or teratogenicity up to and including the highest dose (400 mg/kg bw/day). The NOAELs for maternal and developmental toxicity were 400 mg/kg bw/day, the highest dose.

4.3.8.2 Reprotoxicity

Mantovani (1992) has reviewed literature on reproductive toxicity and found no detrimental effects of Canthaxanthin administration on reproductive function.

Reference	EFSA (2010) and ILSI (1999) and Hallagan (1995) Reported as: Buser S, 1987b. Canthaxanthin in a three-generation study in rats. Unpublished Report No. HLR 138/86755 of Huntingdon Research Centre Ltd. Submitted by F. Hoffmann-La Roche & Co., Basel, Switzerland.
Type	Three-Generation Reprotoxicity Study (2 litters per generation)
Guideline	Comparable to OECD 416, sperm and estrous cycle analysis were not performed
GLP	Yes
Test substance	Canthaxanthin 10% in water soluble beadlet formulation Placebo beadlets containing 0% Canthaxanthin
Species / sex	Rat
Strain	CrL COBS CD (SD) BR
Route of administration	Feed admix (ad libitum)
Period of administration	Throughout treatment

Frequency of administration	Daily
Pre-mating exposure period males	F0 generation: 9 weeks F1A and F2A generation: 12 weeks
Pre-mating exposure period females	F0 generation: 9 weeks F1A and F2A generation: 12 weeks
Doses male	0 (control), 0 (placebo control), 250, 500, and 1000 mg Canthaxanthin /kg bw/day 24 to 32 animals per group
Doses female	0 (control), 0 (placebo control), 250, 500, and 1000 mg Canthaxanthin /kg bw/day 24 to 32 animals per group
Control group	Yes
NO(A)EL parental	250 mg/kg bw/day
LOEL parental	500 mg/kg bw/day
NO(A)EL developmental	1000 mg/kg bw/day
LOEL developmental	> 1000 mg/kg bw/day
NO(A)EL reproduction	1000 mg/kg bw/day
LOEL reproduction	> 1000 mg/kg bw/day

In this assessment of the effect of Canthaxanthin on growth and reproductive performance of the rat, the test material Canthaxanthin was administered as water soluble beadlets in the diet throughout three generations at nominal doses of 250, 500 and 1000 mg/kg bw/day.

There were no treatment-related adverse effects based on reproductive performance (i.e. mating performance, duration of gestation, parturition or ability of dams to lactate and rear their offspring successfully).

However, some treatment-related effects without consequences for reproductive performance were noted in animals of F0, F1 and F2 generation: orange/red coloration of faeces, fur, viscera and adipose tissue; reduced food efficiency and growth; increased levels of serum AST, ALT, AP, and cholesterol in adult females; significantly increased relative liver weights in weanlings at all dosages in each of the six litters attaining statistical significance in nearly every instance; increased spleen weight in adult females at the highest dose in all generations attaining statistical significance for F0 and F2A generations; decrease of adrenal weight in adult females at all doses with the differences from the placebo controls consistently attaining statistical significance but with no indication of a dose related effect; histological changes in the liver with foci of foamy macrophages in the liver sinusoids in F0 and F2 adult females; and increased hepatocyte vacuolation at 500 and 1000 mg/kg bw/day in F2 adults. The above mentioned adverse effects were partially reversed during an eight-week withdrawal phase at the end of the study.

In another three generation study, Wistar rats (20 male and 20 female animals per group) were exposed to 0 or 0.1% Canthaxanthin in a synthetic diet for 2 years. Based on food consumption, the average Canthaxanthin exposure was 30-74 mg/kg bw/day in the Canthaxanthin-treated group. The animals of the first and second generations were mated in order to determine the effect of the treatment on fertility. There was no statistically significant difference between control and Canthaxanthin-exposed animals with regard to fertility (EFSA 2010, reported as Hoffmann-La Roche, 1966. Canthaxanthin. Unpublished report submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland).

In another study Wistar rats (25 male animals and 25 female animals per group) were exposed to 0, 0.5, 2 or 5% Canthaxanthin in semi-synthetic diet for 98 weeks. Six months after the start of the trials, the females of each group were paired with the males from the corresponding groups. The presence of Canthaxanthin in the diet had no negative effect on the size of litters, the number of young weaned and their weights at birth and weaning. No anomalies were found on examination of the skeleton (EFSA 2010, reported as Hoffmann-La Roche, 1966. Canthaxanthin. Unpublished report submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland).

4.3.8.3 Developmental Toxicity / Teratogenicity

Reference	EFSA (2010) Reported as: Eckhardt K, 1982. Embryotoxicity study in rabbits with oral administration of Ro 01-9915. Canthaxanthin. Segment II teratological study. Unpublished report submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland.
Type	Developmental Toxicity Study
Guideline	According to guideline of the American FDA and the English CSM, comparable to OECD 414
GLP	Yes
Test substance	Canthaxanthin
Species / sex	Rabbit / females
Strain	Swiss hare rabbits
Route of administration	Oral by gavage in rape seed oil
Period of administration	From day 7 to 19 of gestation, copulation was considered day 1 of gestation
Frequency of administration	Daily
Doses	0 (vehicle control), 100, 200, and 400 mg/kg bw/day 20 mated females per group
Control group	Yes
NO(A)EL maternal	400 mg/kg bw/day
LOEL maternal	> 400 mg/kg bw/day
NO(A)EL developmental	400 mg/kg bw/day
LOEL developmental	> 400 mg/kg bw/day

Mated female Swiss hare rabbits (20 animals/group) were given doses of 0, 100, 200 or 400 mg Canthaxanthin/kg bw/day by gavage of a suspension of Canthaxanthin in rape seed oil on days 7 through 19 of pregnancy.

No effects were seen on maternal body weight gain in any dose group. No treatment related effects were found on percentage of pregnant dams, average number and location of implantations and average number of corpora lutea; a slight but statistically significant increase in resorptions was noted in the 100 mg/kg bw/day dose group but there was no such effect in the higher dose groups. Therefore this finding was considered as not treatment-related. The average litter size, the number of dead fetuses, the average crown-rump length and the fetal weight as well as the distribution by sex were comparable to control values. Sporadic non-treatment-related malformations of different types occurred in a few fetuses of all groups, including controls. There was no difference from controls in the incidence of skeletal anomalies. The 24 hours offspring survival test did not reveal an adverse effect of treatment.

It was concluded that, under the conditions of the study, Canthaxanthin was neither embryotoxic nor teratogenic and that the highest dose tested of 400 mg/kg bw/day was the NOAEL for developmental and maternal toxicity.

Reference	EFSA (2010) Reported as: Kistler A, 1982. Embryotoxicity study in rats with oral administration (feed admix) of Ro 01-9915, Canthaxanthin. Phase II - teratological study with postnatal evaluation. Unpublished report submitted to WHO by F. Hoffmann-La Roche & Co., Basle, Switzerland.
Type	Segment II study with postnatal evaluation
Guideline	American FDA (1966) Guidelines for Reproduction Studies for Safety Evaluation of Drugs for Human Use English CWS (1974) Committee on Safety of Medicines. Guidelines for Reproduction Studies for Guidance of Applicants for Product Licenses and Clinical Trial Certificates
GLP	Yes
Test substance	Canthaxanthin 10% beadlet formulation Placebo beadlets containing 0% Canthaxanthin
Species / sex	Rats
Strain	Füllinsdorf (FU) albino
Route of administration	Oral by feed (ad libitum)
Period of administration	From day 7 to 16 of gestation, day 1 of gestation = 24hours after copulation
Frequency of administration	Daily via feed
Doses	0, 250, 500, and 1000 mg Canthaxanthin/kg bw/day 40 mated females per group
Control group	Yes
NO(A)EL maternal	1000 mg/kg bw/day
LOEL maternal	> 1000 mg/kg bw/day
NO(A)EL developmental	1000 mg/kg bw/day
LOEL developmental	> 1000 mg/kg bw/day

Pregnant FU-Albino rats (40 animals/group) were given Canthaxanthin in the form of beadlets in the diet at dose levels of 0, 250, 500 or 1000 mg/kg bw/day on days 7 through 16 of pregnancy. On the 21st day of gestation the dams of each group were divided into a necropsy subgroup and a rearing subgroup.

No treatment-related effects were seen in the necropsy subgroup on reproductive parameters (i.e. percentage of pregnant dams, average number and location of implantations and resorptions and the number of corpora lutea). There were no findings demonstrating an increased incidence of gross, skeletal or visceral malformations. There was no indication of any embryotoxic or teratogenic action of Canthaxanthin at any of the dose levels used. The rearing experiment showed neither evidence of effects on lactation nor on postnatal development the offspring (i.e. surviving and weight gain of the offspring as well as the offspring organ weights at sacrificing at postnatal day 23).

The test substance was neither embryotoxic, teratogenic, nor developmental toxic up to and including the limit dose of 1000 mg/kg bw/day. The NOAEL maternal and developmental were considered to be 1000 mg/kg bw/day.

4.3.9 Specific Investigations

4.3.9.1 Mechanistic studies on Canthaxanthin crystalline deposits

Reference	EFSA (2010) and JECFA (1996) Reported as: Bruinink A, Cohn W and Weiser H, 1992. Chick embryonic neuronal retina, RPE, brain and meninges cell cultures as a model for canthaxanthin induced alterations in the eye. Unpublished research report submitted to WHO by F. Hoffmann-La Roche & Co., Basel, Switzerland
Type	In vitro mechanistic study
Guideline	Not applicable
GLP	No
Test substance	Canthaxanthin 10% WS

The formation of Canthaxanthin crystals in embryonic chick neuronal retina reaggregate cell cultures was studied. In addition, the effect of Canthaxanthin on lysosomal and mitochondrial activity, protein synthesis and differentiation in flat sedimented cells of chick embryonic neuronal retina, retinal pigment epithelium, brain and meninges were examined. Canthaxanthin was added to the cell cultures in association with HDL which was obtained from chickens fed Canthaxanthin or placebo. In neuronal retina reaggregate cell cultures, incubation with high doses of Canthaxanthin resulted in the formation of red/brown birefringent entities. The frequency of the birefringent entities induced in the cell cultures was directly proportional to Canthaxanthin concentrations in the medium and occurred at a concentration of 1.2 mg/L of medium and above. Incubation with Canthaxanthin did not affect the cellular viability and differentiation in the cultures.

4.3.9.2 Animal models for retinal Canthaxanthin deposition

In 1980's, the presence of shiny golden crystals in the human retina was observed in persons who had ingested high doses of Canthaxanthin (pharmaceutical or cosmetic use). Since then several attempts were made to find an appropriate animal model for Canthaxanthin crystalline deposition in the retina.

However, toxicological studies showed that standard laboratory animal species were inappropriate models because no crystalline deposits were visible in dogs, rats, and mice especially by standard ophthalmoscopy. Therefore, several other species (including chicken, rabbits, cats, ferrets, and monkeys) were investigated on retinal Canthaxanthin deposition (for reviews please refer to JECFA 1996, SCF 1997, ILSI 1999, and EFSA 2010).

Briefly, it was shown that in almost all investigated species crystalline deposits identical or comparable to those observed in human could not be induced:

- In dogs, no crystalline deposits were found after treatment with Canthaxanthin. Beagles fed Canthaxanthin also showed no abnormalities upon ophthalmoscopy and histopathology.

- In rabbits, no retinal deposits could be induced even with high dietary concentrations of 200 ppm and long-treatment of 11 months. The presence of Canthaxanthin in the rabbit retina could not be proven analytically.
- In cats, some evidence of "orange sheen" was noted in the retina. However, the typical crystals could not be observed.
- Ferrets did not accumulate Canthaxanthin in the retina. Microscopical examination of the eyes of ferrets treated with Canthaxanthin did not reveal any crystalline deposits in the retina or iris, nor choroid or pigmented epithelium.
- Chickens fed diets containing 15000 ppm Canthaxanthin over 6 to 12 weeks showed crystal-shaped birefringent reddish-brown particles which closely resembled synthetic Canthaxanthin crystals. The birefringent entities seen in retina cell cultures were present in the peripheral part of the retina and in the iris. In a subsequent dose-response study, it was shown that a dose level of about 2.1 mg/kg bw/day did not result in Canthaxanthin deposition.

The only species with comparable observations was the monkey. Briefly, birefringent inclusions in the retina and macula were noted using special investigative methods. These findings were, however, without any adverse effect on visual performance. A threshold of 0.6 mg Canthaxanthin/kg bw/day could be established for Canthaxanthin crystalline deposits in the retina of monkey. The NOEL for eye pigmentation in monkey was 0.2 mg/kg bw/day.

4.3.9.3 Canthaxanthin crystals in human retina

In its "classical" presentation, the phenomenon of Canthaxanthin crystalline retinal deposits is characterized by the presence of numerous glistening, golden-yellow crystals containing Canthaxanthin deposited in the inner layer of the retina. In this "classical picture" the crystals are arranged in a ring-shaped form around the macula. In those cases where fewer crystals are present, they may not be arranged in this classical form but may be scattered irregularly. They are easily detected by ophthalmoscopy but quantifying their exact numbers is difficult. These deposits are also present within the peripheral retina and, at a lesser density, within the remaining retina, but they are not seen in the center of the macular area. Ultrastructurally, the birefringent deposits, which are believed to be in the form of lipoprotein complexes, appear to be associated with the Mueller cells, which are structural glial cells within the retina (Daicker et al., 1987).

The phenomenon of Canthaxanthin crystalline retinal deposits in humans has been thoroughly evaluated by JECFA (1997), SCF (1997), ILSI (1999) and EFSA (2010) as well as by ophthalmologists (for a comprehensive review please refer to Arden & Barker (1991)). Therefore, an excerpt of most relevant investigations is given with special emphasis on

- dose-response assessment of Canthaxanthin deposition in the eye,
- investigations on reversibility of Canthaxanthin crystalline deposits in retina after cessation of exposure,
- investigations on visual function,

- on pharmacological behavior of Canthaxanthin in human, and
- further information on possible systemic toxicity.

For detailed information, please refer to the cited literature and the respective evaluations of JECFA 1997, SCF 1997, Arden & Barker 1991, ILSI 1999, and EFSA 2010.

Reference	Köpcke W et al. (1995)
Type	Summary of human case reports and biostatistical evaluation
Guideline	Not applicable
GLP	No
Test substance	Phenoro (15 mg Canthaxanthin per capsule) or Orobronze (30 mg Canthaxanthin per capsule)
Species / sex	Human patients
Route of administration	Oral

All cases of reported high and long-term Canthaxanthin intake, whether published or unpublished, obtained systematically or anecdotally, were collected, analyzed and evaluated statistically. This compilation contains 691 cases who have taken Canthaxanthin for cosmetic or medical purposes, 133 of whom showing crystals. However, the information on dosage was missing in a considerable number of cases, finally 411 cases (95 with crystals) were suitable for evaluation. In this population mean daily doses taken varied from 15 to 240 mg per day. Calculated medium yearly doses ranged from 420 to 50400 mg per year, total doses were from 630 to 201600 mg and the duration of treatment varied from 1 to 14 years.

There was a highly significant correlation ($p < 0.0001$) for the presence of retinal deposits and certain parameters investigated. The descriptive statistical analysis clearly demonstrated the existence of a relationship between daily dose and the observation of crystals in the retina. In delineating this dose-relationship it is important to realize that Canthaxanthin was taken in multiples of 15 mg because the actual content of Canthaxanthin in the pharmaceutical preparations which were used was 15 mg Canthaxanthin (Phenoro) or 30 mg (Orobronze). Therefore the cases were allotted to six groups according to the daily dose: < 30, 30, 45, 60, 75-105 and > 105 mg. The relative incidence of crystal formation in these dose-groups was calculated to be 0, 9.6, 20.3, 23.4, 43.1, and 48.6% respectively. Thus, 30 mg per day was the lowest dose which has been reported to result in crystal formation. There were no crystals at all reported with a dose lower than this value.

Ingestion of Canthaxanthin below a dose of 30 mg/day (0.5 mg/kg bw/day) does not result in crystal formation in the human retina.

Reference	Arden GB et al. (1989)
Type	Human Surveillance
Guideline	Not applicable
GLP	No
Test substance	Phenoro (15 mg Canthaxanthin and 10 mg beta-Carotene per capsule)
Species / sex	Human / both sexes
Route of administration	Oral

Twenty-seven human subjects (suffering from porphyria) were treated with Canthaxanthin at dose levels of 15 mg/day for 5 weeks, increasing to 60 mg/day for 5 weeks, and subsequently receiving 90 to 120 mg/day during the summer months. No treatment was given during the winter months. Some of the patients received Canthaxanthin for the first time while others had been treated for up to 10 years (total dose up to 170 g). Canthaxanthin blood level was determined in patients during winter (off-treatment) and during summer (on-treatment).

Blood levels during winter were $98 \pm 99 \mu\text{g/L}$ (equivalent to blood levels resulting from normal food intake); during summer blood levels were substantially higher: $2277 \pm 1093 \mu\text{g/L}$.

In most cases, no changes occurred in the ERG. Systemic changes were only observed for the scotopic b-wave. One month treatment with 15 mg/day Canthaxanthin produced no systemic change in the ERG scotopic b-wave amplitude while an additional month on a dosage of 60 mg/day produced a reduction in ERG scotopic b-wave amplitude which was more pronounced after a further month at a dose of 90 mg/day. Human subjects with Canthaxanthin crystals in the retina showed an even more marked reduction in the ERG scotopic b-wave amplitude. However, the reduction in the ERG scotopic b-wave amplitude was not correlated with the concentration of Canthaxanthin in blood.

During winter time (off-treatment), the effect on the ERG scotopic b-wave amplitude was reversible. It was suggested that the mechanism for the reduction of the ERG scotopic b-wave amplitude was due to the concentration of Canthaxanthin by the Müller cells, known to generate the scotopic b-wave.

The characteristic refractile retinal crystals reduced during the winter months.

Ingestion of Canthaxanthin results only in changes in the b-wave of the ERGs. A dose-response relationship could be established: The NOEL for b-wave changes in this study was 15 mg/day, equivalent to 0.25 mg/kg bw/day; the LOEL was 60 mg/day (1 mg/kg bw/day). Changes in b-waves were reversible upon discontinuation of the treatment.

Reference	Harnois et al. (1989)
Type	Human surveillance study on reversibility
Guideline	Not applicable
GLP	No
Test substance	Not applicable, follow up study of patients
Species / sex	Nine patients and 7 or 12 control volunteers / not indicated
Route of administration	Oral
Remark	
Date	Not applicable

Nine patients with Canthaxanthin retinopathy were examined two to four times between 1982 to 1987 for reversibility of Canthaxanthin deposits by fundus photographs in a subjective way and by quantitative determination of retinal deposits. Retinal sensitivity was evaluated by static perimetry in an exposed group with typical retinal deposits. The control group consisted of volunteers who had never used Canthaxanthin.

The number of retinal deposits decreased after cessation of Canthaxanthin ingestion as evaluated by the qualitative and quantitative methods: There was no significant difference after a nine-month follow-up. A statistically significant decrease in the number of retinal deposits was found after 26 months. The deposits disappeared slowly, while some remained even after Canthaxanthin ingestion was discontinued. Threshold static perimetry performed did not differ significantly between the control and the patient group at the end of the follow-up period.

Canthaxanthin retinopathy is slowly reversible after cessation of treatment.

Reference	Leyon et al. (1990)
Type	Human Surveillance Study
Guideline	Not applicable
GLP	No
Test substance	Phenoro (15 mg Canthaxanthin and 10 mg beta-Carotene per capsule)
Species / sex	Human
Route of administration	Oral
Remark	
Date	Not applicable

Fifty-three patients having been treated with Phenoro for various photosensitivity disorders were examined ophthalmologically. The patients' ages ranged from 15 to 78 years, and the total amount of Canthaxanthin ingested ranged from 7.5 to 178 g. Most of the patients had been treated intermittently, on the average for 3 to 5 months per year at daily doses of between 60 and 90 mg Canthaxanthin for 1 to 12 years. Fundus pictures were taken and examined thereby counting the deposits independently by two assessors. The results were averaged. The number of retinal deposits was qualitatively assessed by classification into grades: 1-25 deposits, 26-500 deposits, and more than 500 deposits. Treatment was stopped and 14 of 22 patients with deposits were investigated again, 5 years after cessation of Canthaxanthin intake. At this time, an ocular examination included slit-lamp biomicroscopy, visual acuity testing and fundus photographs. In addition, liver function parameters and plasma Canthaxanthin concentrations were carried out.

Out of 53 patients, 22 were found with deposits. Of these, 15 had bilateral deposits. The patient with the lowest dose showing deposits had consumed 15 g Canthaxanthin during 3 years. None of the patients had visual problems. One of the patients (70 years old) had macular degeneration resulting in a slight reduction of visual acuity during the follow-up study. The reversibility study showed a 70% reduction of the number of deposits in all eyes. There were no indications of changes in liver function. Canthaxanthin determination in plasma after 5 years indicated that patients had stopped treatment with Phenoro. The plasma concentrations ranged from 38 to 232 µg/L; typical plasma levels which are known to result from food exposure.

Cessation of treatment with high doses of Canthaxanthin in patients results in substantial decrease of deposits in the eyes, no indications for changes in vision parameters, or hepatotoxicity. Thus, crystalline Canthaxanthin deposition in the retina is reversible.

Reference	Hueber et al. (2011)
Type	Human surveillance study
Guideline	Not applicable

GLP	No
Test substance	Canthaxanthin
Species / sex	13 Patients / not indicated
Route of administration	Oral

Patients that had taken Canthaxanthin between December 1983 and March 1988 were recruited via a newspaper article. Examination for macular Canthaxanthin deposition was performed on 35 patients. Up to 24 years later, a follow-up examination was performed in each patient, including determination of visual acuity, the Amsler grid, slit lamp examination, perimetry, electro-oculography, electroretinography, optical coherence tomography and fluorescein angiography.

Canthaxanthin deposition was found in 13 of the 35 patients examined. The occurrence of the golden particles appeared to be dose dependent. Secondary, retinal pigment epithelium (RPE) changes and a choroidal nevus seem to be predisposing factors for Canthaxanthin deposition. In such cases, also asymmetrical deposition between the left and right eye has been observed. A follow-up examination has been conducted on 7 patients. Whilst after 2 years the Canthaxanthin particles were still visible, complete disappearance could be observed in the patients after a follow-up time of 16-24 years. The patients in the study were asymptomatic and no functional defect definitely related to Canthaxanthin could be detected.

Ingestion of Canthaxanthin causes no long-term adverse effects in humans with regard to ocular endpoints. Complete reversibility of Canthaxanthin crystal deposition was observed after 16-24 years.

The effect of Canthaxanthin ingestion on crystal deposition in human retina has been reviewed by Nadim et al. (2002), Rasquin (2007), Fraunfelder (2004), Fraunfelder (2005) and Santaella & Fraunfelder (2007). Canthaxanthin is deposited in all layers of the retina, especially in the superficial layers of the macula. Deposition of Canthaxanthin into the eye is mainly dependent on the amount ingested and on the age of the patient (the older the more deposits). The crystalline deposits are slowly reversible after cessation of Canthaxanthin uptake. Appearance shows bilateral multiple golden, crystalline-like deposits in the nerve fiber layer surrounding but sparing the macula. Patients are normally asymptomatic with normal visual acuity and color perception. Fluorescein angiography is either normal but can also show a perifoveolar ring of decreased blood flow in the perifoveolar area. In some reviews, abnormalities in static threshold perimetry, electroretinography, and dark adaptation are noticed.

Chan et al. (2006) have reported a case study of a 56-year old man with numerous yellow crystalline deposits throughout the retina in both eyes. The patient had a history of ingestion of a tanning agent containing Canthaxanthin (dose and duration not given). The crystals were approximately 4 to 25 μm in diameter and were located in the outer plexiform layer.

Espallat et al. (1999) give a case report of a 34-year old man. Findings from the eye examination were completely normal except for numerous bilateral inner retinal crystalline deposits arranged around the macula. A fluorescein angiogram showed a perifoveolar ring of decreased blood flow corresponding to the area of the crystalline deposits. It was ascertained

that the patient had been taking 120 mg of oral Canthaxanthin per day for the purpose of self-tanning (period of time not mentioned).

4.3.9.4 Induction of xenobiotic metabolizing enzymes

Reference	Gradelet et al. (1996)
Type	Mechanistic study in vivo on induction of phase I- and phase II-enzymes
Guideline	An OECD guideline is not available for such type of studies.
GLP	No
Test substance	Canthaxanthin cold water-dispersible powder 10% (Hoffmann-La Roche)
Species / sex	Rat / male
Strain	SPF Wistar
Route of administration	Feed admix
Group size	5 animals per group
Period of administration	15 days
Frequency of administration	Daily
Doses	Experiment 1: 300 ppm in diet Experiment 2: 10, 30, 100, and 300 ppm in diet
Control group	Yes, placebo-treated

The aim of this work was to evaluate the possible inducing effect of carotenoids Astaxanthin, Canthaxanthin, Lycopene and Lutein on different xenobiotic-metabolizing-enzymes in rats fed with the respective carotenoids or a placebo diet for 15 days. Activities of various phase I- and phase II-enzymes were measured in liver microsomes and cytosol. Phase I-enzymes included CYP 1A1, 1A2, 2B1/2, 2E1, and 3A1/2; phase II-enzymes measured were 4NP-UGT, 4-HBP-UGT, GST, NADPH-quinone reductase, and ALDH3. Additionally, the activity of NAD(P)H cytochrome P450 reductase was assessed.

Food consumption, bodyweight gain and liver weight (absolute and relative) of the rats were not affected by carotenoid administration. Analysis of the liver revealed that carotenoids and especially Canthaxanthin are deposited in the liver (397 ± 83 nmol/g liver) and liver microsomes (1424 ± 363 pmol/mg protein). Canthaxanthin induced a significant increase in CYP isoenzymes at doses of 10 ppm (1A1 and 1A2) and 100 ppm (2B1/2); NADPH-quinone reductase was also significantly induced (at concentrations of 100 and 300 ppm) as well as 4NP-UGT (at 10 ppm and above), and liver total cytochrome P450 content (at and above 30 ppm). No significant induction was measured of the activities of CYP 2E1, CYP 3A, UGT, GST, and ALDH3; also liver weight, and NAD(P)H cytochrome P450 reductase activity were not affected by Canthaxanthin treatment, compared to vehicle control.

Canthaxanthin is a distinct inducer especially of CYP 1A activity in the rat and co-induces 4NP-UGT and quinone reductase; ALDH3 is only marginally increased.

Reference	Astorg et al. (1997a)
Type	Mechanistic study in vivo on induction of phase I- and phase II-enzymes
Guideline	An OECD guideline is not available for such type of studies.
GLP	No

Test substance	Canthaxanthin cold water-dispersible powder 10% (Hoffmann-La Roche)
Species / sex	mice / male
Strain	SPF Swiss
Route of administration	Feed admix
Period of administration	15 days
Frequency of administration	Daily
Group size	6 animals per group
Doses	300 ppm Canthaxanthin in diet
Control group	Yes, placebo-treated

The aim of this work was to evaluate the possible inducing effect of carotenoids Astaxanthin, Canthaxanthin, β -Carotene and β -apo-8'-Carotenal on different xenobiotic-metabolizing-enzymes in mice, fed with the respective carotenoids or a placebo diet for 15 days. Activities of various phase I- and phase II-enzymes were measured in liver microsomes and cytosol. Phase I-enzymes included CYP 1A1, 1A2, 2B1/2, 2E1, and 3A; phase II-enzymes measured were UGT, GST, NADPH-quinone reductase, and ALDH3. Additionally, the activity of NAD(P)H cytochrome P450 reductase was assessed.

Food consumption and bodyweight gain of the mice was not affected by carotenoid administration. Analysis of the liver revealed that carotenoids and especially Canthaxanthin are deposited in liver microsomes (56 ± 8 ng/mg protein). Canthaxanthin induced a moderate but significant increase in CYP isoenzymes 1A1 (2.7-fold), 1A2 (1.6-fold), and 2B1/2 (2.5-fold); NADPH-quinone reductase was also significantly induced (1.14-fold). No effects were measured on the activities of CYP 2E1, CYP 3A, UGT, GST, and ALDH3. Liver weight, liver P-450 content and NAD(P)H cytochrome P450 reductase activity were comparable to vehicle control.

Canthaxanthin is a moderate inducer of CYP 1A activity but has at best little influence on other CYP isoenzyme activity in mouse liver; NAD(P)H cytochrome P450 reductase activity was not affected by Canthaxanthin feeding.

Reference	Astorg et al. (1994)
Type	Mechanistic study in vivo on induction of phase I- and phase II-enzymes
Guideline	An OECD guideline is not available for such type of studies.
GLP	No
Test substance	Canthaxanthin cold water-dispersible powder 10% (Hoffmann-La Roche)
Species / sex	Rats / male
Strain	SPF Wistar
Route of administration	Feed admix
Period of administration	15 days
Frequency of administration	Daily
Group size	5 animals per group
Doses	300 ppm Canthaxanthin in diet
Control group	Yes, placebo-treated

The aim of this work was to evaluate the possible inducing effect of Canthaxanthin, β -Carotene and excess vitamin A on different xenobiotic-metabolizing-enzymes in rats, fed with the respective carotenoids or a placebo diet for 15 days. Activities of various phase I- and phase II-

enzymes were measured in liver microsomes and cytosol. Phase I-enzymes included CYP 1A1, 1A2, and 2B1/2; phase II-enzymes measured were UGT and GST. Additionally, the activity of NAD(P)H cytochrome C reductase was assessed.

Food consumption and bodyweight gain of the rats was not affected by carotenoid administration. Analysis of the liver revealed that carotenoids and especially Canthaxanthin are deposited in liver ($478 \pm 148 \mu\text{g/g}$ liver) and in liver microsomes ($902 \pm 96 \mu\text{g/g}$ protein). Canthaxanthin induced a significant increase in CYP isoenzymes 1A1 (98-fold), 1A2 (15-fold), and 2B1/2 (6.5-fold); UGT1, UGT2, GST, and NADH cytochrome c reductase activities were also significantly induced (factor 3.4, 1.2, 1.2, and 1.5, respectively). No effects were measured on the activity of NADPH cytochrome c reductase.

Canthaxanthin is a distinct inducer of CYP 1A activity but has at best little influence on other CYP and phase II enzyme activities in rat liver.

Reference	Gradelet et al. (1997b)
Type	Mechanistic study in vivo on induction of phase I- and phase II-enzymes accompanied by an in vitro Ah-receptor binding assay
Guideline	An OECD guideline is not available for such type of studies.
GLP	No
Test substance	Canthaxanthin cold water-dispersible powder 10% (Hoffmann-La Roche)
Species / sex	Mice / male or female
Strain	C57BL/6, male (Ah receptor-responsive) DBA/2, male (Ah receptor-low responsive) Ah-/-, female (Ah receptor-non responsive)
Route of administration	Feed admix
Period of administration	14 days
Frequency of administration	Daily
Group size	3 animals per group
Doses	300 ppm Canthaxanthin in diet
Control group	Yes, placebo-treated

Mice of different Ah receptor-sensible strains were fed a diet containing 300 ppm Canthaxanthin for 14 days. At the end of the experiments, mice were sacrificed and livers were removed. Liver microsomal and cytosolic fractions were prepared and tested on different phase I and II enzyme activities (CYP1A1, CYP1A2, CYP2B1/2, 4NP-UGT, 4HBP-UGT, GST, quinone reductase, ALDH1, ALDH3, and NAD(P)H cytochrome c reductase). The AhR binding assay was performed using C57BL6 mouse liver tissue. Competition experiments were carried out by incubating cytosol with ^3H -labelled TCDD in presence of the carotenoids in 2000-fold excess. After the incubation period, the sample was fractionized and measured by LSC.

In the Ah receptor-responsive C57BL/6 mice, Canthaxanthin induced a significant increase in the liver cytochrome P450 content (1.7-fold) but did not modify the activities of NADH and NADPH cytochrome C reductases. Canthaxanthin also induced activities of CYP 1A1, 1A2, and 2B1/2 (19-, 4.4, and 5.3-fold, respectively, versus control). No induction of the phase II enzyme activities (4NP- and 4HBP-UGT, GST, quinone reductase, ALDH1, and ALDH3) were measured. In the Ah receptor-low responsive DBA/2 and in the AhR knock-out mice, no effect on phase I and II enzyme activities were observed. Canthaxanthin, even in 2000-fold excess

concentration, failed to compete with ^3H -labelled TCDD for the specific binding site of the C57BL/6 Ah receptor.

Results show that the observed induction of CYP1A activity in mouse liver by Canthaxanthin is mediated via the Ah receptor. Results of the competition assay indicate that the induction of the Ah receptor may be mediated by a different mechanism than TCDD.

Reference	Jewell C & O'Brian NM (1999)
Type	Mechanistic study in vivo on induction of phase I-enzymes in different organs
Guideline	An OECD guideline is not available for such type of studies.
GLP	No
Test substance	Canthaxanthin cold water-dispersible powder 10% (Hoffmann-La Roche)
Species / sex	Rats / male
Strain	Wistar
Route of administration	Feed admix
Period of administration	14 days
Frequency of administration	Daily
Group size	8 animals per group
Doses	300 ppm Canthaxanthin in diet, 15g feed/day
Control group	Yes
Remark	
Date	Not applicable, publication

Male Wistar rats (26 d old, mean body weight 46.0 g) were allocated to 8 groups of 8 animals each. Six groups received 300 mg carotenoid/kg feed; the different carotenoids administered were Canthaxanthin, beta-Carotene, Bixin, Lycopene, Lutein, or Astaxanthin. A negative control group received plain diet whereas the positive control group received diet plus 3-Methylcholantrone, a known inducer of phase I-metabolism. After 14 days of treatment animals were sacrificed, the small intestine, liver, lung, and kidney were excised and used for measurement. Specific activity assays were performed on the following enzymes: CYP 1A1, CYP 1A2, CYP 2B1/2, and GST. Additionally, the GSH and carotenoid content of each tissue was determined.

Food intake, body weights, body weight changes and organ weights were not affected by treatment. Canthaxanthin significantly induced liver CYP isoforms 1A1, 1A2, and 2B1/2 by factors of 44, 34, and 15, respectively. In the lung, CYP 1A1 and 1A2 were induced by factors of 3.1 and 1.2, respectively. In the kidney, Canthaxanthin induced kidney microsomal CYP 1A1 and 1A2 by factors of 32 and 6.8, respectively. Activities of all enzymes were undetectable in the small intestine. GST activity and GSH status were not affected by carotenoid treatment. Canthaxanthin was detectable in all organs: liver (1200.8 ± 14.9 nmol/g tissue), lung (582.0 ± 36.6 nmol/g tissue), small intestine (224.6 ± 22.8 nmol/g tissue), and kidney (75.9 ± 0.1 nmol/g tissue).

Cytochrome P450 activity was induced by Canthaxanthin not only in the liver but also in kidney and lung tissue.

In an in vitro experiment with human liver microsomes, Zheng et al. (2013) have assessed the inhibitory effect of carotenoids on inhibition of cytochrome P450 activities. They found that

Canthaxanthin up to 5 μM does not inhibit any of the probed cytochromes (1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4/5). Calculated IC₅₀ values of 11 and 14 μM (for CYP2C19 and 3A4/5, respectively) were much higher than 1) solubility in aqueous solution and 2) the max. plasma concentration found by Pateau et al. (1997).

In their review on Ah receptor regulation, Denison and Heath-Paglusio (1998) mention that Canthaxanthin is a relatively weak ligand and inducer of the Ah receptor compared to TCDD. For further information, the publication of Gradelet et al (1996) is referenced.

4.3.9.5 Immuno-modulatory activities

Chew et al. (1999b) have assessed the effects of different carotenoids including beta-Carotene, Astaxanthin, and Canthaxanthin on lymphocyte function in a feeding study in mice. Whereas beta-Carotene and Astaxanthin enhanced splenic lymphocyte function in mice, Canthaxanthin showed no such effect after treatment of mice for up to weeks at dose levels up to 4000 ppm in diet.

In a study from Bendich and Shapiro (1986) the effect of beta-Carotene and Canthaxanthin on immune response in rats was evaluated. Groups of 8 male Wistar Kyoto rats were fed the respective carotenoids (0.2% in diet) for a period of up to 66 weeks or a comparable placebo. In vitro immune responses of splenocytes to T- and B-lymphocyte mitogens were consistently enhanced in both groups compared to placebo control group.

Chew and Park (2004) have reviewed the effects of different carotenoids including Canthaxanthin on the immune response. Rats fed Canthaxanthin had a heightened mitogen-induced lymphocyte proliferation. In vitro, Canthaxanthin enhanced the expression of activation markers of T helper cells and natural killer cells in human PBMC and also increased cytochrome oxidase and peroxidase activities in macrophages.

ILSI (1999) has thoroughly reviewed the different actions of Canthaxanthin and found that Canthaxanthin enhanced proliferation of T- and B-lymphocytes in the spleen of rats.

EFSA (2010) has also reviewed the effects of Canthaxanthin on immune response in animals and humans. They concluded that the observed effects were usually limited in intensity and were considered to be of no biological significance.

4.3.10 Overall Summary

4.3.10.1 Absorption, Distribution, Metabolism, and Excretion

Absorption of Canthaxanthin via the oral route was investigated in rat, monkey, and human. Existing data show that absorption is incomplete and reciprocal to the administered dose: in human, absorption ranges from 8% for a single dose of 150 mg to 34% at a daily dose of 1 mg. In rats, absorption ranges from 9 to 20%. Absorption rate was not influenced by repeated

administration or dietary fiber but by co-administration of other lipophilic compounds (e.g. beta-Carotene, alpha-Tocopherol) and the amount of lipids in the intestinal lumen.

In blood, Canthaxanthin is transported in lipoproteins. Blood, plasma and serum concentrations peak after 3 to 10 hours when applying a single dose or after repeated administration in rats, monkey, and human. Thereafter, concentrations decline steadily with time with a half-life of approximately 5 days for single or repeated administration.

In rats, Canthaxanthin is distributed all over the body; in all investigated tissues Canthaxanthin derived radioactivity is noted. In rats, highest concentrations are found in liver, spleen, fat, and small intestine, whereas in monkeys highest concentrations are noted in adrenals. In dogs, highest concentrations are found in adipose tissue, adrenals, liver, and skin. Repeated administration results in accumulation in tissues in all investigated laboratory species. After cessation of Canthaxanthin intake, concentrations in tissues decline; in rats, this decline is slowest in adipose tissue with a half-life of about 31 weeks.

Orally applied Canthaxanthin is mainly excreted via faeces (about 85-89% in monkey and about 25% in human); urinary excretion is of minor importance (max. about 20% in rats and max. 5% in monkey). In rats, most ingested Canthaxanthin is excreted within 24 to 48 hours; elimination is nearly complete after 96 hours in rats, monkeys and human. After 7 days less than 2% remain in the body of treated rats.

Comparison of rat and monkey ADME at identical dose levels and under steady-state conditions show in most parameters comparable results. Exceptions are as follows: Tissue concentration tended to be slightly higher in monkey. Retention of Canthaxanthin derived radioactivity in monkey is found in adrenals compared to liver and spleen in rats. Excretion via urine and metabolism is slightly slower in monkey. In rat urine some polar metabolites are found which could not be detected in monkey. Vice versa monkey urine contained some less polar metabolites which are not present in rat urine.

Canthaxanthin is not metabolized to Vitamin A in rats, monkey, and human. Hypervitaminosis A can be therefore excluded. Importantly, repeated administration had no influence on ADME of Canthaxanthin.

In vitro, Canthaxanthin induces CYP3A4 and CYP2B6 at the mRNA, protein and monooxygenase activity levels in human primary hepatocyte culture. Expression of other CYP genes including CYP1As, CYP2C9, and CYP2C19 is not influenced. Monooxygenase activity of CYP1A and CYP2C9 in human hepatocytes is enhanced; this is not accompanied by increased levels of respective CYP protein, mRNA, nor increased activity of NADPH cytochrome P450 reductase. Canthaxanthin does not inhibit activity of human cytochrome 450 isoforms 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4/5.

In vivo, Canthaxanthin increases the activity of isoenzymes CYP1A and CYP2B, but not of CYP2E1, and CYP3A in Canthaxanthin fed rats and mice. However, the extent of CYP activity increase is much more pronounced in rats as compared to mice. No increase in NADPH cytochrome P450 reductase activity was noticed. Induction of phase II enzymes was less

distinct, showing slight induction of quinone reductase but no increase in the activities of GST, ALDH and UGT.

Induction of xenobiotic metabolizing enzyme activity was found to be mediated via the aryl hydrocarbon receptor (AhR) pathway in mice.

4.3.10.2 Acute Toxicity

Canthaxanthin is not toxic when applied via the oral route. Obtained LD₅₀ via the oral route were greater than 10000 mg/kg bw in mice.

4.3.10.3 Effects on Skin and Eye

In recent evaluations by EFSA (2013) and JECFA (1996) Canthaxanthin was considered to be neither a skin nor an eye irritant nor showed it skin sensitizing potential.

4.3.10.4 Repeated Toxicity

Toxicity of Canthaxanthin was studied in different laboratory animal species: dog, rat, mouse, and monkey.

In all species consistently red discoloration of faeces was noted in most cases accompanied by discoloration of fur and skin. Macroscopically, yellow / orange / red staining of adipose tissue was observed and in almost all species also in the liver (rat, mouse, monkey), in the pancreas (rat, mouse) as well as in most cases also in the GIT.

Conventional ophthalmoscopy did not show any effects on eyes in standard as well as non-standard laboratory animal (rats, mice, dogs, rabbit, cats, and ferret). In monkeys, however, birefringent inclusions in the retina and macula were noted using special investigative methods. These findings were without any adverse effect on visual performance. A threshold of 0.6 mg Canthaxanthin/kg bw/day (LOEL) could be established for Canthaxanthin crystalline deposits in the retina of monkey; the NOEL was 0.2 mg/kg bw/day.

In rats, the major target organ was the liver. Liver toxicity manifested itself in clinical chemistry parameters (increased cholesterol and liver enzyme levels) as well as increased liver weights. Histologically, hepatocyte vacuolation, centrilobular hypertrophy, reduced centrilobular glycogen, increased periportal lipid content, iron-positive content of parenchymal and interstitial cells, and pigment deposition in hepatocytes and periportal macrophages were noted. Liver findings increased in incidence and / or severity with increasing duration of exposure, and / or were observed also at lower dose levels with increasing duration i.e. increasing age of the animals. Females were more affected from mild hepatotoxicity when compared to males. Liver toxicity was reversible in low to mid doses and at least showed some evidence of reversibility at high doses. Reversibility of observed liver toxicity gives evidence that the dose-related hepatocellular findings are more an adaptive response to non-physiological conditions and not a

toxic manifestation. Furthermore, older animals exhibit diminished capacity of metabolism with age. It is therefore not unexpected that a lipophilic overload may cause an overload of biological elimination pathways leading to the observed accumulation in hepatocytes and macrophages and fatty change/vacuolation. Overall, the NOAEL in female rats based on liver toxicity in a chronic study was 5 mg/kg bw/day; for males 25 mg/kg bw/day.

In dogs, histologically no adverse findings were noted up to and including a dose level of 500 mg/kg bw/day.

4.3.10.5 Genotoxicity and Mutagenicity

There was no evidence of genotoxicity or mutagenicity in a comprehensive battery of studies:

Canthaxanthin or Canthaxanthin formulations did neither induce reverse mutation in bacteria (Ames test) nor forward mutation in mammalian cells (HPRT test). There was further negative response with regard to mutation in yeast cells. The test for unscheduled DNA synthesis in primary hepatocytes (in vitro UDS) was also negative; the same result was obtained in an in vitro MNT in cultured human lymphocytes and in an in vivo MNT in the bone marrow of the mouse.

There is a body of other studies substantiating the protective potential of Canthaxanthin, i.e. to inhibit genotoxic effects caused by known mutagens.

4.3.10.6 Carcinogenicity

Carcinogenic potential of Canthaxanthin was studied in rats and mice. In both species, no carcinogenicity was observed.

Dietary treatment of male and female mice up to the limit dose of 1000 mg/kg bw/day for up to 98 weeks resulted in no toxicity or treatment-related changes in tumor incidence. Treatment-related findings were discoloration of tissues and pigment deposition in the liver (sinusoidal cells, macrophages, and hepatocytes). However, these were not considered to be adverse in the absence of tissue damage. Canthaxanthin was neither tumorigenic nor carcinogenic in mice. The NOAEL was considered to be 1000 mg/kg bw/day.

Dietary two-year studies in male and female rats resulted in a NOAEL for female rats of 5 mg/kg bw/day; for male rats a NOAEL of 25 mg/kg bw/day was obtained. The NOAELs are based on observed non-neoplastic liver toxicity (see repeated toxicity). In female rats, a slightly increased incidence in benign liver tumours being non-dose responsive was noted at dose levels equivalent or greater than 250 mg/kg bw/day (lowest dose level tested). However, in a second combined long-term carcinogenicity study no increased incidence in benign liver tumours was noted in female rats up to and including a dose level of 250 mg/kg bw/day. There was no indication of increased incidences in malignant tumours, neither in males nor in females up to the limit dose of 1000 mg/kg bw/day.

In contrast, Canthaxanthin was found to exert anticarcinogenic activity in several animal studies.

4.3.10.7 Reprotoxicity and Developmental Toxicity

In a 3-generation reprotoxicity study in rats with 2 matings per generation, there were no adverse effects on mating performance, gestation duration, parturition, or ability of the dams to lactate and rear their progeny successfully up to and including the highest dose level administered (1000 mg/kg bw/day). Orange / red coloration of viscera and adipose tissue was noted in adults and weanlings. There was indication of liver toxicity in adult females as indicated by clinical biochemistry and histopathology (foamy macrophages in liver sinusoids in F₀ and F₁ females and slightly increased hepatocyte vacuolation in F₂ adults at 500 and 1000 mg/kg bw/day). In addition, adult females showed decreased adrenal weight in all dose groups and increased spleen weight at 1000 mg/kg bw/day. Culled weanlings had significantly increased liver weights, a finding which showed considerable tendency for recovery. The NOAEL for reprotoxicity and developmental toxicity was 1000 mg/kg bw/day. The NOAEL for parental toxicity was 250 mg/kg bw/day.

In a combined developmental toxicity and post-natal toxicity study in rats with exposure during organogenesis, no adverse effects were seen on dams. There was no indication of embryotoxicity, developmental toxicity or teratogenicity up to and including the limit dose of 1000 mg/kg bw/day. The rearing experiment showed no evidence of adverse effects on lactation or functional abnormality in the offspring. The NOAELs for maternal and developmental toxicity were 1000 mg/kg bw/day.

In a developmental toxicity study in rabbits, no adverse effects were noted on does. There were no indications of embryotoxicity, developmental toxicity or teratogenicity up to and including the highest dose (400 mg/kg bw/day). The NOAELs for maternal and developmental toxicity were 400 mg/kg bw/day.

4.3.10.8 Immuno-modulatory activities

Enhancement of immune activity after dietary uptake of Canthaxanthin was observed in rats but not in mice. This was reflected by enhanced proliferation of T and B lymphocytes in the spleen. However, EFSA accounts these effects as of limited intensity and considers them to be of no biological significance (EFSA 2010).

4.3.10.9 Canthaxanthin deposition in the eye

In the 1980's, the presence of shiny golden crystals in the human retina was observed in persons who had ingested high doses of Canthaxanthin for pharmaceutical or cosmetic use (tanning). Since that time numerous investigations were performed on the subject of Canthaxanthin crystals in the retina at high doses and after prolonged periods of exposure. Initially, this phenomenon of Canthaxanthin crystalline deposits was characterized as

“Canthaxanthin retinopathy”; however, it has subsequently been shown that there is no retinopathy (i.e. persistent or acute damage to the retina of the eye) from Canthaxanthin crystals.

The glistening, golden-yellow crystals are deposits in the inner layer of the retina and may also be seen in the peripheral retina and at a lesser density within the remaining retina. In most cases they are arranged in a ring-shaped form around the macula. In humans, these crystalline deposits are easily detected using ophthalmoscopy. The birefringent deposits appear to be associated with the Mueller cells.

The presence of Canthaxanthin crystals in human retina, possible visual impairment, dose-response relationship, and reversibility has been thoroughly investigated: A biostatistical evaluation on hundreds of case reports gave evidence of a threshold of Canthaxanthin deposition in the human retina. Below a dose level of 30 mg/day (0.5 mg/kg bw/day), no crystalline deposition of Canthaxanthin in the retina was observed. Although initially the deposits were reported to be permanent long-term surveillance programs finally showed that the deposits disappear slowly after cessation of Canthaxanthin treatment. A recently published follow-up study reported that Canthaxanthin deposition in the human retina is fully reversible within at least 16 years.

In addition, there was great debate on the possible impairment of functional vision due to Canthaxanthin crystals in the retina. Visual function was investigated by various methods (visual acuity testing, dark adaption, perimetry, and electroretinography. Impairment of visual acuity (if any) was not clinically significant. When testing dark adaption of Canthaxanthin users versus age matched controls, no differences between the two groups were noted. Changes in perimetry were not noted. The electroretinogram (ERG) responses consist of a series of waves and their sites of origin can be ascribed to the photoreceptors (a-waves) and the Mueller glial cells (b-waves). These b-waves, however, are not directly indicative of visual function. Indeed, a reduction in the b-waves was the only investigated endpoint in relation to Canthaxanthin exposure which showed a response. For this endpoint a threshold dose could be established: Doses equivalent or lower than 15 mg/day (0.25 mg/kg bw/day) did not result in a change in the b-wave of the ERG. Importantly, none of the other investigated endpoints for visual function showed clinical relevant changes.

Overall, it was concluded that there is no evidence to support the idea that Canthaxanthin contributes to any significant temporary or permanent ocular or visual dysfunction.

4.3.10.10 General conclusions

The lowest NOAEL obtained for systemic toxicity in laboratory animals was 5 mg/kg bw/day (2-year combined chronic / carcinogenicity study in rats which is the most sensitive species for Canthaxanthin hepatotoxicity). However, indications for hepatotoxicity were not evident in human studies.

The most critical endpoint is therefore the observation of crystalline deposits in the retina of human and monkey as well as the reduction of b-waves in the ERG in human. The BMDL₀₅ for Canthaxanthin deposits in human retina is 0.2-0.33 mg/kg bw/d. The NOEL derived for Canthaxanthin deposits in monkey eye was 0.2 mg/kg bw/day i.e. in the same range. At this dose level, no changes in the ERG were evident in monkey. From various human observations the NOEL for reduction of b-waves in the ERG of humans was 0.25 mg/kg bw/day. Importantly, these dose levels were not associated with any systemic toxicity or any clinical relevant changes in visual performance.

The most relevant and most sensitive endpoint for Canthaxanthin (crystalline deposits and reduction in b-waves in the ERG) was investigated in humans. Therefore, an uncertainty factor of 10 is applicable. SCF and JECFA deduced an ADI of 0.025 mg/kg bw/day based on the NOEL of 0.25 mg/kg bw/day and a safety factor of 10. The resulting figure of 0.025 mg/kg bw/day was rounded to 0.03 mg/kg bw/day. This ADI was confirmed in recent re-evaluations by EFSA (EFSA 2010, 2014). Also the U.S. FDA (1998) has evaluated the safety of Canthaxanthin and has determined an ADI of 150 mg/person/day (2.5 mg/kg bw/day).

4.3.11 Acceptable Daily Intake

4.3.11.1 ADI in the US

The acceptable daily intake (ADI) of canthaxanthin in the US is 150 mg/p/d (50 FR 47532 at 47533). This value was derived at the end of the evaluation carried out by FDA in 1968, on the (b) (4).

A memorandum from the Food and Drug administration, summarizing the findings of the toxicology data submitted in that dossier is herewith provided (FDA 1968). This report concluded the following:

“... The toxicological data indicate that canthaxanthin is safe as, a color additive for general use in food and drugs. The “no-effect” level in rat was 0.5% crystalline trans canthaxanthin fed in the diet for 2 years; this level with the 100-fold safety factor formed the basis for estimating the maximum acceptable daily intake for man (ADI) which is 150 mg (100 ppm of the total 1.5 kg diet)”

Subsequent submissions for (b) (4) approval of canthaxanthin as a colour additive for broilers and salmonids didn't provide toxicology data that would challenge this 150 mg/p/d value. Nevertheless, for the approval of Canthaxanthin for Salmonids (FDA 1998), and with JECFA having defined a lower ADI in 1996I, FDA indicated the following:

“..., the agency may determine, in response to a petition for an additional use of canthaxanthin, that a reevaluation of the exposure to this color additive is warranted, including consideration of the studies that JECFA considered in arriving at its 1996 ADI.”

4.3.11.2 Other ADI values

The lowest NOAEL obtained for systemic toxicity in laboratory animals presented in this dossier was 5 mg/kg bw/day (2-year combined chronic / carcinogenicity study in rats which is the most sensitive species for Canthaxanthin hepatic changes). However, indications for hepatotoxicity were not evident in human studies. The most critical endpoint is therefore the observation of crystalline deposits in the retina of human and monkey as well as the reduction of b-waves in the ERG in human. The BMDL₀₅ for Canthaxanthin deposits in human retina is 0.2-0.33 mg/kg bw/d. The NOEL derived for Canthaxanthin deposits in monkey eye was 0.2 mg/kg bw/day i.e. in the same range. At this dose level, no changes in the ERG were evident in monkey. From various human observations the NOEL for reduction of b-waves in the ERG of humans was 0.25 mg/kg bw/day.

Importantly, these dose levels were not associated with any systemic toxicity or any clinical relevant changes in visual performance (See section 4.3.9.3). The most relevant and most sensitive endpoint for Canthaxanthin (crystalline deposits and reduction in b-waves in the ERG) was investigated in human.

Therefore, an uncertainty factor of 10 is applicable. SCF (1997) and JECFA (1996) deduced an ADI of 0.025 mg/kg bw/day based on the NOEL of 0.25 mg/kg bw/day (study from Arden et al. 1989), and a safety factor of 10. The resulting figure of 0.025 mg/kg bw/day was rounded to 0.03 mg/kg bw/day.

Recently, this ADI was confirmed in a re-evaluation by EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) (EFSA 2010) and the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (EFSA 2013). A benchmark analysis was conducted by EFSA (2010) based on the meta-analysis reported by Köpcke et al. (1995). Using the Benchmark Dose Lower Confidence Limit (BMDL) to derive a point of departure from the human data, the EFSA Panel identified a BMDL₀₅ of 12-20 mg/d which equals 0.20-0.33 mg/kg bw/d for a 60 kg person. This value is consistent with the NOEL for b-wave reduction of 0.25 mg/kg bw/d.

4.3.12 Consumer Exposure to Canthaxanthin

4.3.12.1 Fate of culled eggs from poultry breeders

Fertile eggs for hatching are not aimed or destined for human consumption in the US. In the case of the EU, EFSA also confirmed that Eggs produced for breeding purposes are normally not consumed (EFSA 2013). In the US, unfertile and excess fertile eggs culled from the breeder operations are subject to the FDA Prevention of *Salmonella enteritidis* in Shell Eggs During Production, Storage and Transport rule.(Fed. Reg. 74, 33030-33101). The requirement for refrigeration of culled eggs during storage and transport to fluid egg processors has caused breeder operations to use the culled eggs as a feed ingredient because they lack the refrigerated storage facilities. However, in the event that layer operators would comply with the Egg Production Rule, the following calculation (Table 4-5) reveals that there would be minimal impact on human exposure to Canthaxanthin from the consumption of culled eggs.

Table 4-5 Theoretical Exposure from consuming culled eggs from breeder hens

Hatching eggs	Produced*: 1,065 million dozen	CXN contribution from breeder's culled eggs (mg CXN/p/d)
	Used*: 952 million dozen	
	Potentially available for human consumption: 113 million dozen	
USA Population	312,040 million of people in 2011	
	4.35 culled eggs/p/yr	
	@ 50g per culled egg ~ 217.28g of culled eggs/p/yr	
CXN Exposure from culled eggs	Residues = 5.83mg CXN/kg egg** ~ 1.26mg of CXN/p/yr	
Worst case CXN Intake from Culled Eggs		0.0035
* USDA 2011. WASDE Report for egg production, **Weber et al. 2013. Hens fed at 6mg CXN/kg Feed		

4.3.12.2 Calculation from proposed new use as antioxidant

In August 2006 the FDA published a guidance for the calculation of exposure to food ingredients entitled "Guidance for Industry: Estimating Dietary Intake of Substances in Food" (FDA 2006). One of the examples given in the guidance was the calculation for the exposure to the food Color Additive Canthaxanthin. Based upon data supplied by industry indicating that 1,050 pounds of Canthaxanthin were used annually to generally color food, FDA calculated that the per capita intake at the 90th percentile was 0.018 mg/p/d.

DSM Nutritional Products is a (b) (4) manufacturer and marketer of Canthaxanthin for use as a Food Color Additive and Feed Color Additive. Sales data from 2010 indicate that DSM sold (b) (4) of Canthaxanthin in the United States for food and feed applications. A conservative estimate of DSM's share of the total Canthaxanthin business in the United States (b) (4). Therefore it can be reasonably expected that up to 2,066.9 pounds or 1013.2 kg of Canthaxanthin were used in food and feed in 2010. The population of the United States in 2010 per the official census was 308.7 million people. Thus the per capita exposure to Canthaxanthin was 0.00898 mg/p/d; [1013.2 kg/ 365 days / 308.7 million people]. Multiplying by 2 gives the intake at the 90th percentile as 0.0179 mg /p /d. Therefore the exposure to Canthaxanthin from the current uses has not changed from what it was previously in 1985.

The proposed new use will increase human exposure to Canthaxanthin. In August 2006 FDA-CVM published a guidance (FDA 2006) on how to calculate consumption values for materials that are present in edible animal tissues. It is assumed that people will consume a portion of tissue at any eating occasion and that a portion of tissue is defined as: 300 g of muscle, 100 g of liver, 50 g of kidney and 50 g of skin/fat. Tissues from the breeders used in the target animal safety study (Weber et al. 2013) were analyzed for Canthaxanthin content following the analytical procedures explained in this GRAS dossier. The corresponding Canthaxanthin residues in tissues are presented in Table 4-6: muscle 0.00 mg/kg, liver 3.63 mg/kg, kidney 0.79 mg/kg and fat 0.90 mg/kg.

Table 4-6 Canthaxanthin deposition in eggs and tissues of egg breeders, supplemented with Canthaxanthin at 0, and 6 mg/kg feed for 24 weeks, expressed as mean ± SEM.

CXN (mg/kg feed)	Muscle CXN	Egg yolk	Liver CXN	Kidney CXN	Adipose tissue CXN	Skin CXN
0	ND	ND	ND	ND	ND	ND
6	ND	17.5±0.53	3.63±0.19	0.791±0.066	0.903±0.102	0.212±0.017

Source: Weber et al. 2013. ND = Not detectable. Data are means of 9 replicates of 2 breeders per treatment

Breeder hens that are past their usable production life in the United States are utilized for the manufacture of processed chicken products (NCC 2011). According to the USDA Annual Poultry Slaughter Report of January 25, 2011 (USDA 2011); 50,110,980,000 pounds of edible chicken tissue were produced in 2010. The same report stated that 585,511,000 pounds of edible breeder tissue were produced. Breeder tissue was therefore 1.168 % $[(585,511,000/50,110,980,000) \times 100]$ of the total edible chicken tissue in the market place. This calculation assumes that all the edible chicken tissue and breeder tissue were consumed by humans and nothing went to the animal feed or pet food industries. Table 4-7 was constructed utilizing all the above mentioned data and the exposure due to each tissue and the total possible exposure were calculated. Assuming in the worst case scenario that a person ate one portion of each tissue every day yields a consumption of 0.0052 mg/p/d.

Table 4-7 Theoretical exposure from consuming edible breeder tissues*

Consumption of Chicken Organ Meat per FDA Guidance*	Portion Size (g)	CXN Residues in Tissues (mg/kg)**	% of Organs from Breeders***	CXN contribution from breeders (mg CXN/p/d)
Liver	100	3.63	1.1684	0.0042414
Kidney	50	0.79	1.1684	0.0004615
Skin / Fat	50	0.9	1.1684	0.0005258
Muscle meat	300	0	1.1684	0
Worst case CXN Intake from Spent Hens				0.0052

* Per Guidance for Industry: Estimating Dietary Intake of Substances in Food" (FDA 2006).
 ** Weber et al. 2013. Hens fed at 6mg CXN/kg Feed
 *** Per the USDA slaughter report of January 2011, spent breeders were 1.168 % of the total chicken tonnage

4.3.12.3 Cumulative exposure to Canthaxanthin from current and proposed uses:

Consumer exposure to canthaxanthin in the US, from the proposed GRAS use and existing color uses will be below the Acceptable Daily Intake limits in the US, JECFA and the EU (Table 4-8).

Table 4-8 Cumulative canthaxanthin intake and safety margin

Intake (mg CXN/p/d)	Use as a color additive - food and feed (FDA 1998)			0.36	
	Worst case CXN Intake from GRAS use	Culled Eggs		0.0035	
		Spent Hens		0.0052	
	Total exposure from all CXN sources				0.3687
Safety Margin		ADI (mg/p/day)	Intake (mg/p/day)	% of ADI	Margin of Safety (fold)
	US	150	0.3687	0.25%	406.83

DSM1493-001



	JECFA, EU	1.8	0.3687	20.5%	4.88
--	-----------	-----	--------	-------	------

4.4 Overall Safety conclusions

The characterisation of the hazard profile of CAROPHYLL[®] Red products is in direct consideration of Canthaxanthin, its active ingredient. Canthaxanthin has been the object of numerous safety evaluations by different scientific bodies, having been thoroughly assessed worldwide, especially in the US and the EU.

In terms of tolerance, the safety evaluation of Canthaxanthin, in the form of CAROPHYLL[®] Red products, for the target species has shown that the substance is well tolerated up to at least 10 times the maximum recommended dose without any adverse effects.

Regarding consumer safety, the safety profile of Canthaxanthin has been thoroughly evaluated in the US, with an ADI of 150 mg/person/d. The proposed use does not increase the consumer exposure beyond any established ADI.

As such, the use of CAROPHYLL[®] Red for the proposed antioxidant application with poultry breeders does not considerably increase Canthaxanthin intake by the US consumer. For the most part, this is because Canthaxanthin fed to hens is mainly deposited in egg yolk and any potential exposure by consumer's intake of tissues from breeders can be expected to be low. Furthermore, fertile eggs for hatching are not aimed or destined for human consumption.

Based on this general assessment, DSM Nutritional Products considers that there are no safety concerns with regard to the proposed conditions of use for Canthaxanthin and CAROPHYLL Red[®] 10%.

DSM1493-001



5. ENVIRONMENTAL SAFETY

Canthaxanthin and CAROPHYLL Red® 10% are produced in Europe under cGMP and in compliance with local, country and EU environmental regulations. Therefore there is no sustained release of the substance into the environment and procedures are in place for addressing an accidental release.

In addition, Canthaxanthin is an approved color additive and CAROPHYLL Red® 10% is a GRAS substance when used as a nutritive antioxidant in poultry breeders' food. Per 21 CFR 25.32 (k), GRAS substances added directly to food that are intended to remain in the food through ingestion by consumers and that are not intended to replace macronutrients in food are categorically excluded from the preparation of an environmental assessment or environmental impact assessment.

For the purposes of this GRAS Notice, the consumer is a chicken.

6. ANNEXES

(b) (4)



(b) (4)



DSM1493-001



(b) (4)



6. REFERENCES

AAFCO – Official Publication Association of American Feed Control Officials Incorporated (2011): Feed ingredients Definitions 48.8 Corn Flour.

Ajuyah, A.O., Ahn, D.U., Hardin, R.T. and Sim, J.S. (1993): Dietary Antioxidants and Storage Affect Chemical Characteristics of omega-3 Fatty Acid Enriched Broiler Chicken Meats. *Journal of Food Science* 58, 43-46.

Akhtar, P., Gray, J.I., Booren, A.M. and Garling, D.L. (1998): Effect of dietary components and surface application of oleoresin rosemary on lipid stability of rainbow trout (*Oncorhynchus mykiss*) muscle during refrigerated and frozen storage. *Journal of Food Lipids*. 5, 43-58.

Akhtar, P., Gray, J.I., Gomaa, E.A. and Booren, A.M. (1998a): Effect of dietary vitamin E and surface application of oleoresin rosemary on iron/ascorbate-stimulated lipid stability in muscle tissue and microsomes from rainbow trout (*Oncorhynchus mykiss*). *Journal of Food Lipids*. 5:73–86.

Arden GB, Barker FM (1991): Canthaxanthin and the Eye: A Critical Ocular Toxicologic Assessment, *J Toxicol – Cut and Ocular Toxicol* 10 (1and2): 115-155

Arden GB, Oluwole JOA, Polkinghorne P, Bird AC, Barker FM, Norris PG, Hawk JLM (1989) Monitoring of Patients taking Canthaxanthin and Carotene: An Electroretinographic and Ophthalmological Survey, *Human Toxicol* 8: 439-450

Astorg P, Gradelet S, Leclerc J, Canivenc MC, Siess MH (1994) Effects of beta-Carotene and Canthaxanthin on Liver Xenobiotic-Metabolizing enzymes in the Rat, *Food Chem Toxicol.*, 32(8): 735-742

Astorg P, Gradelet S, Bergès R, Suschetet M (1996) No evidence for an Inhibitory effect of beta-Carotene or of Canthaxanthin on the Initiation of Liver Preneoplastic Foci by diethylnitrosamine in the Rat, *Nutr Cancer*, 25(1): 27-34

Astorg P, Gradelet S, Leclerc J, Siess MH (1997a) Effects of Provitamin A or Non-Provitamin A Carotenoids on Liver Xenobiotic-Metabolizing Enzymes in Mice, *Nutr Cancer*, 27(3): 245-249

Astorg P, Gradelet S, Leclerc J, Siess MH (1997b) Dietary Lycopene Decreases the Initiation of Liver Preneoplastic Foci by Diethylnitrosamine in the Rat, *Nutr Cancer*, 29(1): 60-68

Azuine MA, Goswami UC, Kayal JJ, Bhide SV (1992) Antimutagenic and anticarcinogenic effects of carotenoids and dietary palm oil. *Nutr. Cancer*, 17: 287–295.

Baron, C.P., Hyldig, G. and Jacobsen, C. (2009): Does feed composition affect oxidation of rainbow trout (*Oncorhynchus mykiss*) during frozen storage? *Journal of Agricultural and Food Chemistry* 57, 4185-4194.

Bast, A., Haenen, G.R.M.M., Van Den Berg, R. and Van Den Berg, H. (1998): Antioxidant effects of carotenoids. *International Journal for Vitamin and Nutrition Research* 68, 399-403.

DSM1493-001

Bendich A, Shapiro SS (1986) Effect of beta-Carotene and Canthaxanthin on the Immune Responses of the Rat, *J Nutr.* 116: 2254-2262

Bertram, J.S., Pung, A., Churley, M., Kappock, T.J., Wilkins, L.R. and Cooney, R.V. (1991) Diverse carotenoids protect against chemically induced neoplastic transformation. *Carcinogenesis*, 12: 671-678

Blount, J.D., Surai, P.F., Houdston, D.C. and Moller, A.P. (2002): Patterns of yolk enrichment with dietary carotenoids in gulls: the roles of pigment acquisition and utilization. *Functional Ecology* 16: 445- 453.

Blount, J.D., Surai, P.F., Nager, R.G., Houston, D. C., Moller, A. P., Trewby, M. L. and Kennedy, M.W. (2002a): Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: A supplemental feeding study of maternal effects. *Proceedings of the Royal Society B: Biological Sciences* 269, 29-36.

Blount, J.D., Metcalfe, N.B., Birkhead, T.R. and Surai, P.F. (2003): Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science* 300, 125-127.

Böhm, F., Edge, R., Land, E.J., McGarvey, D.J. and Truscott, T.G. (1997): Carotenoids enhance vitamin E antioxidant efficiency. *Journal of the American Chemical Society* 119, 621-622.

Britton, G., Liaaen-Jensen, S. and Pfander, H. (2004): Canthaxanthin. In: *Carotenoids handbook*, Birkhäuser Verlag, Basel; Boston. ISBN 3-7643-6180-8

Burton, G.W. (1989): Antioxidant action of carotenoids. *Journal of Nutrition* 119, 109-111.

Burton, G.W. and Ingold, K.U. (1984): β -Carotene: An unusual type of lipid antioxidant. *Science* 224, 569-573.

Butler, M.W. and McGraw, K.J. (2010): Relationships between dietary carotenoids, body tissue carotenoids, parasite burden, and health state in wild mallard (*Anas platyrhynchos*) ducklings. *Arch Biochem Biophys.* 504: 154-160.

(b) (4)

CFR (1991): 21 CFR 110 cGMP U.S. Government. Revised as of November 6, 1991.

CFR (2010a): 21 CFR 73.75 "Canthaxanthin" Color Additive for Food. U.S. Government. Revised as of April 1, 2010

(b) (4)

(b) (4)

(b) (4)

CFR (2012a): 21 CFR 73.1075 "Canthaxanthin" Color Additive for Drugs. U.S. Government. Revised as of April 1, 2010

Chan A, Ho TH, Duker JS (2006) Ultrahigh-Resolution Optical coherence Tomography of Canthaxanthine Retinal Crystals, *Ophthalmic Surgery, Lasers & Imaging*, 37(2): 138-139

Cherian, G. and Sim, J.S. (1997): Egg yolk polyunsaturated fatty acids and vitamin E content alters the tocopherol status of hatched chicks. *Poult Sci.* 76: 1753-1759.

Cherian, G., Wolfe, F.H. and Sim, J.S. (1996): Feeding dietary oils with tocopherols: Effects on internal qualities of eggs during storage. *Journal of Food Science* 61, 15-18.

Cherian, G., Traber, M. G., Goeger, M. P., and Leonard, W. S.(2007): Conjugated Linoleic Acid and Fish Oil in Laying Hen Diets: Effects on Egg Fatty Acids, Thiobarbituric Acid Reactive Substances, and Tocopherols During Storage. *Poult Sci.* 88:953-958.

Chew BP, Park JS (2004) Carotenoid Action on the Immune Response, *J Nutr* 134: 257S-261S

Chew BP, Park JS, Wong MW, Wong TS (1999a) A comparison of the anticancer activities of dietary β -Carotene, Canthaxanthin and Astaxanthin in mice in vivo. *Anticancer Res*, 19: 1849-1853

Chew BP, Wong MW, Park JS, Wong TS (1999b) Dietary beta-Carotene and Astaxanthin but not Canthaxanthin Stimulate Splenocyte Function in Mice, *Anticancer Res*, 19(6B): 5223-5228

Chopra, M., Willson, R.L. and Thurnham, D.I. (1993): Free radical scavenging activity of lutein in vitro. In: *Annals of the New York Academy of Sciences* (Cranfield, L.M., Olson, J.A. and Krinsky, N.I., eds.), Vol 691, pp. 246-249, New York.

Clark RM., Furr HC. (2001): Absorption of Canthaxanthin by the Rat Is Influenced by Total Lipid in the Intestinal Lumen, *Lipids* 36: 473-475

Clark RM, Yao L, She L, Furr HC (1998): A Comparison of Lycopene and Canthaxanthin Absorption: Using the Rat to study the Absorption of Non-Provitamin A Carotenoids, *Lipids* 33(2): 159-163

Clark, T.H., Faustman, C., Chan, W.K.M., Furr, H.C. and Riesen, J.W. (1999): Canthaxanthin as an antioxidant in a liposome model system and in minced patties from rainbow trout. *Journal of Food Science* 64, 982-986.

Cobb (2012): Accessed Online on 2012.10.25: http://www.poultryhub.org/wp-content/uploads/2012/05/Poster_Chick_Embryo_Dev_English.pdf

Conn, P.F., Schalch, W. and Truscott, T.G. (1991): The singlet oxygen and carotenoid interaction. *Journal of Photochemistry and Photobiology, B: Biology* 11, 41-47.

Costantini, D. and Møller, A.P. (2008): Carotenoids are minor antioxidants for birds. *Functional Ecology* 22: 367-370.

Daicker B, Schiedt K, Adnet JJ, Bermond P (1987) Canthaxanthin retinopathy – an investigation by light and electron microscopy and physiochemical analysis, *Graefe's Arch Clin Exp Ophthalmol* 225(3): 189-197

De Flora S, Bagnasco M, Vainio H (1999) Modulation of genotoxic and related effects by carotenoids and vitamin A in experimental models: mechanistic issues, *Mutagenesis*, 14(2): 153-172

Denison MS, Heath-Pagliuso S (1998) The Ah receptor: a regulator of the biochemical and toxicological actions of structurally diverse chemicals, *Bull Environ Contam Toxicol.*, 61(5): 557-568

DiMascio, P., Kaiser, S. and Sies, H. (1989): Lycopene as the most efficient biological carotenoid singlet oxygen quencher *Archives of Biochemistry and Biophysics*. *Archives of Biochemistry and Biophysics* 274 (2): 532-538.

EC (2008): COMMISSION REGULATION (EC) No 775/2008 of 4 August 2008 establishing maximum residue limits for the feed additive canthaxanthin in addition to the conditions provided for in Directive 2003/7/EC. *Official Journal of the European Union*.

Edge, R., McGarvey, D.J. and Truscott, T.G. (1997): The carotenoids as anti-oxidants - a review. *Journal of Photochemistry and Photobiology* 41B: 189-200.

Edge, R., El-Agamey, A., Land, E.J., Navaratnam, S. and George Truscott, T. (2007): Studies of carotenoid one-electron reduction radicals. *Archives of Biochemistry and Biophysics* 458, 104-110.

EFSA (2007): Opinion of the Scientific Panel of Additives and Products or Substances used in Animal Feed on the Maximum Residue Limits for Canthaxanthin in Foodstuffs coming from Animals fed with Canthaxanthin used as Feed Additive in Accordance with Council Directive 70/524/EEC, adopted on 14 June 2007, *The EFSA Journal* 507: 1-18

EFSA (2010): Scientific Opinion on the re-evaluation of Canthaxanthin (E 161 g) as a food additive, *The EFSA Journal* 8(10): 1852

EFSA (2010a): EFSA Panel on Animal Health and Welfare (AHAW): Scientific Opinion on welfare aspects of the management and housing of the grand-parent and parent stocks raised and kept for breeding purposes. *EFSA Journal* 2010; 8(7):1667. [81 pp.]

EFSA (2013): EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Scientific opinion on the safety and efficacy of CAROPHYLL® Red 10% (preparation of canthaxanthin) for all poultry for breeding purposes (chickens, turkeys and other poultry). *EFSA Journal* 2013;11(1):3047.

EFSA (2014) Scientific Opinion on the Safety and efficacy of Canthaxanthin as feed additive for poultry and for ornamental birds and ornamental fish, *The EFSA Journal*, 12(1):3527

Elmadfa I, Majchrzak D (1999) Absorption und Transport von Astaxanthin und Canthaxanthin beim Menschen nach einer Lachsmahlzeit, *Ernährungs-Umschau*, 46(5): 173-177

EP (2005): *European Pharmacopoeia 5.0*. - 2.2.1. Clarity and degree of opalescence of liquids.

Espaillat A, Aiello LP, Arrigg PG, Villalobos R, Silver PM, Cavicchi RW (1999) Canthaxanthine retinopathy, *Arch Ophthalmol.*, 117(3): 412-413

Everett, S.A., Dennis, M.F., Patel, K.B., Maddix, S., Kundu, S.C. and Willson, R.L. (1996): Scavenging of nitrogen dioxide, thiyl, and sulfonyl free radicals by the nutritional antioxidant β -carotene. *Journal of Biological Chemistry* 271, 3988-3994.

(b) (4)

FDA (1972): Canthaxanthin as a Chicken feed Additive. Color Additive Petition No. 101. Memorandum from December 29, 1972.

FDA (1998): Listing of Color Additives Exempt from Certification; Canthaxanthin, *Federal Register* 63(59): 14814-14817.

FDA (2006): Guidance for Industry: Estimating Dietary Intake of Substances in Food. U.S. Department of Health and Human Services Food and Drug Administration. Center for Food Safety and Applied Nutrition. August 2006.

FEFANA (2010): Canthaxanthin –Analytical method in Poultry tissues. FEFANA.

FEFANA (2011a): Canthaxanthin –Analytical method in CAROPHYLL Red 10%. FEFANA.

FEFANA (2011b): Canthaxanthin –Analytical method in Premix and Feed. FEFANA.

FEFANA (2011c): Canthaxanthin –Analytical method in Egg Yolk. FEFANA.

Foote, C.S. and Denny, R.W. (1968): Chemistry of singlet oxygen. VII. Quenching by β -carotene [14]. *Journal of the American Chemical Society* 90, 6233-6235.

Foote, C.S., Chang, Y.C. and Denny, R.W. (1970): Chemistry of singlet oxygen. X. Carotenoid quenching parallels biological protection. *Journal of the American Chemical Society* 92, 5216-5218.

Franchini, A., Sirri, F., Tallarico, N., Minelli, G., Iaffaldano, N. and Meluzzi, A. (2002): Oxidative stability and sensory and functional properties of eggs from laying hens fed supranutritional doses of vitamins E and C. *Poultry science* 81, 1744-1750.

Frankel, E.N. (1989): The antioxidant and nutritional effects of tocopherols, ascorbic acid and beta-carotene in relation to processing of edible oils. *Bibliotheca nutritio et dieta*, 297-312.

Fraunfelder FW (2004) Ocular Side Effects from Herbal Medicines and Nutritional Supplements, *Am J Ophthalmol*, 138: 639-647

Fraunfelder FW (2005) Ocular Side Effects associated with Dietary Supplements and Herbal Medicines, *Drugs of Today*, 41(8): 537-545

Gaál, T., Mézes, M., Noble, R.C., Dixon, J. and Speake, B.K. (1995): Development of antioxidant capacity in tissues of the chick embryo. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* 112, 711-716.

Galobart, J., Barroeta, A.C., Baucells, M.D., and Guardiola, F. (2001): Effect of dietary supplementation with rosemary extract and alfa-tocoferil acetate on lipid oxidation in eggs enriched with 3-fatty acids. *Poultry Science*. 80: 460-467.

Goralczyk, R., Buser, S., Bausch, J., Bee, W., Zühlke, U., Barker, F.M. (1997): Occurrence of Birefringent Retinal Inclusions in Cynomolgus Monkeys After High Doses of Canthaxanthin, *Investigative Ophthalmology and Visual Science* 38 (3): 741 – 752.

Goralczyk, R., Barker, F.M., Buser, S., Liechti, H., Bausch, J. (2000): Dose Dependency of Canthaxanthin Crystals in Monkey Retinal and Spatial Distribution of its Metabolites, *Investigative Ophthalmology and Visual Science* 41 (6): 1513 – 1522.

Gradelet S, Astorg P, Leclerc J, Chevalier J, Vernevault MF, Siess MH (1996) Effects of Canthaxanthin, Astaxanthin, Lycopene and lutein on liver-metabolizing enzymes in the rat, *Xenobiotica* 26(1): 49-63

Gradelet S, Astor P, Le Bon AM, Berges R, Suschetet M (1997a) Modulation of aflatoxin B₁ carcinogenicity, genotoxicity and metabolism in rat liver by dietary carotenoids: evidence for a protective effect of CYP1A inducers. *Cancer Lett.*, 114: 221–223

Gradelet S, Astorg P, Pineau T, Canivenc MC, Siess MH, Leclerc J, Lesca P (1997b) Ah Receptor-Dependent CYP1A Induction by Two Carotenoids: Canthaxanthin and beta-apo-8'-carotenal, With No Affinity for the TCDD binding site, *Biochem Pharmacol.* 54(2): 307-315

Gradelet S, Le Bon AM, Bergès R, Suschetet M, Astorg P, (1998) Dietary carotenoids inhibit aflatoxin B₁-induced liver preneoplastic foci and DNA damage in the rat: Role of the modulation of aflatoxin B₁ metabolism, *Carcinogenesis* 19(3): 403-411

Grashorn, M.A. and Steinberg, W. (2002): Deposition rates of Canthaxanthin in egg yolks. *Archiv fur Geflügelkunde* 66, 258-262.

Grolier P, Duszka C, Borel P, Alexandre-Gouabau MC, Azais-Braesco V (1997) In Vitro and in Vivo Inhibition of beta-Carotene Dioxygenase Activity by Canthaxanthin in Rat Intestine, *Arch Biochem Biophys.*, 348(2): 233-238

Hageman SH, She L, Furr HC, Clark RM (1999) Excess Vitamin E Decreases Canthaxanthin Absorption in the Rat, *Lipids* 34(6): 627-631

Hallagan JB, Allen DC, Borzelleca JF (1995) The Safety and Regulatory Status of Food, Drug and Cosmetics Colour Additives Exempt from Certification, *Food Chem Toxicol.* 33(6): 515-528

Haq, A.U., Bailey, C.A. and Chinnah, A.D. (1995): Neonatal immune response and growth performance of chicks hatched from single comb White Leghorn breeders fed diets supplemented with beta-carotene, Canthaxanthin, or lutein. *Poultry science* 74, 844-851.

Harnois C, Samson J, Malenfant M, Rousseau, A (1989) Canthaxanthin Retinopathy. Anatomic and Functional Reversibility, *Arch Ophthalmol* 107: 538 – 540

He Y, Campbell C (1990) Effects of carotenoids on aflatoxin B1-induced mutagenesis in *S. typhimurium* TA100 and TA98. *Nutr. Cancer* 13: 243–253.

Hencken, H. (1992): Chemical and physiological behavior of feed carotenoids and their effects on pigmentation. *Poultry science* 71, 711-717.

Hogg, N. and Kalyanaraman, B. (1999): Nitric oxide and lipid peroxidation. *Biochimica et Biophysica Acta - Bioenergetics* 1411, 378-384.

Hueber, A., Rosentreter, A. and Severin, M. (2011): Canthaxanthin retinopathy: Long-term observations. *Ophthalmic research* 46, 103-106.

IARC (1998) IARC Handbooks of Cancer Prevention, Vol. 2, Carotenoids. International Agency for Research on Cancer, Lyon, France.

ILSI (1999): Safety Assessment and potential health benefits of food components based on selected scientific criteria. ILSI North America Technical Committee on Food Components for Health Promotion, *Crit Rev Food Sci Nutri* 39(3): 203-316.

Isler, O., Gutmann, H. and Solm, U. (1971): Introduction. In: *Carotenoids.*, pp. 11-27, Birkhauser Verlag Basel.

JECFA (1996): Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series No. 35 (839).

Jewell C, O'Brian NM (1999) Effect of dietary supplementation with carotenoids on xenobiotic metabolizing enzymes in the liver, lung, kidney and small intestine of the rat, *Brit J Nutr.*, 81(3): 235-242

Jorgensen, K. and Skibsted, L.H. (1993): Carotenoid scavenging of radicals. *Zeitschrift für Lebensmittel Untersuchung und Forschung.* 196: 423-429.

Karadas, F., Pappas, A.C., Surai, P.F. and Speake, B.K. (2005): Embryonic development within carotenoid-enriched eggs influences the post-hatch carotenoid status of the chicken. *Comp Biochem Physiol B Biochem Mol Biol.* 141: 244-251.

Kennedy, T.A. and Liebler, D.C. (1991): Peroxyl radical oxidation of β -carotene: Formation of β -carotene epoxides. *Chemical research in toxicology* 4, 290-295.

Kennedy, T.A. and Liebler, D.C. (1992): Peroxyl radical scavenging by β -carotene in lipid bilayers. Effect of oxygen partial pressure. *Journal of Biological Chemistry* 267, 4658-4663.

Köpcke W, Barker FM, Schalch W (1995): Canthaxanthin Deposition in the Retina. A Biostatistical Evaluation of 411 Patients, *J Toxicol – Cut and Ocular Toxicol* 14(2): 89-104

Koutsos, E.A., Clifford, A.J., Calvert, C.C. and Klasing, K.C. (2003): Maternal carotenoid status modifies the incorporation of dietary carotenoids into immune tissues of growing chickens (*Gallus gallus domesticus*). *Journal of Nutrition* 133, 1132-1138.

Krinsky, N.I. (1989). Carotenoids in medicine. In: *Carotenoids: Chemistry and Biology*, Edited by: Krinsky, N.I., Mathews-Roth, M.M. and Taylor, R.F., Plenum Press, New York and London, pp. 279-291.

Krinsky, N.I. (1989a): Antioxidant functions of carotenoids. *Free Radical Biology and Medicine* 7, 617-635.

Krinsky, N.I. and Deneke, S.M. (1982): Interaction of oxygen and oxy-radicals with carotenoids. *Journal of the National Cancer Institute* 69, 205-210.

Kübler W, von Reinersdorf D (2002) *Praktikum der Nährstoff-Biokinetik* (6), *Ernährungs-Umschau* 49(9): 353-356

Kumar A, Srikanta AH, Muthukumar SP, Sukumaran UK, Govindaswamy V (2011): Antioxidant and lipid peroxidation activities in rats fed *Aspergillus Carbonarius* carotenoid. *Food Chem Toxicol.* 49: 3098-3103

Kumar A, Srikanta AH, Peddha MS, Sukumaran UK, Govindaswamy V (2012): A short-term toxicity study of *Aspergillus Carbonarius* carotenoid. *Int J Toxicol.* 31(2): 158-65

Latour, M.A., Devitt, A.A., Meunier, R.A., Stewart, J.J. and Watkins, B.A. (2000): Effects of conjugated linoleic acid. 2. Embryonic and neonatal growth and circulating lipids. *Poultry science* 79, 822-826.

Leyon H, Ros AM, Nyberg S, Algvere P (1990) Reversibility of Canthaxanthin Deposits within the Retina, *Acta Ophthalmologica* 68: 607 – 611

Lim, B.P., Nagao, A., Terao, J., Tanaka, K., Suzuki, T. and Takama, K. (1992). Antioxidant activity of xanthophylls on peroxy radical-mediated phospholipid peroxidation. *Biochimica et Biophysica Acta* 1126: 178-184.

Laurado, L.I., Francesch, A., Hernandez, J.M. & Brufau, J., (1997): Effect of canthaxanthin supplementation on the hatchability of eggs of broiler breeders. *Proceedings 11th European Symposium Poultry Nutrition, Denmark*: 280- 282.

Mathews-Roth MM. (1982) Antitumor activity of beta-carotene, Canthaxanthin and phytoene. *Oncology.* 39(1): 33-37.

Mathews-Roth MM, Krinsky NI (1987) Carotenoids affect development of UV-B induced skin cancer. *Photochem Photobiol.* 46(4): 507-509

DSM1493-001



Mayne, S.T. and Parker, R.S. (1989): Antioxidant activity of dietary Canthaxanthin. *Nutrition and Cancer* 12: 225-236.

McGraw, K.J. and Toomey, M.B. (2010): Carotenoid accumulation in the tissues of zebra finches: predictors of integumentary pigmentation and implications for carotenoid allocation strategies. *Physiol Biochem Zool.* 83: 97-109.

Monaghan, P., Metcalfe, N.B. and Torres, R. (2009): Oxidative stress as a mediator of life history trade-offs: Mechanisms, measurements and interpretation. *Ecology Letters* 12, 75-92.

Nadim F, Walid H, Adib J (2002) The differential diagnosis of crystals in the retina, *Int Ophthalmol.*, 24(3): 113-121

NCC (2011): Consumer information. About chickens. National Chicken Council. <http://www.nationalchickencouncil.com/consumerInfo/detail.cfm?id=11>

Noble, R.C. and Cocchi, M. (1990): Lipid metabolism and the neonatal chicken. *Progress in lipid research* 29, 107-140.

Noble, R.C., Lonsdale, F., Connor, K. and Brown, D. (1986): Changes in the lipid metabolism of the chick embryo with parental age. *Poultry science* 65, 409-416.

OECD 1995a. OECD 102 Melting Point Method.

OECD 1995b. OECD 105 Water Solubility Method.

Ohkawa, H., Ohishi, N., Yagi, K. (1979): Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry* 95, 351-358.

Okai Y, Higashi-Okai K, Nakamura S-I, Yano Y, Otani S (1996) Suppressive effects of retinoids, carotenoids and antioxidant vitamins on heterocyclic amine-induced umu C gene expression in *Salmonella typhimurium* (TA1535/pSK 1002). *Mutat. Res.*, 368: 133–140.

Ong, A.S.H., Tee, E.S. (1992): Natural sources of carotenoids from plants and oils. *Methods in Enzymology* 213, 142-167.

Paetau I, Chen H, Goh NM, White WS (1997) Interactions in the postprandial appearance of beta-carotene and Canthaxanthin in plasma triacylglycerol-rich lipoproteins in humans, *Am J Clin Nutr.*, 66(5): 1133-1143

Palozza, P. (1998): Prooxidant actions of carotenoids in biologic systems. *Nutrition reviews* 56, 257-265.

Palozza, P. and Krinsky, N.I. (1992): Antioxidant effects of carotenoids in vivo and in vitro: An overview. *Methods in Enzymology* 213, 403-420.

Palozza, P., Luberto, C., Ricci, P., Sgarlata, E., Calviello, G. and Bartoli, G.M. (1996): Effect of beta-carotene and Canthaxanthin on tert-butyl hydroperoxide-induced lipid peroxidation in murine normal and tumor thymocytes. *Archives in Biochemistry and Biophysics* 325: 145-151.

Palozza P, Maggiano N, Calviello G, Lanza P, Piccioni E, Ranelletti FO, Bartoli GM (1998) Canthaxanthin induces apoptosis in human cancer cell lines, *Carcinogenesis* (1998) 19(2): 373-376

Palozza, P., Calviello, G., De Leo, M.E., Serini, S. and Bartoli, G.M. (2000): Canthaxanthin supplementation alters antioxidant enzymes and iron concentration in liver of Balb/c mice. *Journal of Nutrition* 130, 1303-1308.

Papastergiadis, A., Mubiru, E., Van Langenhove, H., De Meulenaer, B. (2012): Malondialdehyde Measurement in Oxidized Foods: Evaluation of the Spectrophotometric Thiobarbituric Acid Reactive Substances (TBARS) Test in Various Foods. *J. Agric. Food Chem.* 2012, 60, 9589–9594

Paust, J. (1996): Synthesis Part VII, In: Britton, G., Liaaen-Jensen, S. and Pfander, H. (eds.) *Carotenoids, Vol 2: Synthesis*, pp. 267-269, Birkhäuser, ISBN 3-7643-5297-3.

Pfander, H. (1992): *Carotenoids: An overview. Methods in Enzymology* 213, 3-13.

Prager, M., Johansson, E.I.A. and Andersson, S. (2009): Differential ability of carotenoid C4-oxygenation in yellow and red bishop species (*Euplectes* spp.). *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* 154, 373-380.

Pung A, Rundhaug JE, Yoshizawa CN, Bertram JS (1988) b-Carotene and Canthaxanthin inhibit chemically- and physically-induced neoplastic transformation in 10T1/2 cells. *Carcinogenesis*, 9: 1533–1539.

Rasquin F (2007) Les Rétinopathies Cristallines, *Bull Soc Belge Ophtalmol.*, 304: 77-82

Rengel, D., Díez-Navajas, A., Serna-Rico, A., Veiga, P., Muga, A. and Milicua, J.C.G. (2000): Exogenously incorporated ketocarotenoids in large unilamellar vesicles. Protective activity against peroxidation. *Biochimica et Biophysica Acta - Biomembranes* 1463, 179-187.

Rice-Evans, C.A., Samson, J., Bramley, P.M. and Holloway, D.E. (1997): Why do we expect carotenoids to be antioxidants in vivo? *Free Radical Research* 26: 381-398.

Riedl J, Lineisen J, Hoffmann J, Wolfram G (1999) Some dietary fibers reduce the absorption of carotenoids in women, *J Nutr.*, 129: 2170-2176

Robert, F., Panheleux- Le Bastard, M., Hamelin, C. and Boulard, C. (2007): Effect of Canthaxanthin supplementation in the ROSS breeder diet on oxidative stress in chick. *Proc. 16th European Symposium on Poultry Nutrition, France*, pp. 731-734.

Rocha, J.S.R., Lara, L.J.S., Baiao, N.C., Vasconcelos, R.J.C., Barbosa, V.M., Pompeu, M.A. and Fernandes, M.N.S. (2010): Antioxidant properties of vitamins in nutrition of broiler breeders and laying hens. *World's Poultry Science Journal* 66: 261- 270.

Rocha, J., Baiao, N., Lana, A., Cancado, S. (2011): The Impacts Produced by Dietary Canthaxanthin added to the Diet of Broiler Breeders at Old Age and by the Egg Storage Period on Fertility, Incubation Yield, yolk nutrients and embryonic development. Original: Thesis for Doctor, Federal University of Minas Gerais, Brazil.

Rocha, J., Barbosa, V., Lara, L., Baiao, N., Cançado, S. Lana, A., Pompeu, M., Vasconcelos, R., Machado, A., Miranda, D., Fernandes, M., Mendes, P. (2013): The effect of storage and dietary canthaxanthin on fertile egg quality and embryonic development. *Arq. Bras. Med. Vet. Zootec.*, v.65, n.3, p.792-800.

Rock, C.L. (1997): Carotenoids: Biology and treatment. *Pharmacology and Therapeutics* 75, 185-197.

Rosa, A.P., Scher, A., Sorbara, J.O.B., Boemo, L.S., Forgiarini, J. and Londero A. (2012): Effects of Canthaxanthin on the Productive and Reproductive Performance of Broiler Breeders. *Poultry Science* 91, 660–666

Royle, N.J., Surai, P.F., McCartney, R.J. and Speake, B.K. (1999): Parental investment and egg yolk lipid composition in gulls. *Functional Ecology* 13, 298-306.

Royle, N.J., Surai, P.F. and Hartley, I.R. (2001): Maternally derived androgens and antioxidants in bird eggs: Complementary but opposing effects? *Behavioral Ecology* 12, 381-385.

Rubolini, D., Romano, M., Bonisoli Alquati A. and Saino, N. (2006): Early maternal, genetic and environmental components of antioxidant protection, morphology and immunity of yellow-legged gull (*Larus michahellis*) chicks. *Journal of Evolutionary Biology* 19: 1571-1584.

Ruiz, J.A., Perez-Vendrell, A.M., Esteve-Garcia, E. (1999): Effect of B-Carotene and Vitamin E on Oxidative Stability in Leg Meat of Broilers Fed Different Supplemental Fats. *Journal of Agricultural and Food Chemistry* 47, 448-454.

Santaella RM, Fraunfelder FW (2007) Ocular Adverse Effects Associated with Systemic Medications, *Drugs* 67(1): 75-93

Santamaria L, Bianchi A, Arnaboldi A, Andreoni L, Bermond P (1983) Dietary carotenoids block photocarcinogenic enhancement by benzo(a)pyrene and inhibit its carcinogenesis in the dark. *Experientia*. 39(9): 1043-1045.

Santamaria L, Bianchi A, Arnaboldi A, Ravetto C, Bianchi L, Pizzala R, Andreoni L, Santagati G, Bermond P (1988) Chemoprevention of indirect and direct chemical carcinogenesis by carotenoids as oxygen radical quenchers. *Ann. NY Acad. Sci.*, 534: 584–596.

Satoh, K (1978): Serum lipid peroxide in cerebrovascular disorders determined by has new colorimetric method. *Covering joint Chim Acta* 15; 90(1), 37-43.

SCAN (2002): Opinion of the Scientific Committee on Animal Nutrition on the use of Canthaxanthin in feedingstuffs for salmon and trout, laying hens, and other poultry. May 3rd, 2002. http://ec.europa.eu/food/fs/sc/scan/out81_en.pdf

SCF (1997): Scientific Committee on Food, Opinion on Canthaxanthin, expressed on 13 June 1997

(b) (4)

Schiedt, K. (1998): Absorption and Metabolism of carotenoids in birds, fish and crustaceans. In: Carotenoids. (Britton, G., Liaaen-Jensen, S. and Pfander, eds.), H. Birkhauser Verlag, Basel-Boston-Berlin.

Schwartz JL, Shklar G (1997) Retinoid and Carotenoid Angiogenesis: A Possible Explanation for Enhanced Oral Carcinogenesis, *Nutr Cancer*, 27(2): 192-199

SCMPMD (1998): Scientific Committee on Medicinal Products and Medical Devices, Scientific "Opinion" on Colouring Agents Amaranth, Erythrosine and Canthaxanthine, *Drugs made in Germany* 41 (4): 117

Shih, C.K., Chang, J.H., Yang, S.H., Chou, T.W. and Cheng, H.H. (2008): β -Carotene and Canthaxanthin alter the pro-oxidation and antioxidation balance in rats fed a high-cholesterol and high-fat diet. *British Journal of Nutrition* 99, 59-66.

Souza et al., (2008): Effect of the use of CAROPHYLL® Red on reproductive indices in broiler breeders APINCO – Poultry Science and Technology Conference Proceedings. 27-29 of May 2008. Santos. SP.

Speake, B.K., Murray, A.M.B. and C. Noble, R. (1998a): Transport and transformations of yolk lipids during development of the avian embryo. *Progress in lipid research* 37, 1-32.

Speake, B.K., Noble, R.C. and Murray, A.M.B. (1998b): The utilization of yolk lipids by the chick embryo. *World's Poultry Science Journal* 54, 331-334.

Stahl, W. and Sies, H. (1993): Physical quenching of singlet oxygen and cis-trans isomerization of carotenoids. *Annals of the New York Academy of Sciences* 691, 10-19.

Stich HF, Stich W, Rosin MP, Vallejera MO (1984) Use of the micronucleus test to monitor the effect of Vitamin A, beta-Carotene and Canthaxanthin on the buccal mucosa of betel nut / tobacco chewers, *Int J cancer*, 34: 745-750

Sujak, A., Gabrielska, J., Milanowska, J., Mazurek, P., Strzałka, K. and Gruszecki, W.I. (2005): Studies on Canthaxanthin in lipid membranes. *Biochim Biophys Acta*. 1712: 17-28.

Surai, P.F. (1999a): Vitamin E in Avian Reproduction. *Avian and Poultry Biology Reviews* 10, 1-60.

Surai, P.F. (1999b): Tissue-specific changes in the activities of antioxidant enzymes during the development of the chicken embryo. *British Poultry Science* 40, 397-405.

Surai, P.F. (2000): Effect of selenium and vitamin E content of the maternal diet on the antioxidant system of the yolk and the developing chick. *British Poultry Science* 41, 235-243.

Surai, P.F. (2002): Selenium in poultry nutrition 1. Antioxidant properties, deficiency and toxicity. *World's Poultry Science Journal* 58, 333-347.

Surai, P.F. (2012a): The antioxidant properties of Canthaxanthin and its potential effects in the poultry eggs and on embryonic development of the chick. Part 1. *Worlds Poult Sci J* 68, 465-475.

Surai, P.F. (2012b): The antioxidant properties of Canthaxanthin and its potential effects in the poultry eggs and on embryonic development of the chick. Part 2. *Worlds Poult Sci J* 68, 717-726.

Surai, P.F. and Fisinin, V.L. (2012): Feeding breeders to avoid oxidative stress in embryos. *World's Poultry Congress August 2012*.

Surai, P.F. and Sparks, N.H.C. (2001): Comparative evaluation of two maternal diets on fatty acids, vitamin E and carotenoids in the chick embryo. *British Poultry Science* 42: 252-259.

Surai, P.F. and Speake, B.K. (1998): Distribution of carotenoids from the yolk to the tissues of the chick embryo. *Journal of Nutritional Biochemistry* 9: 645-651.

Surai, P.F., Noble, R.C. and Speake, B.K. (1996): Tissue-specific differences in antioxidant distribution and susceptibility to lipid peroxidation during development of the chick embryo. *Biochim Biophys Acta* 1304: 1-10.

Surai, P.F., Speake, B.K., Noble, R.C. and Sparks, N.H.C. (1997): Antioxidant systems of the developing chicken embryo: Glutathione peroxidase. *British Poultry Science* 38, S19-S20.

Surai, P.F., Royle, N.J. and Sparks, N.H.C. (2000): Fatty acid, carotenoid and vitamin A composition of tissues of free living gulls. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* 126, 387-396.

Surai, P.F., Speake, B.K., Wood, N.A.R., Blount, J.D., Bortolotti, G.R. and Sparks, N.H.C. (2001): Carotenoid discrimination by the avian embryo: A lesson from wild birds. *Comp. Biochem. Physiol.* 128B, 4: 743-750.

Surai, P.F., Speake, B.K. and Sparks, N.H.C. (2001a): Carotenoids in avian nutrition and embryonic development. 2. Antioxidant properties and discrimination in embryonic tissues. *Journal of Poultry Science* 38: 117-145.

Surai, A.P., Surai, P.F., Steinberg W., Wakeman, W.G., Speake, B.K. and Sparks, N.H.C. (2003): Effect of Canthaxanthin content of the maternal diet on the antioxidant system of the developing chick. *British Poultry Science* 44: 612-619.

Tanaka T, Makita H, Ohnishi M, Mori H, Satoh K, Hara A (1995) Chemoprevention of Rat Oral carcinogenesis by Naturally Occurring Xanthophylls, Astaxanthin and Canthaxanthin, *Cancer Res* 55: 4059-4064

Tanaka T, Shnimizu M, Moriwaki H (2012) Cancer chemoprevention by carotenoids. *Molecules* 17(3):3202-3242

Terao, J. (1989): Antioxidant activity of β -carotene-related carotenoids in solution. *Lipids* 24: 659-661.

Terao, J., Boey, P.L., Ojima, F., Nagao, A., Suzuki, T. and Takama, K. (1992): Astaxanthin as a chain-breaking antioxidant in phospholipid peroxidation. In: *Oxygen Radicals*, Edited by : Yagi, K., Kondo, M., Niki, E. and Yoshikawa, T., New York: Elsevier Science Publishers; pp. 657-660.

Thomas P, Wu J, Dhillon V, Fenech M (2011) Effect of dietary intervention on human micronucleus frequency in lymphocytes and buccal cells, *Mutagenesis*, 26(1): 69-76

Tullett, S.G. (1990): Science and the art of incubation. *Poultry science* 69, 1-15.

Tyczkowski, J. K., Hamilton, P. B., (1986): Canthaxanthin as a model for the study of utilization of oxycarotenoids by chickens. *Poultry Sci.* 65, 1335-1356.

Tyczkowski, J.K., Yagen B. and Hamilton P.B. (1988): Metabolism of canthaxanthin, a red diketocarotenoid, by chickens. *Poultry Sci.* 67, 787-793.

USDA (2011): *Poultry Slaughter* (Released January 25, 2011) National Agricultural Statistics Service (NASS), Agricultural Statistics Board, United States Department of Agriculture (USDA). ISSN: 1949-1581

Vainio, H. and Rautalahti, M. (1998): An international evaluation of the cancer preventive potential of carotenoids. *Cancer Epidemiology Biomarkers and Prevention* 7, 725-728.

Vleck, C.M. and Bucher, T.L. Energy metabolism, gas exchange, and ventilation. In: *Avian growth and development: evolution within the altricial-precocial spectrum.*, pp. 89-116, Oxford University Press, Oxford.

Wilson HR. (1997): Effects of maternal nutrition on hatchability. *Poult Sci.* 76: 134-43.

Wilson, J.X., Lul, E.M.K. and Del Maestro, R.F. (1992): Developmental profiles of antioxidant enzymes and trace metals in chick embryo. *Mechanisms of ageing and development* 65, 51-64.

Zhang, L.X., Cooney, R.V. and Bertram, J.S. (1991): Carotenoids enhance gap junction communication and inhibit lipid peroxidation in C3H/10T1/2 cells: relationship to the cancer chemopreventative action. *Carcinogenesis* 12: 2109-2114.

Zhang, W., Zhang, K.Y. Ding, X.M., Bai, S.P., Hernandez, J.M., Yao, B., and Q. Zhu, Q. (2011): Influence of Canthaxanthin on broiler breeder reproduction, chick quality, and performance. *Poultry Science* 90: 1516–1522.

Zhao, W., Han, Y., Zhao, B., Hirota, S., Hou, J. and Xin, W. (1998): Effect of carotenoids on the respiratory burst of rat peritoneal macrophages. *Biochimica et Biophysica Acta - General Subjects* 1381, 77-88.

Zheng YF, Bae SH, Kwon MJ, Park JB, Choi HD, Shin WG, Bae SK (2013) Inhibitory effect of astaxanthin, β -cryptoxanthin, Canthaxanthin, lutein, and zeaxanthin on cytochrome P450 enzyme activities. *Food Chem Toxicol.* 59: 78-85

7. GRAS EXPERT PANEL OPINION

7.1 Background

This GRAS notification is an updated version of a GRAS conclusion reviewed by a GRAS experts in December, 2011. Compared to the 2011 dossier, this 2014 version for the use of canthaxanthin as a nutritive antioxidant for poultry breeder diets, takes into consideration data on the safety and utility of canthaxanthin published since December 2011 and includes refined consumer exposure calculations.

Based on the safety assessment from 2011, the GRAS Panel considered that there are no safety concerns with regard to the proposed conditions of use for Canthaxanthin and CAROPHYLL[®] Red 10% and that the intended use of this product is safe and GRAS. No data published from 2011 until 2014 contradict the panel's conclusions from 2011. The panel had access to unpublished study reports and DSM has included a list of those reports in the [Annex Unpublished Studies Reports](#) and are available for review by FDA upon request as discussed at our meeting at the CVM offices in Rockvill, MD on 11 March 2014.

The 2011 Opinion from the Panel on the proposed GRAS use of canthaxanthin is provided below.

DSM1493-001



This Page Intentionally Blank

DSM1493-001



This Page Intentionally Blank

DSM1493-001



This Page Intentionally Blank

This Page Intentionally Blank

This Page Intentionally Blank

Summary of the GRAS Panel

REPORT OF THE EXPERT PANEL ON THE SAFETY AND THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF THE PROPOSED USES OF CANTHAXANTHIN (CAROPHYLL® RED) (β-CAROTENE-4,4'-DIONE) IN THE FOOD OF BREEDING HENS

Introduction

DSM Nutritional Products convened 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") to evaluate the safety and Generally Recognized as Safe (GRAS) status of the proposed use in certain poultry breeder feed rations of Canthaxanthin (trade name Carophyll® Red) (β-Carotene-4,4'-Dione) as an antioxidant as described in Title 21 of the Code of Federal Regulations (21CFR§170.30) (U.S. FDA, 2007). Canthaxanthin will be used at a level no greater than 6 mg/kg feed (60 mg/kg feed for Carophyll® Red). Canthaxanthin is currently authorized for use as a Color Additive in the US, where its safety and use have been confirmed by the FDA for the following applications per 21 CFR §73.75: *"to enhance the yellow color of broiler chicken skin; to enhance the pink to orange-red color of flesh of salmonids fish; for use as a general food color in solid and semi-solid food and liquid food; and per 21 §CFR 73.1075, for coloring ingested drugs generally in amounts consistent with good manufacturing practice"*. DSM does not intend to exceed the current statutory limits for use of Canthaxanthin and merely seeks to expand its permitted use in breeder poultry rations.

A comprehensive search of the scientific literature for safety and toxicity information on Canthaxanthin was conducted by DSM Nutritional Products through August, 2011. The following databases were searched: NCBI Pubmed, SciFinder, Scopus, TOXNET, OECD eChem Portal, RTECS, IPCS INCHEM, NTP (National Toxicology Program), ILSI (International Life Sciences Institute) and BIBRA toxicity profiles and the DSM RDR for internal reports. The result of these searches can be found in the Annex as search 1 and search 2. All relevant publications were reviewed, summarized and incorporated into a GRAS dossier, "THE SAFETY AND THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF THE PROPOSED USES OF CANTHAXANTHIN (CAROPHYLL® RED) (β-CAROTENE-4,4'-DIONE) IN THE FOOD OF BREEDING HENS," assimilated by The (b) (4) Group, and submitted to the Expert Panel. Copies of the literature were available for the Expert Panel.

The GRAS Panel also received information pertaining to the method of manufacture, product specification, analytical data, intended use levels in specified food formulation rations, anticipated residues and resulting consumption estimates from all intended uses, safety studies conducted with Canthaxanthin, and any other relevant data on safety and tolerance-related information. The members of the Expert Panel were (b) (4)

(b) (4). 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.

The GRAS Panel reviewed the safety of CAROPHYLL[®] Red products, which is based on Canthaxanthin, its active ingredient. Canthaxanthin has been the object of numerous safety evaluations by different scientific bodies, having been thoroughly assessed worldwide, particularly in the US and the EU. In terms of tolerance in the targeted species, the safety evaluation of Canthaxanthin, in the form of CAROPHYLL[®] Red products, for the target species (*Gallus gallus*) demonstrates that the Canthaxanthin is well tolerated up to at least 10 times greater than the maximum recommended dose without any adverse effects.

The FDA thoroughly evaluated the available safety information on Canthaxanthin and established an ADI of 150 mg/p/d. The proposed use of CAROPHYLL[®] Red as an antioxidant in certain breeder rations will not result in an appreciable increase in canthaxanthin intake by the US consumer. Canthaxanthin fed to hens is mainly deposited in egg yolk and fertile eggs for hatching are not intended or destined for human consumption. Any potential exposure by consumer's intake of tissues from breeders will be low.

The Expert Panel convened via telephone conference call on 19 December 2011, and unanimously concluded that Canthaxanthin and Carophyll[®] Red produced consistent with current good manufacturing practice (cGMP) and meeting appropriate specifications, is safe for its intended use as an antioxidant in certain breeder rations. The Expert Panel further concluded that the intended use is safe and GRAS based on scientific procedures. It is 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

DSM Nutritional Products Ltd. is the manufacturer and distributor of Carophyll[®] Red and other carotenoid materials for use in human and animal nutrition around the world. Carophyll[®] Red is a carotenoid preparation that contains Canthaxanthin, CAS number 514-78-3, (β -Carotene-4,4'-dione). Canthaxanthin is an approved color additive that has been in the human food supply in the United States since 1969 and in certain animal feeds since 1985.

Canthaxanthin (Carophyll[®] Red) will be sold in (b) (4) forms containing typically between 10 and 15% Canthaxanthin and marketed under the name Carophyll[®] Red. The 10% form marketed as CAROPHYLL[®] Red 10% will be the predominant product in the marketplace and also contains other

materials that are GRAS for feed use (b) (4).
Canthaxanthin is also a synthetic antioxidant approved by the Food and Drug Administration following the submission of an extensive color additive petition (CAP) by Hoffman LaRoche in 1971.

The safety package supporting the 1971 Color Petition contained a comprehensive assessment of all animal nutritional and toxicological studies conducted on Canthaxanthin in support of its safety and use, and product residue data along with theoretical exposure estimates from human intake of products derived from animals consuming feed rations containing Canthaxanthin.

Current U.S. Regulatory Approvals for Canthaxanthin Uses

Canthaxanthin is currently authorized as a Color Additive in the US, where its safety and use has been confirmed by the FDA for the following applications:

- *“to enhance the yellow color of broiler chicken skin 4.41 mg/kg of complete feed to supplement other known sources of xanthophyll and associated carotenoids to accomplish the intended effect”.* (21 §CFR 73.75)(Color Additive Petition (CAP) submitted in 1971, submitted by Hoffman-La Roche)
- *“to enhance the pink to orange-red color of flesh of salmonids fish at not more than 80 mg/kg of feed salmonids fish”.* (21 CFR §73.75) (CAP approved in 1999 for BASF)
- *“General food color at not more than 30 mg/lb of solid and semi-solid food and not more than 30 mg/pint of liquid food”.* (21 CFR §73.75) (CAP approved in 1969, submitted by Hoffman-La Roche)
- *“may be safely used for coloring ingested drugs generally in amounts consistent with good manufacturing practice”.* (21 CFR §73.1075)

Canthaxanthin can lawfully be added as a color additive in the US, only if the applicable standards of identity (Specifications), as described in Title 21 of the Code of Federal Regulations (CFR § 73.75), are met. The Canthaxanthin that is the subject of this GRAS notification meets the applicable specifications; it is the same material that has been approved as a color additive.

Manufacturing Process

(b) (4)



Intended Use

Canthaxanthin as CAROPHYLL[®] Red is intended to be added to breeder feed ration formulations as an antioxidant to protect nutrients that otherwise would have been subject to oxidative stress and lipid peroxidation. Optimizing the antioxidant and nutritional status of the egg yolk-embryo-chick system favors chick embryo development, and lowers embryonic and/or post-hatch mortality of the offspring. DSM Nutritional Products proposes to use Canthaxanthin at a level no greater than 6 mg/kg feed (60 mg/kg feed for Carophyll[®] Red) as described in Title 21 of the Code of Federal Regulations (21CFR§170.30) (U.S. FDA, 2007). Canthaxanthin is currently permitted for use at levels up to 4 mg/kg feed in broiler chickens, 80 mg/kg feed in salmonid fish diet, as a general food color at not more than 30 mg/lb of solid and semi-solid food and not more than 30 mg/pint of liquid food, and for coloring ingested drugs generally in amounts consistent with good manufacturing practice (cGMP). DSM does not intend to exceed the current statutory limits for Canthaxanthin and merely seeks to expand its permitted use to include breeder poultry rations. Any increase in exposure will not result in any safety concern to consumers.

Exposure

The current exposure to Canthaxanthin from food and feed is 0.018 mg/p/d. . A conservative exposure estimate by DSM Nutritional Products which considers the proposed new use and incorporates a ten-fold safety factor is 0.053 mg/p/d for a total exposure of 0.071 mg/p/d.

In the Federal Register of Tuesday November 19, 1985, FDA published the final rule for the use of Canthaxanthin as a color additive exempt from certification. Within the supplementary information of the rule, FDA noted that the Acceptable Daily Intake (ADI) from all sources for Canthaxanthin was 150 mg/p/d based upon the No Observable Effect Level (NOEL) in a 2-year feeding study in rats.

There usually is no concern for adverse health effects of a food or feed ingredient when the estimated daily intake (EDI) is less than the ADI. The EDI of 0.071 mg/p/d provides a safety factor of more than 2100-fold when compared to the ADI of 150 mg/p/d. There should be no concern for over exposure to Canthaxanthin from the proposed new use.

This is consistent with the EFSA (EFSA, 2010) evaluation that noted the following: "*Total anticipated combined exposure from both sources has been estimated for children with a mean of 9.6 µg/kg bw and a 95th percentile of 23.6 µg/kg bw and 6.1 and 16.1 µg/kg bw for adults for mean and 95th percentile intakes, respectively. Intake estimates due to feed application are based on the Irish population. Some European populations potentially consume more fish than the Irish population, however, exposure estimates are based on the assumptions that all fish consumed contains canthaxanthin as a result of its use as an additive in fish feed, which is unlikely due to the availability of alternative colours in feed application. It can therefore be deduced, that for both adults and children, anticipated combined exposure to canthaxanthin from application as food and feed additive is unlikely to exceed the ADI.*"

In August 2006, the FDA published guidance for the calculation of exposure to food ingredients entitled "Guidance for Industry: Estimating Dietary Intake of Substances in Food". One of the examples given in the guidance was the calculation for the exposure to the food color additive canthaxanthin. Based upon data supplied by industry indicating that 1,050 pounds of canthaxanthin were used annually to color food, FDA calculated that the per capita intake at the 90th percentile was 0.18 mg/p/d.

DSM Nutritional Products is a (b) (4) manufacturer and marketer of canthaxanthin for use as a food color additive and feed additive. Sales data from 2010 indicate that DSM sold (b) (4) of canthaxanthin in the United States for food and feed applications. A conservative estimate of DSM's share of the total canthaxanthin business in the United States (b) (4). Therefore it can be reasonably expected that up to 2,066.9 pounds or 1013.2 Kg of canthaxanthin were used in food and feed in 2010. The population of the United States in 2010 per the official census was 308.7 million people. Thus the per capita exposure to canthaxanthin was 0.00898 mg/p/d; [1013.2 Kg/ 365 days / 308.7 million people]. Multiplying by 2 gives the intake at the 90th percentile as 0.0179 mg /p /d. Therefore the exposure to canthaxanthin from the current uses has not changed from what it was previously in 1985.

The proposed new use will increase human exposure to canthaxanthin. In August 2006 FDA published guidance (FDA 2006) on how to calculate consumption values for materials that are present in edible animal tissues. It is assumed that people will consume a portion of tissue at any eating occasion and that a portion of tissue is defined as: 300 gm of muscle, 100 gm of liver, 50 gm of kidney and 50 gm of skin/fat.

Tissues from the breeders used in the target animal study were analyzed for canthaxanthin content following the analytical procedure explained in section 2 of the GRAS dossier. The corresponding canthaxanthin residues in tissues are presented in Table 4-141: muscle N.D., liver 3.63 mg/Kg, kidney 0.79 mg/Kg and fat 0.90 mg/Kg.

Table 1 Residues in tissues of broiler breeders, supplemented with canthaxanthin (CXN) at 0, and 6 ppm Feed over 24 weeks.

Treatment	Canthaxanthin (mg/kg feed)	Leg muscle CXN*	Liver CXN*	Kidney CXN*	Abdominal adipose tissue CXN*	Skin CXN*
T-1	0	ND*	0 b	0 b	0 b	0 b
T-2	6	LLQ*	3.63 a	0.79 a	0.90 a	0.21 a
Pooled St.Err.		-	0.14	0.05	0.07	0.01
Pr>F		-	0.0001	0.0001	0.0001	0.0001

* *Means within a column not sharing a common superscript differ significantly (P<0.05)
 †Data are means of 9 replicates of 2 breeders per treatment
 †P<0.1; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS:P>0.1.

Breeder hens that are past their usable production life in the United States are utilized for the manufacture of processed chicken products (NCC 2011). According to the USDA Annual Poultry

Slaughter Report of January 25, 2011 (USDA 2011), 50,110,980,000 pounds of edible chicken tissue were produced in 2010. The same report stated that 585,511,000 pounds of edible breeder tissue were produced. Breeder tissue was therefore 1.168 % [(585,511,000/50,110,980,000) x100] of the total edible chicken tissue in the market place.

This calculation assumes that all the edible chicken tissue and breeder tissue were consumed by humans and nothing went to the animal feed or pet food industries.

The following table was constructed utilizing all the above data and the exposure due to each tissue to calculate total possible exposures. In the worst case scenario, a person eating one portion of each tissue every day would consume 0.0053 mg canthaxanthin. Incorporating a ten-fold safety factor would increase the exposure from breeder tissue to 0.053 mg/p/d. These calculations are summarized in Table 2.

Table 2. Theoretical Exposure from Consuming Edible Chicken and Breeder Tissue

Consumption of Chicken Organ Meat per FDA Guidance	Portion Size	Tissue Residue	% of Organs from Breeders	Canthaxanthin contribution from breeders
Liver	100 gm	3.63 mg/Kg	0.011684	0.0042414 mg
Kidney	50 gm	0.79 mg/Kg	0.011684	0.0004615 mg
Skin / Fat	50 gm	0.9 mg/Kg	0.011684	0.0005258 mg
Muscle meat	300 gm	0 mg/Kg	0.011684	0
TOTAL				0.0052287 mg
Assuming the worst case basis where every organ is consumed every day				0.0052287 mg/p/d

Summary of Cumulative exposure to Canthaxanthin from current and proposed uses

The current exposure to Canthaxanthin from food and feed is 0.018 mg/p/d. The conservative exposure estimate due to the proposed new use with a ten fold safety factor is 0.053 mg/p/d. Adding the two values yields a total exposure of 0.071 mg/p/d.

In the Federal Register of Tuesday November 19, 1985 FDA published the final rule for the use of Canthaxanthin as a color additive exempt from certification. Within the supplementary information of the rule, FDA noted that the Acceptable Daily Intake (ADI) from all sources for Canthaxanthin was 150 mg/p/d based upon the No Observable Effect Level (NOEL) in rats after a 2 year feeding study.

The conservative total exposure estimate of 0.071 mg/p/d provides a safety factor of more than 2,100 fold when compared to the ADI of 150 mg/p/d. Thus there is no concern for over exposure to canthaxanthin due to the proposed new use.



Safety Data

Carophyll® Red is a carotenoid preparation that contains 10% Canthaxanthin, CAS number 514-78-3, β -Carotene-4,4'-dione and other materials that are GRAS for feed use (b) (4) (b) (4)). Canthaxanthin is an approved color additive that has been in the human food supply in the United States since 1969 and animal feed since 1985.

The Canthaxanthin will be sold in (b) (4) forms containing typically between 10 and 15% Canthaxanthin and marketed under the name Carophyll®. The 10% form marketed as CAROPHYLL® Red 10% will be the predominant product in the marketplace. Additional product forms may be developed in the future with feed grade ingredients according to marketing needs. Carophyll® is produced by DSM Nutritional Products Ltd. at its plant in (b) (4) under GMP (21 CFR 110) via a (b) (4)

The specifications for canthaxanthin the color additive and for canthaxanthin in Carophyll Red are identical. Therefore, the safety data for the color additive canthaxanthin are appropriately applied to the canthaxanthin in Carophyll Red. The other ingredients in Carophyll Red are all approved for use in animal feed by the FDA or the Association of American Feed Control Officials (AAFCO).

Canthaxanthin has been reviewed by the European Food Safety Authority, FDA's Center for Food Safety And Applied Nutrition and FDA's Center of Veterinary Medicine. An Acceptable Daily Intake of 150 mg/person/day has been established for humans by the U.S. FDA. A summary of these safety evaluations is presented in Table 3.

Table 3. Summary of Safety Evaluations for Canthaxanthin in the US and the EU

Evaluator	Year	Title of Evaluation	Relevance	Link
European Food Safety Authority. (EFSA 2010)	2010	Scientific Opinion on the re-evaluation of canthaxanthin (E 161 g) as a food additive	ADI re-confirmed at 0.03 mg/kg bw/day, by the EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)	http://www.efsa.europa.eu/en/scdocs/scdoc/1852.htm?WT.mc_id=EFSA_HL01&emt=1
European Food Safety Authority. (EFSA 2007)	2007	Opinion of the Scientific Panel on Additives and Products or substances used in Animal Feed on the Maximum Residue Limits of Canthaxanthin coming from animals fed Canthaxanthin used as a feed additive in accordance with Council Directive 70/524/EEC	Proposal of Maximum Residue Limits (MRL's) in tissues for control within the food chain and based on the maximum permitted limits in feed set in 2003. (implemented in 2008 by EC)	http://www.efsa.europa.eu/en/science/feedap/feedap_opinions/ej507_canthaxanthin.html
European Commission. (SCAN 2002)	2002	Opinion of the Scientific Committee on Animal Nutrition on the use of Canthaxanthin in feedingstuffs for salmon and trout, laying hens, and other poultry.	Review of maximum permitted levels of canthaxanthin in complete feed (implemented in 2002 by EC) Trigger for the setting of MRL's	http://ec.europa.eu/food/fs/sc/scan/out81_en.pdf
ILSI North America Technical Committee on Food Components for Health Promotion. (ILSI 1999)	1999	Safety Assessment and potential health benefits of food components based on selected scientific criteria. "Canthaxanthin"	Comprehensive review of data of Canthaxanthin where the committee found that Canthaxanthin has been marketed in the U.S. and the EU as a direct and indirect food additive for more than 30 years and during that time, there have been no problems of safety with regard to the use of Canthaxanthin for either purpose.	Crit Rev Food Sci Nutri 39(3): 203-316
European Commission. Scientific Committee on Food (SCF 1997)	1997	SCF Opinion on Canthaxanthin.	Setting an Acceptable Daily Intake (ADI) based on laboratory animals and data on humans – the ADI was set by SCF at 0.03 mg / kg body weight/ d (i.e. for a 60 kg person: 1.8 mg per day)	http://ec.europa.eu/food/fs/sc/oldcomm7/out10_en.html
Joint Expert Committee on Food Additives (JECFA 1996)	1996	Toxicological evaluation of certain food additives and contaminants. Canthaxanthin.	The Committee allocated an ADI of 0-0.03 mg/kg bw to canthaxanthin, based on a NOEL of 0.25 mg/kg bw/day in humans and a safety factor of 10.	http://www.inchem.org/documents/jecfa/jecmono/v35je08.htm WHO food additives Series No. 35. 839.
FDA	1985	Color Additive Petition	Approved as a color additive for salmonoid fish at no more than 80 mg/kg of feed	21 CFR § 73.75
FDA	1971	Color Additive Petition	Approved as a color additive for chicken skin at 4.41 mg/kg of feed	21 CFR § 73.75

Evaluator	Year	Title of Evaluation	Relevance	Link
FDA	1969	Color Additive Petition	Approved as a general food color additive at no more than 30 mg/lb of food	21 CFR § 73.75

Canthaxanthin is authorized as a food additive in the EU. JECFA evaluated the safety data on canthaxanthin in 1974, 1987, and 1995. The SCF evaluated canthaxanthin in 1983, 1987 and 1997 (SCF 1984, 1989, 2000). At their latest evaluation of the safety of canthaxanthin, both Committees established an ADI of 0.03 mg/kg bw/day (rounded up from 0.025) based on a NOAEL of 15 mg canthaxanthin/day which corresponded to 0.25 mg/kg bw/day for a 60 kg person and using the uncertainty factor of 10 for the systemic change in the ERG scotopic b-wave amplitude in a human study (Arden et al., 1989, EFSA, 2010).

Canthaxanthin is not toxic when ingested. The oral LD₅₀ is greater than 20 000 mg/kg bw in rats and mice after a single or multiple doses.

In animals, only 3 to 9% of orally administered canthaxanthin is absorbed and fecal excretion is the major route of elimination (85 to 89% of the dose in monkeys). Canthaxanthin is distributed to liver, spleen, adipose tissue and adrenals. In monkeys, the concentration of canthaxanthin in the retina is dose-related, but nonlinear, suggesting that saturation could occur. In animals, elimination from adipose tissue is very slow, while canthaxanthin is eliminated from other tissues soon after removal of canthaxanthin from the diet. In humans, a part of the orally ingested canthaxanthin is absorbed (8 to 34% of the dose). Of the absorbed dose, 60% was transferred to fat tissue as assumed by measuring the concentration of canthaxanthin in the chylomicrons after fractionation of serum lipids. In plasma, canthaxanthin was associated with VLDL, LDL and HDL fractions, and 52% is transferred from chylomicrons to fat tissues. Remobilization from fat was very slow and the half-life was estimated to be approximately 5 days. No data on canthaxanthin metabolism were reported in humans.

Metabolism was studied in the target species for feed use. In fish, Canthaxanthin metabolites detected were echinenone, 4-hydroxy-beta,beta-carotene-4-one which is transformed to isozeaxanthin; the end product being β,β -carotene. In poultry, 4-hydroxyechinenone and isozeaxanthin as well as negligible amounts of 4-oxoretinol were found. The presence of 4-hydroxyechinenone and isozeaxanthin as metabolites was demonstrated in monkeys. EFSA concluded in 2007, that "Canthaxanthin is by far the major component of residues in target tissues of poultry and fish". The European FEEDAP Panel also considers Canthaxanthin as the only residue of concern and therefore retains Canthaxanthin (measured as the all-trans isomer) as the marker residue.

Studies in laboratory animals and human volunteers indicate that the amount and types of lipids and carotenoids ingested with the diet can interfere with the absorption of canthaxanthin, and vice-versa.

In subchronic (13-week) studies with canthaxanthin, doses of 125 mg/kg bw/day (male mice), of 1000 mg/kg bw/day (male and female mice) and of 2000 mg/kg bw/day in rats were associated with small decreases in body weight compared with placebo controls. At necropsy discoloration of some internal tissues, a finding typical of carotenoids, was recorded in mice and rats but no toxicologically relevant lesions were seen (Steiger and Humler, 1981; Steiger and Buser, 1982). In rats, the administration of canthaxanthin in doses ranging from 125 to 2000 mg/kg bw/day was associated with statistically significant increases in plasma cholesterol levels compared to the concurrent

controls but the cholesterol levels remained within the physiological limits for this species and strain in this laboratory. These are summarized in Table 4 below.

Table 4. Subchronic Toxicity Studies (Unpublished)

Authors	Study Type	Test Substance	Species/ sex	Route of Delivery	Result
Steiger & Hummler (1981)	90 Day Dose-range finder [how long?]	0 (negative control), 0 (placebo control 1), 0 (placebo control 2), 125, 250, 500, 1000, and 2000 mg Canthaxanthin/kg bw/day	Albino-SPF Mice 10/sex/dose	Oral – Feed Admix	NOAEL 2000 mg/kg bw/day LOAEL > 2000 mg/kg bw/day
Steiger & Buser (1982)	90 Day Dose-range finder [how long?]	0 (negative control), 0 (placebo control 1), 0 (placebo control 2), 125, 250, 500, 1000, and 2000 mg Canthaxanthin/kg bw/day	Albino-SPF Rats 10/sex/dose	Oral – Feed Admix	NOAEL 2000 mg/kg bw/day LOAEL > 2000 mg/kg bw/day
(b) (4) (1968)	13-Week study	0, 25, 50 mg Canthaxanthin/kg/day	Beagle hound 3/sex/dose	Oral – gelatin capsule	Evidence of storage particles in spleen & liver NOAEL 50 mg/kg bw/day
Buser & Hummler (1980)	90 Day Dose-range finder	0 (placebo control), 250, and 500 mg Canthaxanthin /kg bw/day	Beagle hound 1/sex/dose	Oral – Feed Admix	No adverse findings NOAEL 500 mg/kg bw/day
Buser S (1986)	26-week study	0 (standard control), 0 (placebo control), 0 (vehicle control), and 1000 mg Canthaxanthin / kg bw/day (Canthaxanthin 10% WS as feed admix) and 1000 mg Canthaxanthin/kg bw/day (Canthaxanthin 40% SD as gavage application)	Füllinsdorf Albino-SPF Rat 16/ sex/ dose	Oral- Feed Admix And gavage	Via Feed and Gavage NOAEL <1000 mg/kg bw/day LOAEL 1000 mg/kg bw/day

The available data from *in vitro* and *in vivo* studies indicate that canthaxanthin is not genotoxic. (Chételat, 1981 and 1986; Strobel, 1986; Gallandre, 1980).

Canthaxanthin was not carcinogenic in doses amounting to 1000 mg/kg bw/day in laboratory rodents. In two carcinogenicity studies in rats, increased plasma cholesterol (especially in females) and increases in other clinical-chemistry parameters along with increased relative liver weights (females only) and histological changes in the liver were indicative of hepatocellular changes. Also, treated mice exhibited increased plasma cholesterol levels and microscopical changes in the liver tissue (Buser, 1987a; Buser and Banken, 1988). Buser and Banken (1988) considered the lowest dose tested (250 mg canthaxanthin/kg bw/day) a LOAEL based on a dose-related increase in cholangiofibrosis observed in female rats only. The incidence of cholangiofibrosis was low and the dose related trend was weak. These data are summarized in Table 5.

Table 5. Carcinogenicity Studies (Unpublished)

Authors	Study Type	Test Substance	Species/ sex	Route of Delivery	Result
Schärer K & Studer F (1961)	Combined long-term / carcinogenicity / reprotoxicity study 1 st generation: 2-years 2 nd generation: 2-years 3 rd generation: 1-year	0.1% in feed (approx. 30 – 70 mg Canthaxanthin /kg bw/day)	Wistar Rat 1 st gen. 20/sex/dose 2 nd gen. 14 -16 /sex/dose 3 rd gen. 11 – 13 /sex/dose	Oral – Feed Admix	No toxic effects no effect on reproductive performance NOAEL 30 – 70 mg/kg bw/day
Schärer K (1964)	Chronic study 93 and 98 weeks	Growth phase (first 20 weeks): 0, 325 – 700, 1250 – 2500, 3200 – 7000 mg Canthaxanthin/kg bw/day From 20 weeks onwards: 0, 325, 1250, and 3200 mg/kg bw/day	Wistar Rats Both sexes 25 animals for control, low, and mid dose group 15 animals for high dose	Oral – Feed Admix	No negative influence on test animals NOAEL 3200 and 4000 mg/kg bw/day for males and females, respectively
Buser S (1987)	One Year Study	0 (negative control), 0 (placebo control), 50, 100, and 250 mg Canthaxanthin /kg bw/day	Beagle hound 4/sex/dose	Oral – Feed Admix	No Adverse Effects, organstaining of adipose and liver NOAEL 250 mg/kg bw/day

Authors	Study Type	Test Substance	Species/ sex	Route of Delivery	Result
Goralczyk R, Barker FM, Buser S, Liechti H, Bausch J (2000)	Long-term study with interim kills	0 (#12), 0.2 (#4), 0.6 (#4), 1.8 (#4), 5.4 (#4), 16.2 (#4), 48.6 (#6), 200 (#4), 500 (#4), 1000 (#2) mg Canthaxanthin /kg bw/day	Cynomolgus monkey 36 weeks: 1/ sex at 48.6 mg/kg bw/day 54 weeks: 1/sex at control and 48.6 mg/kg bw/day 83 weeks: 1/sex at control and 48.6 mg/kg bw/day 137 weeks: 4/ sex at control, 5.4, 16.2, 48.6 mg/kg bw/day 156 weeks: 1-4 / sex at control, 0.2, 0.6, 1.8 mg/kg bw/day For remaining animals, test substance was administered for 5 years: 3 control animals/sex, 2 males and 1 female at 1000 mg/kg bw/day, 4 animals per sex at 500 mg/kg bw/day and 1 animal per sex at 48.6 mg/kg bw/day.	Oral - gavage	NOAEL Systemic: 1000 mg/kg bw/day Birefringent inclusions in the eye: 0.2 mg/kg bw/day
Buser S (1988)	Combined long-term / carcinogenicity study with interim kill after 12 months	0 (negative control), 0 (placebo control), 250, 500, and 1000 mg Canthaxanthin/kg bw/day	Sprague-Dawley Rats 70/sex/dose	Oral - Feed Admix	NOAEL < 250 mg/kg bw/day based on observed liver toxicity in both sexes

Authors	Study Type	Test Substance	Species/ sex	Route of Delivery	Result
Buser (1992) S	Long-term study with interim kills after 52 and 78 weeks	0 (negative control), 0 (placebo control), 5, 25, 75, and 250 mg Canthaxanthin/kg bw/day	CrI: CD (SD) BR Male Rats 50 animals / dose level: 30 animals for 2-year and 10 animals for each of the 2 interim sacrifices	Oral – Feed Admix	Neither malignant nor benign liver tumors noted NOAEL 25 mg Canthaxanthin/kg bw/day
Buser (1992) S	Long-term study with interim kills after 52 and 78 weeks and recovery period	0 (negative control), 0 (placebo control), 5, 25, 75, and 250 mg Canthaxanthin /kg bw/day	CrI: CD (SD) BR Female Rats 80-105 / dose	Oral – Feed Admix	Neither malignant or benign liver tumors noted NOAEL 5 mg Canthaxanthin/kg bw/day
Buser (1987) S	Combined long-term / carcinogenicity study with interim kill after 12 months	0 (negative control), 0 (placebo control), 250, 500, and 1000 mg Canthaxanthin/kg bw/day	CD-1 Mice 60/sex/dose	Oral – Feed Admix	Neither tumorigenic and carcinogenic NOAEL 1000 mg/kg bw/day

In beagle dogs, 13-week feeding with canthaxanthin at doses amounting to 500 mg/kg bw/day (Chesterman et al., 1979; Buser and Hummler, 1980) or 52-week feeding at doses amounting to 250 mg canthaxanthin/kg bw/day (Harling et al., 1987, Buser 1987a) were not associated with any adverse effects but red to orange discoloration of feet, muzzle, feces, abdominal fat and some other tissues was recorded. The histopathological changes recorded after 52 weeks in the liver were indicative of canthaxanthin presence in the organ. However these changes were not associated with impairment of the liver function as no toxicologically significant changes in clinical chemistry parameters or pathology related to the liver were recorded in both dog studies.

There were no indications that canthaxanthin had adverse effects on reproduction or the developing rat fetus in doses up to 1000 mg/kg bw/day (Buser 1987a; Kistler 1982), and in doses up to 400 mg/kg bw/day on the developing fetus in rabbits (Eckhardt, 1982). Also there were no indication of maternal toxicity in the developmental studies in rats and rabbits (Kistler 1982, Eckhardt, 1982). However, in a three-generation reproduction study in rats (Buser, 1987b) other treatment-related effects such as increased levels of serum enzymes and plasma cholesterol in adult animals, changes in liver weights in weanlings and adult animals, histological changes in the liver (such as foci of foamy macrophages and increased hepatocyte vacuolisation in adult animals) and decreases

in adrenal weight of adult females were reported. These hepatocellular changes were partially reversible after withdrawal of the test compound followed by a recovery period. These studies are summarized in Table 6.

Table 6. Reproductive and Developmental Toxicity Studies (Unpublished)

Authors	Study Type	Test Substance	Species/ sex	Route of Delivery	Result
Buser (1987)	Three- Generation Reprotoxicity Study (2 litters per generation)	0 (control), 0 (placebo control), 250, 500, and 1000 mg Canthaxanthin /kg bw/day	CrL COBS CD (SD) BR Rats 24-32 /sex/dose	Oral – Feed Admix	Not Reprotoxic NOAEL parental 250 mg/kg bw/day NOAEL developmental 1000 mg/kg bw/day NOAEL reproduction 1000 mg/kg bw/day
Eckhardt (1982)	Developmental Toxicity Study	0 (vehicle control), 100, 200, and 400 mg/kg bw/day canthaxanthin	Female Swiss Hare Rabbits 20/dose	Oral by gavage in rape seed oil	Not embryotoxic, not foetotoxic, not teratogenic NOAEL maternal & developmental 400 mg/kg bw/day
Kistler (1982)	Segment II study with postnatal evaluation	0, 250, 500, and 1000 mg Canthaxanthin /kg bw/day	Füllinsdorf albino Female Rats 40/dose	Oral – Feed Admix	Not embryotoxic, not foetotoxic, not teratogenic, not developmental toxic NOAEL maternal & developmental 1000 mg/kg bw/day

There are no indications that canthaxanthin has an allergenic potential by the oral route.

As summarized in Table 7, a number of studies have evaluated the proposed use of 6 mg canthaxanthin per kg of feed and its effect on the performance of poultry breeders with no adverse effects. While these studies were not specifically designed to evaluate target animal safety, they do

provide supportive evidence that no adverse effects are expected from canthaxanthin when used at the proposed dose level with breeder hens.

Table 7. Studies with poultry breeders fed Canthaxanthin (CAROPHYLL® Red) at different doses in feed.

Author	Dose (mg/kg feed)	Description	Study Length (weeks)	Productivity	Reference
(Llaurado et al. 1997)	0, 2, 4, 6	Effect of Canthaxanthin supplementation on the hatchability of eggs of broiler breeders.	13	Poultry breeders were not adversely affected by canthaxanthin supplementation in the feed, at any of the doses tested.	Proceedings of the 11th European Symposium on Poultry Nutrition, Denmark, 1997. pp. 280—282.
(Surai et al. 2003)	0, 3, 6, 12, 24	Effect of Canthaxanthin content of the maternal diet on the antioxidant system of the developing chick	4		British Poultry Science Volume 44, Number 4 (September 2003), pp. 612—619
(Robert et al. 2007)	0, 6	Effects of Canthaxanthin supplementation in the ROSS breeder diet on oxidative stress of chicks	12		World Poultry Science Association, Proceedings of the 16th European Symposium on Poultry Nutrition. August 26 - 30, 2007 Strasbourg, France
(Rocha et al. 2011)	0, 6	The Impacts Produced by Dietary Canthaxanthin added to the Diet of Broiler Breeders at Old Age and by Egg Storage Period on Fertility and Incubation Yield (submitted to Poultry Science). (Original: Thesis for Doctor, Federal University of Minas Gerais, Brazil)	13		Thesis. Federal University of Minas Gerais. Veterinary School. Belo Horizonte. 2011 (submitted to Poultry Science).
(Rosa et al. 2011)	0, 6	Effects of Canthaxanthin on the Productive and Reproductive Performance of Broiler Breeders	21		(submitted to Poultry Science).
(Souza et al. 2008)	0, 6	Effect of the use of CAROPHYLL® Red on reproductive indices in broiler breeders	10		APINCO – Poultry Science and Technology Conference Proceedings. 27-29 of May 2008. Santos. SP
(Zhang et al. 2011)	0, 6	Influence of canthaxanthin on broiler breeder reproduction, chick quality, and performance	24		Poultry Science 2011 90: 1516-1522

Several animal studies investigated ocular toxicity related to pigment deposition in the eye. Feeding canthaxanthin at 15,000 ppm in the diet for 6 to 12 weeks led to crystalline deposits in the retina of the chicken with a NOAEL of 2.1 mg/kg bw/day (Goralczyk and Weiser, 1992; Goralczyk, 1993a) but ferrets fed 50 mg/kg bw/day for 12 months and rabbits fed 200 ppm for 11 months did not develop these lesions readily (JECFA 1997, data submitted by Hoffmann- La Roche).

In Cynomolgus monkeys prolonged (2.5 to 5 years) daily oral administration of canthaxanthin, (0.6 mg/kg bw/day) was associated with crystalline deposits in the retina, detectable microscopically. The crystalline deposits were not observed in animals receiving 0.2 mg canthaxanthin/kg bw/day (Buser et al., 1993, 1994). The canthaxanthin-induced retinal inclusions were not accompanied by adverse effects on visual function as measured by ERG (Goralczyk et al., 1997; 2000).

In humans, a meta-analysis of the published studies and the available unpublished reports on crystal formation by Köpcke et al. (1995) indicated a significant dose response relationship for both total and daily doses of canthaxanthin. The incidence rates were, for the following daily doses (per subject), <30 mg = 0%, 30 mg = 9.6%, 45 mg = 20.3%, 75-105 mg = 43.1%, > 105 mg = 52%. Crystal formation was reversible as evidenced by their disappearance albeit slowly over time in patients that had ceased high-dose consumption of canthaxanthin.

The EFSA Panel (EFSA, 2010) performed a BiModal Distribution analysis on the data reported by Köpcke et al. (1995). Using the BMDL to derive a point of departure from these human data, the EFSA Panel identified a BMDL₀₅ of 12-20 mg/day which equals 0.20 - 0.33 mg/kg bw/day for a 60 kg person.

Crystal formation in the retina from canthaxanthin ingestion is not associated with any detectable functional changes or any temporary or permanent visual loss even in those subjects who had taken canthaxanthin for extended periods. However, changes in ERG in patients taking high doses of canthaxanthin have been reported by several authors as electrophysiological evidence for an effect of canthaxanthin on the retina. One month dosage of 15 mg canthaxanthin and 10 mg β -carotene/day produced no systemic change in the ERG scotopic b-wave amplitude while an additional month on a dosage of 60 mg canthaxanthin and 40 mg β -carotene caused a reduction in ERG scotopic b-wave amplitude which was slightly more pronounced after a further month at a dose of 90 mg canthaxanthin and 60 mg β -carotene. Human subjects with canthaxanthin crystals in the retina showed a more marked reduction in the ERG scotopic b-wave amplitude (Arden et al., 1989).

The EFSA Panel noted that the NOAEL for the change in the ERG scotopic b-wave amplitude reported by Arden et al. (1989) was 15 mg canthaxanthin/day (1 tablet) which corresponded to 0.25 mg/kg bw/day for a 60 kg person. However, the Panel noted that Arden and Barker (1991) subsequently stated that the effects on ERG were not reproducibly observed and that when observed, the effects were 'mild and clinically not significant'.

The EFSA Panel also noted that the hepatocellular changes, the changes in the levels of serum enzymes and the increases in serum cholesterol observed in rats treated with canthaxanthin were not reported in humans. The Panel noted that the pivotal effects of canthaxanthin are on the eye. These were manifested as crystalline deposits in the retina of monkeys and in humans and electrophysiological alterations recorded by ERG in humans.

Based on the NOAEL of 0.25 mg/kg bw/day for scotopic b-wave changes (without impairment of vision) in a human study by Arden et al. (1989) and the BMDL₀₅ of 12-20 mg/day, amounting to 0.20-0.33 mg/kg bw/day for a 60 kg person, derived in a worst case BMD analysis of the data from the meta-analysis reported by Köpcke et al. (1995) on the crystal incidence in human eyes with increasing daily doses of canthaxanthin, the Panel derived a point of departure of 0.30 mg/kg bw/day and allocated an ADI of 0.03 mg/kg bw/day using an uncertainty factor of 10. This ADI is consistent with the ADI derived previously by JECFA and the SCF.



The current exposure to Canthaxanthin from food and feed in the USA is 0.018 mg/p/d. The conservative exposure estimate due to the proposed new use in breeders with a ten-fold safety factor is 0.053 mg/p/d for a total exposure of 0.071 mg/p/d.

In the Federal Register of Tuesday November 19, 1985 FDA published the final rule for the use of Canthaxanthin as a color additive exempt from certification. Within the supplementary information of the rule, FDA noted that the Acceptable Daily Intake (ADI) from all sources for Canthaxanthin was 150 mg/p/d based upon the No Observable Effect Level (NOEL) in rats after a 2 year feeding study.

The total exposure estimate of 0.071 mg/p/d provides a safety factor of more than 2,100-fold when compared to the ADI of 150 mg/p/d. Thus there is no concern for over exposure to canthaxanthin due to the proposed new use. This is consistent with the EFSA (EFSA, 2010) evaluation as noted in the exposure section.

Safety Conclusions

The safety assessment of CAROPHYLL® Red products is based on Canthaxanthin, its active ingredient. Canthaxanthin has been the object of numerous safety evaluations by different scientific bodies worldwide, and particularly in the US and the EU. FDA established an ADI of

150 mg/p/d. The proposed use does not increase consumer exposure beyond the established ADI. As such, the use of CAROPHYLL® Red for the proposed nutritive antioxidant application with poultry Breeders does not considerably increase canthaxanthin intake by the US consumer. This is because canthaxanthin fed to hens is mainly deposited in egg yolk, and any potential exposure by consumer's intake of tissues from breeders can be expected to be low. Furthermore, fertile eggs for hatching are not intended or destined for human consumption.

The long history of widespread use of CAROPHYLL® Red products with Canthaxanthin as the active constituent as a color additive for poultry and salmonid fish feeds and also as a general food and drug color, combined with the independent regulatory reassessments that have been conducted to ensure its safety, all provide further support and confirmation for the safety of Canthaxanthin (CAROPHYLL® Red) under the conditions of its intended use in poultry breeder feed rations.

The available data on Canthaxanthin, in the form of CAROPHYLL® Red products, for the target species (*Gallus gallus*) demonstrate that the Canthaxanthin is well tolerated up to at least 10 times greater than the maximum recommended dose without any adverse effects.

Based on this safety assessment, the Expert Panel considers that there are no safety concerns with regard to the proposed conditions of use for Canthaxanthin and CAROPHYLL Red® 10% and that the intended use of this product is safe and GRAS.

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 use of the CAROPHYLL® Red form of Canthaxanthin added at a maximum use level no greater than 6 mg/kg feed (60 mg/kg feed for Carophyll® Red) as an antioxidant to preserve and optimize yolk nutrient stability and availability in poultry breeder feed rations, 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 use of the CAROPHYLL® Red form of Canthaxanthin, manufactured consistent with current Good Manufacturing Practice and meeting appropriate feed grade specifications as described in the dossier, is Generally Recognized as Safe (GRAS) based on scientific procedures, corroborated by history of safe use, substantial equivalence to canthaxanthin approved as a color additive, and extensive unpublished safety data.

We, the members of the Expert Panel, conclude that there are no safety concerns with regard to the proposed conditions of use for Canthaxanthin and CAROPHYLL Red® 10% and that the intended use of this product is safe and GRAS.

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



(b) (4)

21 December 2011
Date

23 December 2011
Date

12/20/11
Date

T-2



DSM Nutritional Products
45 Waterview Boulevard
Parsippany
NJ 07054
United States of America

phone +1 973 257-8347
email: james.lamarta@dsm.com

Mr. Geoffrey Wong
Center for Veterinary Medicine
Ingredient Safety Team (HFV 224)
7519 Standish Place
Rockville, Maryland 20855
CC: Dr. Manisha Das

April 8, 2015

Re: Question regarding DSM's GRAS Notice for Canthaxanthin, AGRN 17

Dear Mr. Wong and Dr. Das

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, Canthaxanthin, market under the Carophyll® Red brand name.

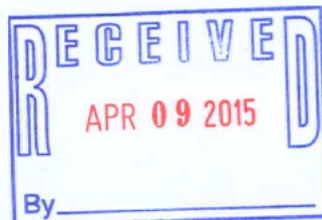
DSM Nutritional Products has determined that all the substances utilized in the manufacture and formulation of Carophyll® Red products are suitable for use in animal food in compliance with a regulation of the Food and Drug Administration, a listing in the Official Publication of the American Association 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 (b) (4) to be generally recognized as safe for their intended use in the manufacture and formulation of the Carophyll® Red products.

Please do not hesitate to contact me if you have any additional questions regarding this matter.

Kind regards,

DSM Nutritional Products

James La Marta, Ph.D., CFS
Sr. Manager, Regulatory Affairs



T-3



DSM Nutritional Products
45 Waterview Boulevard
Parsippany
NJ 07054
United States of America

phone +1 973 257-8347
email: james.lamarta@dsm.com

Mr. Geoffrey Wong
Center for Veterinary Medicine
Ingredient Safety Team (HFV 224)
7519 Standish Place
Rockville, Maryland 20855
CC: Dr. Manisha Das

April 27, 2015

Re: Question regarding DSM's GRAS Notice for Canthaxanthin, AGRN 17

Dear Mr. Wong

In response to the Center's request for clarification of the GRAS Exemption Claim expressed by you during our conversation of 24 April 2015, DSM is providing a revised claim. We have maintained the numbering of the section that was used in the original submission and I have signed the revised claim in accordance with the proposed rule of 17 April, 1997.

The revised GRAS Exemption Claim is attached.

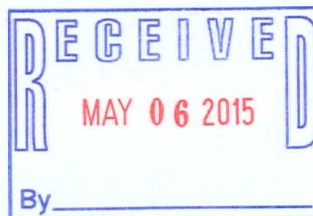
Please do not hesitate to contact me if you have any additional questions regarding this matter.

Kind regards,

DSM Nutritional Products

A handwritten signature in blue ink that reads "James La Marta".

James La Marta, Ph.D., CFS
Sr. Manager, Regulatory Affairs



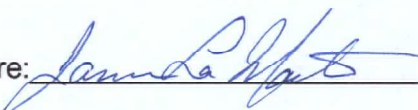


1.2. GRAS Exemption Claim

DSM Nutritional Products hereby notifies FDA of its conclusion that Canthaxanthin is exempt from the definition of a "food additive" and thus from the premarket approval requirements outlined in section 201(s) of the Federal Food, Drug and Cosmetic Act because its use in animal feed at levels up to 6 milligrams per Kg of feed as a nutritive antioxidant in chicken breeders' food to support the development of chicks, is generally recognized as safe (GRAS) by qualified experts, as shown through scientific procedures, corroborated by a history of safe use, substantial equivalence to canthaxanthin approved as a color additive, and extensive safety data.

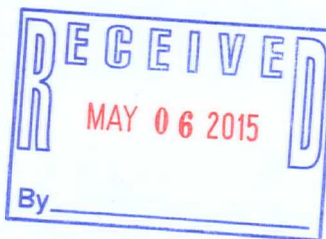
To make the GRAS conclusion, DSM Nutritional Products compiled information regarding the nature of the substance, specifications, manufacturing, proposed conditions of use and technical evidence of safety into a comprehensive technical dossier (GRAS dossier) and sought the opinion of a GRAS Panel specifically convened for the purpose of reviewing the information therein to determine whether there is consensus among qualified experts that the use of the Canthaxanthin as described entails a reasonable certainty of no harm and is generally recognized as safe.

All data and information that are the basis for the GRAS conclusion are available for FDA's review and copying at reasonable times at DSM Nutritional Products, 45 Waterview Blvd, Parsippany, NJ 07054, and will be sent to FDA upon request.

Signature: 

Date: 27 April 2015

James La Marta, Ph.D., CFS
Senior Manager Regulatory Affairs
DSM Nutritional Products
45 Waterview Blvd
Parsippany, NJ 07054



T-4



DSM Nutritional Products
45 Waterview Boulevard
Parsippany
NJ 07054
United States of America

phone +1 973 257-8347
email: james.lamarta@dsm.com

Mr. Geoffrey Wong
Center for Veterinary Medicine
Ingredient Safety Team (HFV 224)
7519 Standish Place
Rockville, Maryland 20855
CC: Dr. Manisha Das

May 13, 2015

Re: Question regarding DSM's GRAS Notice for Canthaxanthin, AGRN 17

Dear Mr. Wong

During a conference call on 24 April 2015, CVM requested that DSM again provide the rationale for a lack of potential ocular toxicity in breeding chickens due to the use of canthaxanthin in their food.

In the GRAS Notice that was reviewed by a panel of toxicologists and supported by a letter from the study center director where the target animal study was performed (noted in the annexes as (b) (4) 2014), there was no observed adverse effect in visual acuity of the animals during the course of the study. If there had been, it would have been recorded in the daily animal inspection logs and duly noted in the final report and in the publication of the study. (b) (4) et al. 2013)

The maximum use level of canthaxanthin in breeding chicken food is 6 mg/ Kg of food. This concentration is equivalent to 0.3 mg/Kg BW/ day for a breeding hen based upon the feed consumption rate reported by the NRC on page 21 in their 1994 publication of 430 gm /wk and a body weight of 1.2 Kg in the 18th week, when egg production typically begins. Ocular toxicity, the presence of canthaxanthin crystals in the retina was reported in humans consuming excess quantities of canthaxanthin by European ophthalmologists in response to clinical observations. Individuals affected had been consuming 15 - 240 mg/day from 1 to 14 years. Controlled studies as reported by Arden et al. 1989 revealed that a dose below 0.5 mg/ Kg BW/ day did not result in crystal formation in humans. Thus the consumption level in chickens is 40% below the lowest concentration expected to possibly cause crystal formation.

In section 4.3.9 of the dossier it was noted that attempts to find animal models for elucidation of the cause and effect were fruitless due to the lack of crystal formation in mice, rats, rabbits, cats, and dogs. Only monkeys exhibited a dose response but not in a reproducible manner and with no adverse effect on vision. We also reported on page 101 that a dose response study in chickens did not result in crystal formation at a dose of 2.1 mg/Kg BW/day; 7 times the maximum GRAS use level.



Canthaxanthin has been an approved additive for animal food for the purpose of coloring the yolk of chicken eggs in the EU, and several countries in Asia and South America since 1995, at up to 8 mg/Kg feed, 33% higher than the maximum use level in the GRAS Notice. There have been no reports of vision impairment nor crystal formation in the retina of the consuming animals over the 20 years since the substance was approved for this use.

DSM therefore concludes that there is no potential for the formation of crystals of canthaxanthin in the eyes of breeding chickens that consume food contain the substance at a use level of no more than 6 mg/Kg of food.

Please do not hesitate to contact me if you have any additional questions regarding this matter.

Kind regards,

DSM Nutritional Products

A handwritten signature in blue ink, appearing to read "James La Marta", is written over a light blue horizontal line.

James La Marta, Ph.D., CFS
Sr. Manager, Regulatory Affairs

