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1. Introduction: Chemotherapeutics in Seafood

Over the past decade, there has been a significant increase in the commercial production and consumption of aquacultured products. As this industry grows, so does the use of approved and non-approved chemicals. Fish or seafood raised in a controlled environment can be better protected from catching wild parasites and fed to promote growth. On the other hand, fish/seafood raised in a high-density setting can cause quick exchange of disease, resulting in the need to pro-actively or actively treat with drugs. The use of non-approved chemical compounds on aquaculture products, or the misuse of approved chemicals, may have an impact on the safety of consumers. Some therapeutic agents are toxic and have been prohibited for use in aquaculture due to concerns for human health. Antibiotic residues can also find their way into the environment (by pond run-off, surviving sewage treatment, etc.) contributing to the development of antibiotic-resistant strains of bacteria; or by the chronic ingestion of antibiotics in our diet giving rise to antibiotic-resistant strains of bacteria.

The FDA routinely monitors domestic and imported aquaculture and seafood products for drug residues under Compliance Program Guidance Manual 7304.018 “Chemotherapeutics in Seafood.” The drugs (and species) are on surveillance status and may change from year to year. The analytical methods are developed primarily by researchers in the Office of Regulatory Affairs (ORA) labs and may or may not be published in a scientific journal or the

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AOAC Official Methods of Analysis. Some methods are only circulated by the Office of Regulatory Science (ORS) or published in FDA's Laboratory Information Bulletin (LIB). However, the compliance program will serve as guidance on which method is correct to use. Mass spectrometric methods described in this section provide either quantitative determination with confirmation of residue identity or provide qualitative screening for multiple classes of therapeutic residues above an established threshold concentration. The chemistry and instrumental analysis involved with these methods are some of the most difficult the analyst will find in the field laboratory because of the challenge of finding residue levels of analyte within a complex matrix. These methods are considered intermediate and advanced training.

2. Triphenylmethane Dyes

2.1. Background

The triphenylmethane dyes malachite green (MG), crystal violet (CV), and brilliant green (BG) have antiseptic and antifungal properties with known uses in human and veterinary medicine. In aquaculture, dyes are primarily used as a treatment for fungal and external parasite infections in fish, and to protect incubating eggs from fungus. Though toxicity and mutagenicity of these compounds have led to worldwide restrictions and discontinuation of therapeutic dye treatments for aquaculture, the long history, efficacy and ready availability of these inexpensive dyes for infection control suggest that regulatory monitoring must continue (1-3). The cationic dyes and the leuco base form of the dyes are susceptible to oxidation/reduction and demethylation reactions in the presence of air and light. Following fish treatment by bath exposure, dyes are metabolized in fish to their reduced leuco forms, which have a several-month residence time in muscle compared to the cationic dye residue. Therefore, methods to determine prohibited residues of the triphenylmethane dyes must include detection of the leuco metabolites of MG and CV in addition to the dye forms, since the concentration of leucomalachite green (LMG) and leucocrystal violet (LCV) residues found will be significantly higher than the concentration of MG and CV. At low concentrations of residues, it is common to observe only LMG or LCV without any MG or CV present. Analytical methods have been developed for these compounds to either determine the total residue present (dye + leuco) following an oxidation reaction to convert the leuco form to the dye, or to measure the dye and leuco components individually. As shown in the compliance program, determinative methods for these compounds are required to quantify 1.0 ng/g as the sum of the contribution by the dye and the leuco metabolite (4).

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2.2. Exercise

Review the references. The trainer will provide a sample that has been either fortified or incurred with an unknown amount of a triphenylmethane dye and/or its leuco metabolite. Determine the quantity and confirm the identity of each residue present, using the AOAC Official Method of Analysis 2012.25 (1-3). Insure all controls are extracted according to laboratory procedure. Determine the percent recovery of the residues in the laboratory positive control. Report all findings on a training analytical worksheet.

2.3. Questions

1. What is the Target Testing Level (TTL) for leucomalachite green?
2. Are there any special handling conditions for the triphenylmethane dyes and metabolites? If so, what are they?
3. What type of calibrants are used in AOAC Official Method of Analysis 2012.25?
4. If an analyst needs to test samples of shrimp, salmon, and eel on the same day, must six extracted calibrants be prepared in shrimp, salmon, and eel (18 calibrants total)?
5. What steps can be taken to minimize the detection of crystal violet residues in negative control samples?

2.4. References

1. Andersen, W.C., et. al. "Triphenylmethane Dye Residue Analysis in Raw and Processed Aquaculture Products by AOAC Official Method of Analysis 2012.25 (CARTS Projects IR01183 and IR01444)" *Laboratory Information Bulletin* #4646, U.S. Food and Drug Administration 34 (2018) 1-23.
2. Andersen, W.C., et al. "Dye Residue Analysis in Raw and Processed Aquaculture Products: Matrix Extension of AOAC International Official Method 2012.25" *J. AOAC Int.*, 101 (2018) 1927-1939. DOI: <https://doi.org/10.5740/jaoacint.18-0015>.
3. Schneider, M.J., et al. "Determination of Triphenylmethane Dyes and Their Metabolites in Salmon, Catfish, and Shrimp by LC-MS/MS Using AOAC First Action Method 2012.25: Collaborative Study" *J. AOAC Int.*, 98 (2015) 658-670.
4. Compliance Program 7304.018, Chemotherapeutics in Seafood Compliance Program (Issue Date 6/9/17).

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3. Nitrofurans/Chloramphenicol

3.1. Background

Nitrofurans (nitrofurantoin, furazolidone, nitrofurazone, and furaltadone) and chloramphenicol (CAP) are antimicrobial drugs that are widely used to treat bacteria in food-producing animals. CAP is used as an agent against both Gram-positive and Gram-negative bacteria and nitrofurans are used for treatment of bacterial and protozoan infections (1-2). Currently, the U.S. Food and Drug Administration requires 12 individual subsamples to be analyzed for each sample collected for nitrofurans and CAP analysis (3). While previous methods for nitrofurans and CAP had labor and time intensive liquid-liquid extraction procedures followed by analyses, the microwave-assisted derivatization using automated solid phase extraction simultaneously extracts all the analytes together.

3.2. Exercise

Review the references. The trainer will provide a sample that has been either fortified or incurred with an unknown amount of nitrofurans and/or CAP. Determine the quantity and confirm the identity of each residue present using the Laboratory Information Bulletin #4597 (2). Ensure all controls are extracted according to laboratory procedure. Determine the concentration of the residues in the laboratory positive control. Report all percent recoveries for matrix spikes and unknown findings.

3.3. Questions

1. What is the Target Testing Level (TTL) for the nitrofurans class of antibiotics? What is the TTL for chloramphenicol?
2. How often should 100 mM 2-nitrobenzaldehyde (2-NBA) be prepared?
3. While nitrofurans and CAP are simultaneously extracted, they are analyzed on a LC coupled with a triple quadrupole MS (MS/MS) using two different ionization modes. What are the ionization modes and source for nitrofurans and CAP?
4. The LIB #4597 extraction uses a microwave assisted derivatization that takes approximately 6 minutes. In previously used nitrofurans metabolite extractions, what was an approximate derivatization time?
5. When utilizing the ASPEC system, samples are loaded on to SPE cartridges, washed, dried, and subsequently eluted with what solvent?
6. When preparing a breaded shrimp sample for analysis, what precautions should be taken before homogenization?

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3.4. References

1. Veach, B.T., et al. "Quantitation of Chloramphenicol and Nitrofurantoin Metabolites in Aquaculture Products using Microwave-Assisted Derivatization, Automated SPE, and LC-MS/MS" *J. AOAC Int.*, 98 (2015) 588-594.
2. Veach, B.T., et al. "Quantitation of Chloramphenicol and Nitrofurantoin Metabolites in Aquaculture Products using Microwave-Assisted Derivatization, Automated Solid-Phase Extraction and LC-MS/MS" *Laboratory Information Bulletin #4597*, U.S. Food and Drug Administration 31 (2015) 1-27.
3. Compliance Program 7304.018, Chemotherapeutics in Seafood Compliance Program (Issue Date 6/9/17)

4. Multiple Residues Method

4.1. Background

Sulfonamide, quinolone, fluoroquinolone, amphenicol, and tetracycline antibiotics are effective for the treatment of bacterial infections in aquacultured products, and these drugs have been monitored in single class regulatory analysis methods for years. Though these historical methods are quantitative; they are also labor intensive with respect to man hours and materials since the majority of regulatory samples are found to be negative. Multi-residue screening methods have been developed to combine the analysis of individual classes of antibiotics with other therapeutic agents (e.g. hormones, antiparasitic or antifungal drugs) for time and cost savings (2-4). In a screening analysis, if a drug residue is identified with a response above a given threshold response, an additional analysis is performed to quantify the amount of the drug residue present.

4.2. Exercise

Review all references. The trainer will provide a sample that has been either fortified or incurred with an unknown amount of an analyte of interest. By utilizing a screening method, determine the presence of the unknown compound, using the method described in Compliance Program 7304.018 (1). Insure all controls are extracted according to laboratory procedure. Additionally, perform a quantitative method on the unknown compound and determine the amount found. Report all findings on a training analytical worksheet.

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4.3. Questions

1. What is the Target Testing Level (TTL) for the sulfonamide class of antibiotics? What is the TTL for ciprofloxacin?
2. If your sample consisted of 12 sub samples; how many grams from each sub sample do you weigh for a representative composite?
3. During sample extraction, how many times do you transfer the upper organic layer?
4. What is the flow rate for the referenced method?
5. Give an example of a situation (analyte/matrix) where the screening method does not provide suitable results for regulatory analysis.
6. Explain the main differences between a screening method and a quantitative method?

4.4. References

1. Compliance Program 7304.018, Chemotherapeutics in Seafood Compliance Program (Issue Date 6/9/17)
2. Clark, S. B., et al. "Analysis of sulfonamides, trimethoprim, fluoroquinolones, quinolones, triphenylmethane dyes (and their leuco metabolites) and methyltestosterone in fish and shrimp using liquid chromatography mass spectrometry" *Laboratory Information Bulletin #4562*, U.S. Food and Drug Administration 30 (2014) 1-23.
3. Storey, J.M., et al. "Analysis of sulfonamides, trimethoprim, fluoroquinolones, quinolones, triphenylmethane dyes and methyltestosterone in fish and shrimp using liquid chromatography–mass spectrometry" *J. Chromatogr. B.* 972 (2014) 38–47.
4. Nickel, T. J., et al. "Method transfer and Optimization of LIB 4562 (Multi-Residue LC-MS/MS Screening Method for Veterinary Drugs in Aquaculture Tissues) from the Agilent 6490 LC-MS/MS to a SCIEX 5500 QTRAP LC-MS/MS" *Laboratory Information Bulletin #4614*, U.S. Food and Drug Administration 32 (2016) 1-30.
5. Bunch, E. A., et al. "Homogeneous sample preparation of raw shrimp using dry ice" *J. AOAC Int.*, 78 (1995) 883-887.

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5. Other Residue Methods

5.1. Background

The Chemotherapeutics in Seafood Compliance Program Guidance Manual 7304.018 also describes methodology and target testing levels for the analysis of additional therapeutic agents (1). Avermectin drugs such as ivermectin, emamectin, abamectin, and doramectin are macrocyclic lactone compounds that are used therapeutically to control parasites in veterinary medicine (2-4). Isoeugenol is used as a fish sedative in aquaculture operations (5). The stilbene drugs diethylstilbestrol, dienestrol, and hexestrol are non-steroidal synthetic estrogens that have been previously used in veterinary medicine for growth promotion; they are now banned for use in food producing animals as the stilbenes are known endocrine disruptors (6). Tetracycline antibiotics have also been used in aquaculture operations. In addition to these specific drug classes, wide scope screening methods have been developed to simultaneously identify hundreds of veterinary drug residues in seafood products (7-9). Training on avermectin, isoeugenol, stilbene, and wide scope screening methods should be completed as needed.

5.2. References

1. Compliance Program 7304.018, Chemotherapeutics in Seafood Compliance Program (Issue Date 6/9/17)
2. Casey, C.R., et al. "Liquid Chromatography-Tandem Mass Spectrometry Method for the Confirmation and Quantitative Analysis of Avermectin Residues in Salmon" *Laboratory Information Bulletin* #4496, U.S. Food and Drug Administration 27 (2011) 1-12.
3. Casey, C.R., et al. "Liquid Chromatography-Tandem Mass Spectrometry Method for the Determination and Confirmation Analysis of Emamectin Residues in Salmon Tissue" *Laboratory Information Bulletin* #4567, U.S. Food and Drug Administration 30 (2014) 1-15.
4. Casey, C.R., et al. "A Rapid Liquid Chromatography-Fluorescence Detection (UPLC/FLD) for the Quantitative Analysis of Avermectin Residues in Salmon and Trout (CARTS No. IR01392)" *Laboratory Information Bulletin* #4636, U.S. Food and Drug Administration 34 (2018) 1-13.
5. Ye, L. "Development and validation of a LC/MS/MS method for the determination of isoeugenol in finfish with rapid extraction and simple off-line derivatization" *Laboratory Information Bulletin* #4619, U.S. Food and Drug Administration 32 (2016) 1-19.

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6. Lohne, J.J., et al. "Analysis of Stilbene Residues in Aquacultured Finfish using LC-MS/MS" *Laboratory Information Bulletin* #4535, U.S. Food and Drug Administration 29 (2013) 1-16.
7. Storey, J.M., et al. "Screening for veterinary drug residues in fish, shrimp and eel using LC-HRMS. Part 1. Optimization of a cleanup and extraction procedure" *Laboratory Information Bulletin* #4615, U.S. Food and Drug Administration 32 (2016) 1-16.
8. Turnipseed, S.B., et al. "Screening for veterinary drug residues in fish, shrimp and eel using LC-HRMS. Part 2: Optimization of MS acquisition and validation of method" *Laboratory Information Bulletin* #4616, U.S. Food and Drug Administration 32 (2016) 1-31.
9. Turnipseed, S.B., et al. "Wide-scope screening method for multiclass veterinary drug residues in fish, shrimp, and eel using liquid chromatography–quadrupole high-resolution mass spectrometry" *J. Agric. Food Chem.* 65 (2017) 7252-7267.

6. Answer Key

6.1. Triphenylmethane Dyes

1. CP 7304.018: The TTL for the sum of leucomalachite green and malachite green is 1.0 ng/g (1 ppb). If only LMG is present, the TTL for LMG is 1.0 ng/g. If both LMG and MG are present, the method must be able to quantify LMG below 1.0 ng/g.
2. AOAC 2012.25: Samples, standards, and solutions need to be protected from light exposure by storing standard solutions, samples, and extracts on a dark cabinet, in amber colored glass or covered with foil when not in use. Triphenylmethane dyes and metabolites have mutagenic and teratogenic properties; proper personal protective equipment is worn while preparing standards and performing the extraction steps to minimize one's exposure to these compounds.
3. AOAC 2012.25: The calibrants are internal standard corrected, matrix-matched extracted calibrants. Despite internal standard correction, differences in method accuracy may occur for some analytes if the calibration curve is prepared from extracted samples of a different type of negative control fish than the fish that is being tested.
4. AOAC 2012.25: Not necessarily. In addition to the full quantitative procedure, AOAC 2012.25 has been also validated as a screening method. Samples can be analyzed, and the response compared to one matrix-matched extracted

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calibrant at the 1.0 ng/g level; further quantitative analysis with the full matrix matched extracted calibration curve is only required for suspected positive samples.

5. AOAC 2012.25: Take care to clean bench areas after standard preparation. Avoid using black permanent markers which may contain crystal violet. Use an injection needle wash protocol between sample injections. Inject water blanks between sample injections.

6.2. Nitrofurantoin/Chloramphenicol

1. CP 7304.018: 1.0 ng/g (ppb) is the TTL for the nitrofurantoin antibiotic residues. 0.3 ng/g (ppb) is the TTL for chloramphenicol.
2. LIB 4597: 100 mM 2-NBA should be prepared fresh daily.
3. AOAC 2015:98: Positive atmospheric pressure chemical ionization (APCI) was used for the nitrofurantoin metabolites and negative electrospray ionization (ESI) was used for CAP analysis to achieve quantitation.
4. AOAC 2015:98: Most current methodologies for nitrofurantoin metabolites analysis utilize a mild acid hydrolysis and about a 16-hour derivatization step.
5. LIB 4597: The SPE cartridges are eluted with ethyl acetate.
6. CP 7304.018: Homogenize only edible portion. Thoroughly remove skin, bones, shell, any breading or seasoning before analysis.

6.3. Multiple residue methods

1. CP 7304.018: 10.0 ng/g (ppb) is the TTL for sulfonamide antibiotic residues. 5.0 ng/g (ppb) is the TTL for the combined contribution of ciprofloxacin and enrofloxacin. Ciprofloxacin is the metabolite of enrofloxacin treatment, so these residues are often found together.
2. CP 7304.018: 50g for a 600g composite
3. LIB 4562/4614: 2 times
4. LIB 4562: 250 µL/min. LIB 4614: 450 µL/min
5. LIB 4562/J. Chromatogr. B paper: Although the 4562 method provides accurate results for the tilapia matrix, this method does not perform well for residues of malachite green, crystal violet, or brilliant green in matrices such as salmon, trout, eel, or shrimp.
6. LIB 4562: The 4562-screening method utilizes a single point calibrant to establish a threshold response for each drug included in the method. The retention time and mass spectrometric product ion transition ratios can be used to identify the compounds of interest. These methods are also referred to as qualitative methods, or when threshold response data is used, as semi-

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quantitative methods. A quantitative method utilizes a calibration curve over a concentration range to calculate the concentration of the unknown sample.

Note: Screening methods DO NOT accurately determine the concentration of a drug residue in a sample.

7. Chemical Indices of Decomposition

7.1. Introduction

While sensory examinations are the primary method used by the agency to determine decomposition in seafood products, chemical indices provide a significant support mechanism to sensory findings in some products.

Histamine development in fish with high free histidine levels and indole in canned, cooked, and fresh/frozen shrimp are two important decomposition indicators supported by the agency. These indices may be used to support sensory findings in these products. Furthermore, since histamine can develop in the absence of detectable odors of decomposition and since the shrimp industry has employed treatments that mask odors of decomposition, chemical indices meeting the established criteria may be used to support regulatory action in the absence of sensory evidence in some cases. However, since decomposition pathways may develop which do not yield these metabolites, chemical analyses should not substitute or eliminate the need for sensory examination. Product guidance should be referred to for more information on the criteria used to support actions based on chemical indicators of decomposition. The original and check analyses should be used when supporting regulatory action based on chemical indices.

In addition to histamine and indole, there are a number of other indices that may have utility in determining the decomposed state of seafood. Two of the more promising ones are the diamines, putrescine and cadaverine. There may be other decomposition metabolites that may also be supportive if problems arise using the established methods. However, the laboratories should consult with the Office of Regulatory Science (ORS) and the Center for Food Safety and Nutrition (CFSAN) before employing any of these methods in the course of their regulatory activities.

The formation of chemical indicators of decomposition is associated with seafood that was not held at a proper temperature for storage after being caught or from mishandling during subsequent storage or processing. Most time-temperature abuse of histamine-susceptible fish occurs soon after catch. At that point the enzyme produced by bacteria is present and can produce more histamine at any point in the supply chain when fish is thawed, until fish products are cooked. In restaurant situations, storage of "good" product at improper temperatures can result in histamine (scombrotoxin) formation. Other

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chemical markers of decomposition have been found in spoiled fish, but their relationship to scombrototoxin fish poisoning has not been determined. Histamine can form in both high and low temperature storage conditions, and even before the associated odors of decomposition are apparent. Histamine-forming bacteria seem to be more sensitive to freezing than spoilage-producing bacteria. According to the FDA's Compliance Policy Guide 540.525, significant decomposition and histamine formation can be avoided by following good handling practices. This includes icing or rapid immersion of the fresh catch in chilled water (at -1°C) followed by continuous frozen storage. Leaving fresh catch lying about on deck of a fishing vessel for an extended period of time or interruption of frozen storage are common occurrences in the histories of histamine-contaminated products. The canning of fish provides additional opportunity for problems associated with poor handling. Frozen fish are received at the cannery and thawed prior to processing, at which point temperature abuse (letting the product get too warm or inadvertently allowing it to thaw) has another chance to occur. Additionally, temperature abuse can occur during transportation or retail display if cooling equipment is not held at the correct temperature.

The seafood industry has implemented programs to establish Hazard Analysis Critical Control Point (HACCP) plans to help producers prevent cases of contamination and foodborne illness. HACCP plans delineate the most likely locations and scenarios for something to go "wrong" in a process that would result in the food product becoming unfit for consumption. The HACCP theory can simply be summarized as: if it is known where the problems are most likely to occur, then a prevention and monitoring plan can be put in place to effectively control them. It is a proactive approach that places the burden on industry, not a reactive approach to be countered by the government and tax dollars.

7.2. Histamine

7.2.1. Background

Scombrototoxin fish poisoning is otherwise known as histamine or scombroid poisoning. The name "scombroid poisoning" was coined because histamine ("scombrototoxin") was first associated with fish species of the family Scombridae. However, it is now known that histamine can be produced in any fish species containing high levels of free L-histidine, which are typically dark meat fish and also include some non-scombroid fish (e.g., from the families Coryphaenidae and Pomatomidae). Histamine-producing fish include tuna, mahi-mahi, escolar, bonito, bluefish, sardine, pilchard, swordfish, marlin, and mackerel, to name a few. Histamine survives processing, and thus can be

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present in fresh, canned, or cooked product. The formation of histamine is typically associated with decomposed product. However, decomposed product (determined organoleptically) does not always produce histamine, and the presence of histamine does not always occur in decomposed product – thus sensory analysis cannot ensure the presence or absence of histamine. Histamine can reliably be quantitated by chemical analysis down to 1-3 ppm (an acceptable level often found in fresh fish) (CPG 540.525). However, LOQs vary based on instrument and analytical method.

The aforementioned fish are inherently high in levels of free L-histidine, from which histamine is formed in the muscle after death. The amino acid L-histidine is decarboxylated by histidine decarboxylase (HDC), an enzyme produced by certain bacteria common in fish. Since histamine-producing bacteria are commonly found in the viscera and gills of the fish, a fillet from the anterior section is more likely to be contaminated as the intestine decomposes. Formation of histamine is dependent upon the growth of these bacteria, which is a function of time and temperature. However, it is critical to understand that once the bacteria produce HDC, more histamine can be produced even when the bacteria are no longer viable. This is true until the enzyme is destroyed by cooking or denaturation (e.g., during a methanolic extraction). Excess L-histidine may also be produced by proteolysis during the advanced stages of spoilage, which can further contribute to the formation of histamine. In this case, however, sensory analysis would likely detect decomposition. Interestingly, histamine can also be found in cheeses (such as Swiss cheese) that rely on the action of bacteria to form the product. The distribution of histamine within an individual fish fillet is heterogeneous. One portion of the fish may cause poisoning, while another causes no reaction. It follows then that cans of processed product can have inconsistent histamine levels even within the same case lot (FDA, 1998).

Scombrototoxin fish poisoning manifests as an allergic reaction. Onset of the reaction can be immediate to within one hour. Symptoms may include tingling/burning mouth and lips, rash, headache, or nausea and vomiting. The symptoms may last for several hours and recovery is generally rapid. Antihistamine drugs are an effective treatment, however sensitive individuals may need further medical treatment. When multiple cases occur in an outbreak, attack rate can also be a useful clue in the differential diagnosis of scombrototoxin fish poisoning versus seafood allergy: Multiple cases support scombrototoxin fish poisoning while seafood allergies will impact a low percentage of those consuming the suspect fish. Patient histories are important also. The suspect food should be collected and analyzed as soon as possible to confirm the presence of histamine. A good indicator of undesirable

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fish is a sharp, metallic or peppery taste. Also, fish with an “off-smell” should be avoided (FDA, 1998).

Scombrototoxin fish poisoning knows no geographic boundaries. The network for harvesting, processing and distributing fisheries products is worldwide. Finished seafood products are sold fresh, frozen or processed to homes, restaurants or various institutions. That adds up to a lot of opportunities for decomposition to occur. The FDA monitors fresh, frozen and canned seafood for decomposition through organoleptic analysis. Products that might form histamine can be subjected to further chemical testing. Aside from the results of organoleptic analysis, product is also considered decomposed if it contains at least 50 ppm of histamine. However, regulatory action is considered on a case by case basis (CPG 540.525).

Methods used for histamine analysis include AOAC fluorometric method 977.13 for original and check analysis, and the ELISA-based Veratox Quantitative Histamine Test for rapid screening purposes.

Caution: When preparing fish flesh composites for testing, those prepared from unprocessed fish products should be considered thermally labile due to the potential presence of active histidine decarboxylase.

7.2.2. Exercise

The student should familiarize him/herself with the analytical guidance for histamine analysis: AOAC fluorometric method 977.13; Veratox for Histamine test kit; CPGM 7303.842 and 7303.844; the ORS-wide SOP for histamine analysis; and local laboratory's SOP for histamine analysis, as appropriate. The student will analyze either canned or fresh/frozen tuna. Both may be analyzed if time and resources permit. The trainer spikes duplicate samples at 50 ppm histamine [spiking done after the trainee has measured out an aliquot of the sample composite]. Alternatively, previously prepared canned tuna packs of known histamine concentration can be used. Analyze duplicate samples using the AOAC method, and following any additional analytical directives in the CPGM and SOP.

7.2.3. Questions

1. How many sub-samples are needed for histamine analysis when no odors of decomposition are present? When is it optional to do histamine analysis on product found by organoleptic analysis to be decomposed?
2. Why does histamine analysis need to be performed immediately following organoleptic analysis? If that is not possible, how should the sample be handled?

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3. For fresh/frozen fish, why is the anterior portion preferred for histamine analysis?
4. What is the purpose of the ion-exchange column? What chemistry is involved?
5. What is the purpose of the OPT reagent? What chemistry is involved?
6. Why is it important to “read” derivatized samples in a timely fashion?
7. Why does the slit width of the xenon lamp need to be less than 6 nm?

7.2.4. References

1. AOAC official methods of analysis (current ed.). Method 977.13 Histamine in Seafood, Fluorometric Method. Gaithersburg, MD: AOAC International.
2. U.S. Food & Drug Administration. Compliance program guidance manual. Compliance Programs 7303.842 Domestic fish & fishery products, and 7303.844 Imported seafood products. Washington DC: U.S. Government Printing Office. Available via Internet at: <http://www.cfsan.fda.gov/~comm/cp-toc.html>.
3. Staruszkiewicz, W., Jr., Waldron, E. and. Bond, J. (1977). Fluorometric determination of histamine in tuna: development of method. Journal of Association of Official Analytical Chemists, 60, 1125-1130.
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6. Hungerford, J.M., 2010. Scombroid poisoning: a review. *Toxicon*, 56(2), pp.231-243.
7. Lupo A, Mozola M. Validation study of a rapid ELISA for detection of histamine in tuna. Journal of AOAC International. 2011 May 25;94(3):886-99.
8. Veratox for Histamine kit insert

7.3. Indole

7.3.1. Background

The presence of indole may serve as a chemical indicator for the evaluation of incipient spoilage of crustaceans and other seafood products. Indole analysis can be used to enhance and reinforce sensory data. Indole is formed in

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shrimp, crabmeat, and other seafood products by bacterial decomposition of proteins in seafood products.

7.3.2. Exercise

The student should familiarize him/herself with the analytical guidance for indole analysis: AOAC LC-fluorometric method 981.07; CPGM 7303.842 and 7303.844; and the local laboratory's SOP for indole analysis. The student will analyze either canned or fresh/frozen shrimp. Multiple sample types may be analyzed if time and resources permit. The trainer spikes duplicate samples at 10 ug indole /100 g tissue (= 0.1 ug/g = 0.1 ppm) [spiking done after the trainee has measured out an aliquot of the sample composite].

Alternatively, previously prepared canned shrimp packs of known indole concentration can be used. Analyze duplicate samples using the AOAC method, and following any additional analytical directives in the CPGM and SOP.

7.3.3. Questions

1. What is the purpose of spiking samples with 2-methylindole?
2. What is the logic behind making standard solutions A, B and C first, instead of making calibrations solutions directly?
3. How does the detector type influence the extent of the extraction chemistry?
4. If a matrix is too "dirty" to allow baseline separation of the indole peak, what other options might the analyst have? Hint: see LIB#4016.

7.3.4. References

1. AOAC official methods of analysis (current ed.) Method 981.07 Indole in seafood, liquid chromatographic fluorometric method. Gaithersburg, MD: AOAC International.
2. U.S. Food and Drug Administration. Compliance program guidance manual. Compliance Programs 7303.842 Domestic fish and fishery products, and 7303.844 Imported seafood products. Washington DC: U.S. Government Printing Office. Available via Internet at: <http://www.fda.gov/ICECI/ComplianceManuals/default.htm>
3. Berg, R. and Carley, C. Modification of HPLC method for indole in shrimp. FDA Laboratory Information Bulletin, No. 4016.
4. Local laboratory's SOP for Indole Analysis.

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7.4. Answer Key: Histamine

1. How many subsamples are needed for histamine analysis when no odors of decomposition are present? When is it optional to do histamine analysis on product found by organoleptic analysis to be decomposed? Six sub-samples are needed. Analysis is optional when a confirmation-qualified organoleptic analyst performs a check exam, or if additional sample is sent to another servicing laboratory for check exam. The remaining sub-sample should be analyzed if histamine is detected at greater than or equal to 35 ppm in any of the initial sub samples. Alternatively, if a product was processed with chemical treatment (e.g., chlorine dip, CO, salt, smoke, etc.) that could mask odors of decomposition, all sub- samples should be analyzed for histamine.
2. Why does histamine analysis need to be performed immediately following organoleptic analysis? If that is not possible, how should the sample be handled? Because the enzymes that cause histamine formation can be still active. If can't analyze immediately, the sample should be frozen.
3. For fresh/frozen fish, why is the anterior portion preferred for histamine analysis? This is the portion of the fish near the gut. It is the gut bacteria that provide the enzyme to form histamine. So, if histamine is present in the fish, it is more likely to be at the anterior end.
4. What is the purpose of the ion-exchange column? What chemistry is involved? It functions as a clean-up mechanism: the anion-exchange column traps the amino acid contaminants but allows histamine to pass through.
5. What is the purpose of the OPT (o-phthaldialdehyde) reagent? What chemistry is involved? OPT converts histamine to a fluorophore making it visible for quantitative analysis. OPT is phthalate ortho-substituted with aldehyde. The two aldehyde groups reach out like arms to form a new ring with the amine function of an amino acid (in the presence of an organic acid), giving form to a fluorescent conjugated system which is water soluble. [Note that OPT is also abbreviated as OPA by chemists and may be written as o-phthalaldehyde.]
6. Why is it important to "read" derivatized samples in a timely fashion? Because the OPT-histamine complex breaks down over time from UV light energy which would give a false low reading.
7. Why does the slit width of the xenon lamp need to be less than 6 nm? Because a larger slit allows too much energy through, which degrades the OPT-histamine complex.

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7.5. Answer Key: Indole

1. What is the purpose of spiking samples with 2-methylindole? This compound is an internal standard with similar extraction efficiencies and absorbance characteristics to indole, yet is able to be chromatographed separately. An internal standard is used to help determine extraction recovery of the analyte, while providing a correction for extraction losses from the method.
2. What is the logic behind making standard solutions A, B and C first, instead of making calibrations solutions directly? Because the solutions are so dilute, it would be difficult to accurately weigh out indole standard in such a small amount. By starting with a large amount in a large volume, any error is minimized by subsequent dilutions (assuming no dilution errors!).
3. How does the detector type influence the extent of the extraction chemistry? Because the fluorescence detector is very specific to the histamine-OPT fluorophore, it eliminates the need to do extensive clean-up of the sample. If using UV detection, minimal clean-up could leave behind matrix components that could interfere with detection of the analyte.
4. If a matrix is too “dirty” to allow baseline separation of the indole peak, what other options might analyst have? Hint: see LIB#4016. Use a SPE-column to help clean-up matrix interferences.

8. Marine Toxins

8.1. Background

Most human intoxications by marine toxins in the US are due to the accumulation and metabolism of planktonic algae by filter-feeding marine organisms especially molluscan shellfish (e.g., mussels, clams, cockles, oysters). Among these microalgae, certain dinoflagellates produce the majority of marine toxins with the exception of the rare amino acid domoic acid and tetrodotoxin, which are produced by certain species in a specific genus of diatoms and bacteria, respectively.

The toxic microorganisms responsible for these seafood intoxications often form as algal blooms, referred to generally as harmful algal blooms (HABs), that occur when regional environmental conditions exist that are favorable to rapid proliferation of their populations. These conditions may include water temperature, sunlight, sea currents, and nutrients. Control factors, such as predation, also play a role in whether a bloom develops. Historically these HABs have been termed “red tides”, due to discoloration of the water caused by the presence of large numbers of these organisms. However, that term is a misnomer: HABs can occur without any noticeable change in the appearance

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of the water, and the presence of a red tide does not mean that the organisms are necessarily toxic.

Marine toxins are extremely diverse in their structures and mechanisms of action. The most toxic are neurotoxins exerting their physiologic effect by specific binding to voltage gated sodium channels (VGSCs) which are integral membrane proteins expressed in neurons, glia, and muscle cells.

Saxitoxins and Tetrodotoxins

Channel blockers such as saxitoxins (STXs) and tetrodotoxins (TTXs) bind to site 1 of the VGSCs and occlude the extracellular pore of the channel. This results in a polarization of net charge of the cell culminating in the toxins' respective pathologies. Among the toxins presenting the greatest risk to human health, they are small water-soluble neurotoxins. These guanidinium-based toxins block nerve function to cause respiratory paralysis. Saxitoxins are produced by certain species of dinoflagellates (e.g., *Alexandrium* spp., *Pyrodinium bahamense*, *Gymnodinium catenatum*) but studies have shown that the toxin profiles observed in dinoflagellates reflect both the dinoflagellate species of their origin and their subsequent metabolism in shellfish. Consequently, the very diverse toxin profiles observed in shellfish vary by shellfish species. Of all the marine toxins, the saxitoxins have the greatest impact on shellfish safety due to their global distribution, frequent occurrence, and well documented fatalities. Thus, the STXs are extensively monitored in US waters and around the world. TTXs, well known for their occurrence in pufferfish and occasionally fatal Fugu poisoning incidents in Japan, have only in recent years been reported in molluscan shellfish (initially in Europe). Tetrodotoxins have similar toxicity to saxitoxin, and yet they have an endosymbiotic bacterial origin. Their occurrence in molluscan shellfish is considered an emerging issue for consideration which may be correlated with climate change.

Ciguatoxins and Brevetoxins

Channel activators such as the brevetoxins (BTXs) and ciguatoxins (CTXs), bind to site 5 of the channel, leaving it in an open state, destroying the ion gradient, and depolarizing the cell.

Ciguatera fish poisoning is caused by the CTXs and is another seafood intoxication associated with certain species of toxic dinoflagellates. The commodity impacted is fish rather than shellfish, especially top predators like grouper, barracuda, and amberjack. Second only to Scombrototoxin fish poisoning, ciguatera is among the leading causes of illness (approximately 50,000/year) associated with consumption of fish. Ciguatera may present with gastroenteritis, cardiovascular issues including bradycardia (slow heart rate) and hypotension. There is also accompanying fatigue, insomnia and malaise.

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The most diagnostically relevant symptoms are neurologic; temperature reversal (cold allodynia), paresthesia (tingling or numbness) most often in the extremities, dental pain or a sensation of loosening teeth. Fatal intoxications are uncommon but do occur especially in the case of ciguatoxins originating in the Pacific Ocean. The potency of ciguatoxins is illustrated by the FDA advisory level of 0.01 ug Pacific ciguatoxin (CTX1B) equivalents per kg. Currently the only assay capable of detecting these low levels for ciguatoxins is a cell bioassay (N2A-MTT) using a mouse neuroblastoma cell line (N2A) and a cell viability indicator (MTT) developed at the Applied Technology Center (ATC) formerly the Seafood Products Research Center at the Pacific Northwest Laboratory. Collaborations between FDA, Japan, and Australia have been critical in addressing the ciguatoxins threat. Regardless of origin, investigations of ciguatera outbreaks using LC-MS/MS methods require gifted purified toxins from researchers as no analytical standards are commercially available. Cell bioassay of ciguatoxins can use commercially available standards such as CTX3C to determine CTX-like activity. Currently two laboratories in FDA (NFFL and GCSL/CFSAN) are equipped to perform the assay.

The BTXs cause Neurotoxic Shellfish Poisoning (NSP) and are unusual in that they have been found only in the Gulf of Mexico, the southern Atlantic seaboard of the US, and New Zealand. The BTXs are far less toxic than ciguatoxins and there are no documented fatalities. In addition to the usual gastrointestinal symptoms, NSP does share two noteworthy symptoms with CFP, temperature reversal and paresthesia which likely reflect their shared receptor, site 5 on the VGSC. Long monitored by using a mouse bioassay, the BTXs are also the subject of studies of validation efforts applying enzyme linked immunosorbent assay (ELISA) and LC-MS/MS confirmation. Although BTXs can also be detected using the N2A-MTT assay as with CFP, applying this assay to the BTXs is plagued by matrix effects due to their lower potency versus the CTXs.

Diarrhetic Shellfish Toxins (DSTs) and Azaspiracids (AZAs)

Like the brevetoxins and ciguatoxins, the DSTs and AZAs are polyether compounds and are produced by certain dinoflagellates. DSTs and AZAs which cause diarrhetic shellfish poisoning (DSP) and Azaspiracid Shellfish Poisoning (AZP) have much lower toxicity and are not neurotoxic. Further, the DSTs and AZAs can be found in temperate waters in contrast to the tropical waters where BTXs and CTXs occur. DSTs have a much wider global distribution than AZAs. Like the brevetoxins, the DSTs and AZAs are not associated with human fatalities. DSTs have a global distribution. In contrast,

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while AZAs have been detected globally, their largest commercial impact is in harvest waters off the coast of Ireland. The symptoms of both DST and AZA intoxications are primarily gastrointestinal and include vomiting, diarrhea, body aches, fever and chills. The onset of symptoms occurs from 30 minutes to 14 hours of eating contaminated shellfish. Although both cause primarily diarrhea, the mechanisms of action are different. Detection methods for both the DSTs and AZAs use predominantly LC-MS/MS. The DSTs can be screened using an enzymatic assay based on protein phosphatase subunit 2A (PP2A). The DSTs impacted Europe and Asia long before they were reported in the US. The DSTs caused a recreational harvest-related outbreak in the US Pacific Northwest while US illnesses due to AZAs have thus far only been due to imported mussels harvested off the coast of Ireland.

Domoic Acid

Domoic acid is a small hydrophilic and neurotoxic amino acid which can cause brain damage manifesting as loss of short-term memory. It was first discovered as a shellfish toxin in Canada in 1987 and first impacted the US in 1993. Many cases of permanent memory impairment resulted from the Canadian outbreak, and several fatalities occurred among the elderly and the immune-compromised. Other neuropathologic symptoms (confusion, memory loss, disorientation, seizure, and coma) were also manifest during the outbreak. The symptomatic memory loss observed in the illness led to the intoxication to be called amnesic shellfish poisoning (ASP). Accompanying the previously described neurologic symptoms, ASP is characterized by gastrointestinal disorders such as vomiting, diarrhea, and abdominal cramping. There have been no documented outbreaks of ASP in the US, and it is noteworthy that the first occurrence of domoic acid contamination in the US was signaled by its impact not on humans but on wildlife (pelicans and marine mammals) in California waters. Defying the narrow classification implied by ASP as a shellfish toxin, the vector for these intoxications in the wildlife intoxications was anchovies. Like shellfish, anchovies are filter feeders and bioaccumulate the toxins. Since the Canadian and US events, the occurrence of domoic acid in both molluscan and crustacean shellfish has caused numerous harvest closures and is closely monitored.

Commercial harvest of molluscan shellfish in the US is monitored by shellfish-producing states under the National Shellfish Sanitation Program (NSSP), which is the federal/state cooperative program recognized by the FDA and Interstate Shellfish Sanitation Conference (ISSC) for the sanitary control of shellfish produced and sold for human consumption. Although shellfish producing states have programs to post harvest warnings, most shellfish

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intoxications that occur in the US are due to recreational harvesters who ignore posted warnings and closures.

All humans are susceptible to intoxications by marine toxins. They are heat stable non-protein molecules, not inactivated by usual food preparation practices, i.e., cooking, freezing, pickling, etc. Ingestion of contaminated shellfish results in a wide variety of symptoms, depending upon the toxin(s) present, their concentrations in the shellfish, and the amount of contaminated shellfish consumed. Diagnosis of shellfish poisoning is based initially on observed symptomatology and recent dietary history, followed by analysis of unconsumed consumer portions if available.

At the time of writing, there are no active surveillance assignments to collect and analyze seafood for marine toxins. While import and domestic compliance programs (7303.844 and 7303.842, respectively) have provisions for ASP and PSP sample collection and analysis, only local field and center work is being done on other toxins. In both compliance programs, two methods are mentioned: the mouse bioassay (MBA) for PSP and HPLC for ASP. Indeed the “gold standard” method for the detection of marine toxins has traditionally been the MBA. However, in the past few decades, many technologies have been exploited, and validated methods have been developed using them. Some of these methods have been adopted for regulatory use, such as AOAC 2011.02 (RBA) and AOAC 2011.27 (Post-column HPLC), both of which have been tailored and adopted for specific use in some cases by the NSSP.

Currently, for the analysis of regulatory samples, ORS suggests the use of the RBA in the analysis for PSTs (NSSP recognizes this method for mussels and limited use for clams and scallops). For domoic acid the HPLC method described in Quilliam et al.1995, is approved by the NSSP and for use in ORA laboratories. Prior to the implementation of an alternative, validated method, a suitable laboratory verification should be performed, consistent with the servicing laboratory’s Quality Management plan.

Procedures for performing the RBA are given in AOAC 2011.27 and useful information is also found in the AOAC interlaboratory study. In addition, experience in ORA laboratories has shown that it is advantageous to have the following considerations in mind. First, ensure that the ORA laboratory is cleared for use of radioactive materials, and that proper radiation safety use and disposal protocols are adhered to. The amount of tritium used for the assay is within Nuclear Regulatory Commission Exempt status levels. However, if the performing laboratory already operates under an NRC License, the license should be amended to include tritium. Prior to purchasing tritium labeled saxitoxin, contact the ORA RSO and follow guidelines in the ORA Radiation Safety Manual. Next, rat brain synaptosome aliquots should be

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prepared and tested in advance of sample arrival. During preparation of the synaptosomes, all reagents should be kept on ice, and the completed aliquots should be stored at -80°C. Furthermore, protein concentration determination of the synaptosome preparation is best done by the bicinchoninic acid (BCA) protein assay. Bradford-based methods are not advisable due to interference, presumably by cell membrane lipid components, leading to an overestimation of protein concentration. Finally, to ensure well-to-well uniformity during the assay, it is advised to combine the following components into multiwell tubes: sample extract (or standard) dilution, ³H-STX working stock, and synaptosome prep. The tube contents should be mixed well by multichannel micropipettor aspiration prior to, and throughout plating.

Safety Levels in FDA regulated commodities (FDA, 2020, and FDA and EPA, 2020, Appendix 5)		
PSP		≥ 80µg STX equivalents/100g
NSP		≥ 20 MU/100g or 0.8 mg brevetoxin-2 equivalents/kg
AZP		≥ 0.16 mg AZA-1 equivalents/kg (0.16 ppm)
DSP		≥ 0.16 µg OA equivalents/kg
ASP		≥ 20 mg DA/kg
		> 30 mg DA/kg in Dungeness crab viscera
CFP		≥ Caribbean: 0.1 µg C-CTX-1 equivalents/kg
		≥ Pacific: 0.01 µg P-CTX-1 equivalents/kg

Laboratory analysis for marine toxins is restricted to specialized FDA field laboratories; training in this area is therefore up to the discretion of each servicing laboratory. Laboratories may contact the Applied Technology Branch of Pacific Northwest Laboratory or the Northeast Food and Feed Laboratory. Historically the Gulf Coast Seafood Laboratory branch of CFSAN has also provided assistance. These laboratories should be contacted for additional guidance and training suggestions.

8.2. Exercise

Review references, as well as the analytical portions of Compliance programs 7303.842 and 7303.844. Instructor will assign trainee with a PST-spiked sample at three toxin levels; below action level, action level and above action level, as well as unspiked. Trainee should perform extractions, prepare

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dilutions (along with standard dilutions), run the RBA, and analyze data with Graph Pad Prism (or equivalent software)

8.3. Questions

1. What are the criteria for an acceptable Standard Dilution curve in the competitive Receptor Binding Assay?
2. What is the purpose of Tryptophan as a reagent in the Domoic acid HPLC analysis?

8.4. References

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8.5. Answer Key: Marine Toxins

1. The standard curve should have a slope of -1 ± 0.2 and an EC_{50} of $2 \text{ } \mu\text{M} \pm 30\%$
2. Tryptophan is present in shellfish extracts and will elute close to domoic acid, potentially resulting in a false positive result. The inclusion of Tryptophan ensures that the method can adequately address this potential interference.

9. Document History

Revision #	Status* (D, I, R)	Date	Author Name and Title	Approving Official Name and Title
1.3	R	01/17/2008	LMEB	LMEB
1.4	R	02/02/2010	LMEB	LMEB
1.5	R	02/13/2014	LMEB	LMEB
02	R	06/15/2020	LMEB	LMEB

* - D: Draft, I: Initial, R: Revision

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10. Change History

Revision #	Change
1.3	9.2, para 2, line 3 – changed “confirmation” to “confirmatory” 9.3.2. A. – updated website 9.3.2. C. – changed “OK” to “permissible” 9.5, 9.2.1 1. – revised answer 9.5, 9.3.2 5. – changed “spectra” to “spectrum”
1.4	9.2 D. 2. – updated web link 9.3.1 D. 2. – updated 9.3.2 A. – deleted last sentence 9.3.2 D. – revised 9.5 9.3.1 1. and 3. – chemical names corrected Footer – updated web link
1.5	Header – Division of Field Science changed to Office of Regulatory Science 9.2 – Division of Field Science (DFS) changed to Office of Regulatory Science (ORS)
02	Areas shaded gray were revised: <ul style="list-style-type: none"> • Reformatted document which led to change in numbering and bullets • Chemotherapeutics: complete revision • Marine toxins: complete revision • Chemical Indices of Decomposition: updated information related to time/temperature abuse, fish species, enzyme action and implications for analytical testing, outbreaks, Veratox kits, ORS-wide SOPs, literature references, and other highlighted information.

11. Attachments

None