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9.1 Introduction

Seafood analysis within FDA encompasses food safety concerns arising from a number of issues. Chemical indices of decomposition are a measure of product abuse and can be found at the harvesting, distribution and retail levels. This chemical monitoring is done in conjunction with FDA's sensory (organoleptic) monitoring of seafood offered into commerce. The FDA also gets involved with chemically confirming histamine poisoning at the consumer level, especially when the State Health Department has been involved with an illness associated with a restaurant. Other issues center on chemical therapeutic practices at aquaculture farms, or marine toxins in shellfish. Seafood analysis also provides some of the most challenging chemistry encountered as a bench analyst. Both aquaculture drug residue analysis and seafood toxin analysis involve looking for analytes near the limit of detection and within a complex matrix. Extraction and ultimately the interpretation of results are not always straightforward. In addition, sample processing and handling calls for keen attention for reasons of both personal safety as well as sample integrity. This section provides training guidance for the important and common

analyses of chemical indices of decomposition (for all analysts), as well as the advanced and specialized analyses of aquaculture drug residues and marine toxins (for use in specialized laboratories).

9.2 Chemical Indices of Decomposition [Basic]

While sensory examinations are the primary method used by the agency to determine the decomposed state of seafood, 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/fresh/frozen shrimp and canned crab products are two important decomposition indicators supported by the agency. These indices may be used to support original and/or confirmatory 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 Division of Field Science (DFS) 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 properly refrigerated after being caught or from mishandling during subsequent storage or processing. In restaurant situations, storage of "good" product at improper temperatures can result in histamine (scombrotoxin) formation. Other chemical markers of decomposition have been found in spoiled fish, but their relationship to scombroid 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 7108.24, 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 has another chance to occur. Additionally, temperature abuse (letting the product get too warm or inadvertently allowing it to thaw) 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 Hazards Analysis and Critical Control Points (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 pro-active approach that places the burden on industry, not a reactive approach to be countered by the government and tax dollars.

9.2.1 Histamine

A. Background

Histamine poisoning is otherwise known as scombroid poisoning. The name "scombroid poisoning" was coined because histamine ("scombrotoxin") is produced in fish species of the families *Scombridae and Scomberesocidae*, as well as some non-scombroid fish like *Coryphaena* and *Pomatomus*. Histamine-producing species include tuna, mahi mahi, escolar, bonito, yellowtail, bluefish, sardine, pilchard, abalone, and mackerel, to name a few. Fresh product typically has barely detectable levels of histamine. Histamine can be present in fresh, canned and cooked product – the toxin survives processing. 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 5 ppm (an acceptable level often found in fresh fish) (CPG 7108.24).

The aforementioned species of fish are 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, an enzyme produced by certain bacteria common in fish. Since the associated bacteria are found in the fish gut, 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. Excess L-histidine may also be produced by proteolysis during spoilage, which can further contribute to the formation of histamine. 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 not necessarily consistent. 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).

Histamine 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. The suspect food is analyzed within a few hours to confirm the presence of histamine. A good indicator of undesirable fish is a sharp, metallic or peppery taste. Also, fish with an "off-smell" should be avoided (FDA, 1998).

Scombroid 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 spoilage 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 7108.24).

B. Exercise

The student should familiarize him/herself with the analytical guidance for histamine analysis: AOAC fluorometric method 977.13; CPGM 7303.842 and 7303.844; and the local laboratory's SOP for histamine analysis. 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.

C. 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?

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?

D. 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.

4. Local laboratory's SOP for Histamine Analysis.

9.2.2 Indole

A. Background

The presence of indole may serve as a chemical indicator for the evaluation of incipient spoilage of shellfish and other fisheries products. Indole analysis can be used to enhance and reinforce sensory data. Indole is formed in shrimp, crabmeat, oysters, clams, lobsters, salted anchovies, and other fisheries products by bacterial decomposition of fish proteins.

B. 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, or canned crabmeat. 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 or crabmeat 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.

C. 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.

D. 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.cfsan.fda.gov/~comm/cp-toc.html.

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.

9.3 Chemotherapeutics in Seafood

Over the past several years, 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. The main public health concern is with antibiotic residues finding their way into the environment (by pond run-off, surviving sewage treatment, etc.) and influencing 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 ORA and may or may not be published in a journal or the AOAC Official Methods of Analysis, but only circulated by DFS or published in FDA's Laboratory Information Bulletin. However, the compliance program will serve as guidance on which method is correct to use. For the listed specie-drug combinations, a determinative analytical method is specified, but only some have an additional confirmation method listed (typically LC or GC with MS). The training methods discussed in this section focus on the determinative methods only. Training for the associated confirmation methods may be done on an individual basis. The chemistry 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.

9.3.1 Malachite/Leucomalachite Green [Advanced]

A. Background

Malachite green is a cationic triphenylmethane dye used as a topical fungicide in the aquaculture industry to control the growth of fungi on fish and incubating eggs. Studies have shown that malachite green excretes rapidly, but the base, leucomalachite green, has a long residence time in muscle tissue. This exercise instructs the trainee to determine the amount of malachite green present in a sample of catfish tissue.

B. Exercise

Review all references. The trainer will provide a sample that has been fortified with 10 ppb with malachite green and leucomalachite green. Determine the amount of malachite green and leucomalachite green present, using the method described in Compliance Program 7304.018. In addition to the training sample, prepare and take through the method a fortified sample (20 ppb) and a negative control sample. Determine the % recovery of the fortified sample. Report all findings on an analytical worksheet.

C. Questions

Consider the chemical structures of malachite green (MG) and leucomalachite green (LMG):

1. Why does MG absorb energy in the visible range and LMG does not?

2. Why does MG and LMG adsorb onto the propylsulfonic acid cation exchange solid phase extraction (PRS-SPE) column during the extraction procedure?

3. What is the elution solvent for the PRS-SPE column and why does it elute the analytes?

4. Why did the method developers choose a cyano (CN) analytical column?

5. What effects would varying the composition (ratio of acetonitrile to acetate buffer) of the mobile phase have on peak retention? Why?

6. The detector is set at 618 nm, the maximum Absorbance wavelength for MG, which is blue-green in color. How is it LMG, which is colorless, is also detected at this wavelength?

7. Why is it important to "pre-treat" the mobile phase with PbO_2 prior to use?

8. The method System Suitability requirements state that the LMG peak height should be >90% of that for MG for equivalent weight (ng) injected. What is the probable cause for failure to meet this requirement?

9. A chromatogram for an injection of a LMG/MG standard shows two peaks, but they are misshapen: broad, with shoulders, or "fronting". What could be causing this?

D. References

1. U.S. Food & Drug Administration, Center for Food Safety and Applied Nutrition. *Compliance program guidance manual*, Compliance Program 7304.018, Chemotherapeutics in seafood. Washington DC: U.S. Government Printing Office.

2. Roybal et.al. (1995). *Journal of AOAC International, 78*(2), 453-457.

9.3.2 Chloramphenicol [Intermediate]

A. Background

Chloramphenicol (CAP) is a powerful antibiotic often reserved for cases of resistant bacterial infections in humans; and in rare cases is implicated in the development of aplastic anemia – thus

a possible danger in its therapeutic use or by exposure as an unexpected food adulterant. It is also effective against common shellfish diseases, thus its use in veterinary practice. Testing of shrimp, crab, or crayfish for chloramphenicol has waxed and waned over the years. Originally CAP testing was strictly a GC/MS method, but now ORA utilizes several different LC/MS/MS methods for simultaneous determination and confirmation. Regulatory testing is based on the premise that the presence of CAP in seafood is by human addition and may render a food unsafe. Declarations from experts in the field, stating that CAP is not being absorbed by the shrimp from indigenous CAP-producing biota in their environment, are available for view at http://intranet.cfsan.fda.gov/OC/pages/seachlor.htm.

B. Exercise

Review all references. The trainer will provide a homogenized shrimp sample that has been fortified at 0.3 ppb with CAP, as well as additional blank homogenized shrimp meat for use as described below. (The trainee should also gain practice with the dry-ice homogenization process by assisting an experienced analyst.) Determine the amount of CAP present, using a method described in Compliance Program 7304.018. In addition to the training sample, prepare and take through the method a fortified sample (0.3 ppb or as appropriate) and a negative control sample. Determine the % recovery of the fortified sample. Report all findings on an analytical worksheet.

C. Questions

1. Why is it permissable to use plastic centrifuge tubes plus aspiration for liquid-liquid extraction in the Rupp and Stuart methods (see below) versus traditional separatory funnels, such as in the Neuhaus method? What are the advantages?

2. What is the advantage of performing shrimp homogenization using dry ice? What precautions should be taken?

3. Why are standards made up in blank matrix extract?

4. What are criteria for successful confirmation of the presence of CAP in crab meat?

5. How does one best use mass spectral data to mathematically confirm the presence of a target analyte?

D. References

CFSAN lists approved chloramphenicol LC/MS methods (for both seafood and honey) on their website: http://www.cfsan.fda.gov/~frf/capintro.html. These methods were developed within ORA, primarily at the Pacific Regional Laboratory Northwest (Seattle) and the Animal Drugs Research Center (Denver). Check with your laboratory management or CPGM 7301.018 for

which method to use based on the equipment available in your laboratory. As of FY 2004, the following methods were included for use in regulatory analyses:

Shrimp:

- Storey, J. M., et al. Determination of Chloramphenicol Residues in Shrimp and Crab Tissuesby Electrospray Triple Quadrupole LC/MS/MS: *Laboratory Information Bulletin*, No. 4306, June 2003 No. 6.

- Neuhaus, B. K., et al. LC/MS/MS Analysis of Chloramphenicol in Shrimp: *Laboratory Information Bulletin*, No. 4290, September 2002 No. 9.

Crab:

- Rupp, H. S., et al. LC/MS/MS Analysis of Chloramphenicol in Crab Meat: *Laboratory Information Bulletin*, No. 4302, April 2003 No. 4.

- Storey, J. M., et al. Determination of Chloramphenicol Residues in Shrimp and Crab Tissuesby Electrospray Triple Quadrupole LC/MS/MS: *Laboratory Information Bulletin*, No. 4306, June 2003 No. 6.

Crawfish:

- Stuart, J. S., et al. LC/MS/MS Analysis of Chloramphenicol in Crawfish Meat: *Laboratory Information Bulletin*, No. 4303, April 2003 No. 4.

Dry ice homogenization:

- Bunch, E. A., et al. "Homogenous sample preparation of raw shrimp with the aid of dry ice, " *JAOAC Int.*, 78:883-887.

Other:

- U.S. Food & Drug Administration, Center for Food Safety and Applied Nutrition. *Compliance program guidance manual*, Compliance Program 7304.018, Chemotherapeutics in seafood. Washington DC: U.S. Government Printing Office.

- Roybal, J. E. (1998) Chloramphenicol and related drugs, in Turnipseed & Long (Eds.), *Analytical procedures for drug residues in food of animal origin*. West Sacramento, CA: Science Technology System.

9.3.3 Oxolinic Acid [Intermediate]

A. Background

Oxolinic acid (OA) is a quinolone antibacterial useful in the treatment or prevention of aquaculture diseases. It is reported that OA residues may persist in fish many days post dosing, especially in the skin and bones. Cooking may release OA residues from bone and skin into the muscle and water. (Zomer & Charm, 1998).

B. Exercise

Review references. The trainer will provide a homogenized salmon sample that has been fortified at 20 ppb with OA, as well as additional blank homogenized salmon meat for use as described below. (The trainee should also gain practice filleting a salmon by assisting an experienced analyst.) Determine the amount of OA present, using the determinative method described in Compliance Program 7304.018. In addition to the training sample, prepare and take through the method a fortified sample (20 ppb or as appropriate) and a negative control sample. Determine the % recovery of the fortified sample. Report all findings on an analytical worksheet.

C. Questions

1. State the general difference between oxolinic acid and oxalic acid. In which part of the method is oxalic acid used and what is its function?

D. References

1. Larocque, L., et al. (1991). Determination of oxolinic acid residues in salmon muscle tissue by liquid chromatography with fluorescence detection. *J. AOAC Int.* 74(4):608-611.

2. Pfenning, A.P., et al. (July 1996). Confirmation of incurred residues of flumequine, nalidixic, oxolinic, and piromidic acids in shrimp and salmon. FDA *Laboratory Information Bulletin*, No. 4039.

3. U.S. Food & Drug Administration, Center for Food Safety and Applied Nutrition. *Compliance program guidance manual*, Compliance Program 7304.018, Chemotherapeutics in seafood. Washington DC: U.S. Government Printing Office.

4. Zomer, E., and Charm, S. E. (1998) Quinolones, in Turnipseed & Long (Eds.), *Analytical procedures for drug residues in food of animal origin*. West Sacramento, CA: Science Technology System.

9.4 Seafood Toxins [Advanced]

Shellfish poisoning is caused by a group of toxins elaborated by planktonic algae (dinoflagellates, in most cases) upon which the shellfish feed. The toxins are accumulated and sometimes metabolized by the shellfish. Amnesic shellfish poisoning (ASP) is caused by the unusual amino acid, domoic acid, as the contaminant of shellfish. The 20 toxins responsible for paralytic shellfish poisonings (PSP) are all derivatives of saxitoxin. Diarrheic shellfish poisoning (DSP) is presumably caused by a group of high molecular weight polyethers, including okadaic acid, the dinophysis toxins, the pectenotoxins, and yessotoxin. Neurotoxic shellfish poisoning (NSP) is the result of exposure to a group of polyethers called brevetoxins. FDA only includes ASP and PSP in the current compliance program.

All humans are susceptible to shellfish poisoning. Elderly people are apparently predisposed to the severe neurological effects of the ASP toxin. A disproportionate number of PSP cases occur among tourists or others who are not native to the location where the toxic shellfish are harvested. This may be due to disregard for either official quarantines or traditions of safe consumption, both of which tend to protect the local population.

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. In the case of PSP, the effects are predominantly neurological and include tingling, burning, numbness, drowsiness, incoherent speech, and respiratory paralysis. ASP is characterized by gastrointestinal disorders (vomiting, diarrhea, abdominal pain) and neurological problems (confusion, memory loss, disorientation, seizure, and coma). Diagnosis of shellfish poisoning is based entirely on observed symptomatology and recent dietary history.

All shellfish (filter-feeding mollusks) are potentially toxic. However, PSP is generally associated with mussels, clams, cockles, and scallops; and ASP with mussels.

[The foregoing was adapted from the "Bad Bug Book: Foodborne Pathogenic Microorganisms and Natural Toxins Handbook," U.S. Food & Drug Administration**,** CFSAN, on-line at: http://www.cfsan.fda.gov/~mow/intro.html.]

Laboratory analysis for marine toxins is restricted to specialized FDA field laboratories. Therefore training in this area is up to the discretion of each servicing laboratory. Laboratories may contact the Seafood Products Research Center, co-located with Pacific Regional Laboratory Northwest and Seattle District, for additional guidance and training suggestions.

9.5 Answer Key

9.2.1 Chemical Indices of Decomposition: 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 lets histamine 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.

9.2.2 Chemical Indices of Decomposition: 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.

9.3.1 Aquaculture Drugs: Malachite Green

1. **Why does MG absorb energy in the visible range and LMG does not?** LMG does not have a high degree of conjugation. Oxidation with Lead Dioxide (PbO_2) gives the molecule an extended conjugated system, making it a chromophore. Most dyes or colorants are chromophores.

2. **Why does MG and LMG adsorb onto the Propylsulfonic Acid Cation Exchange Solid Phase Extraction (PRS-SPE) column during the extraction procedure?** The amino functional groups on the MG and LMG molecules are electrochemically bound to the sulfonic acid functional groups of the SPE substrate in the presence of neutral acetonitrile.

3. **What is the elution solvent for the PRS-SPE column and why does it elute the analytes?** Mobile phase, followed by Hydroxylamine Hydrochloride/ Methanol/p-Toluene Sulfonic Acid (HAH/MeOH/p-TSA) is used to elute the SPE column. In this relatively acidic medium, the analytes easily go into solution, rather than stay bound to the solid substrate.

4. **Why did the method developers choose a cyano (CN) analytical column?** A CN column is a mid-range polarity column that can be used in either normal or reverse phase. The ionic

nature of MG (it has a positive charge) will cause it to be retained on a CN column longer than LMG in the reverse phase, thus achieving good separation.

5. **What effects would varying the composition (ratio of acetonitrile to acetate buffer) of the mobile phase have on peak retention? Why?** Increasing the percentage of acetonitrile in the mobile phase will shorten the retention times of the analytes. Acetonitrile (as used in reverse phase chromatography) is slightly more polar than the CN column, causing MG and LMG to interact less with the column.

6. **The detector is set at 618 nm, the maximum Absorbance wavelength for MG, which is blue-green in color. How is it LMG, which is colorless, is also detected at this wavelength?** LMG and MG are separated on the analytical column, and then each passes through the PbO_2 Post Column Reactor. LMG is oxidized by the PbO_2 to MG before passing through the detector. What the detector "sees" at 618 nm is actually two MG peaks.

7. **Why is it important to "pre-treat" the mobile phase with PbO_2 prior to use?** The solvents and reagents in the mobile phase may contain reducing agents, which could rapidly consume PbO_2 in the post-column oxidizing chamber.

8. **The method System Suitability requirements state that the LMG peak height should be >90% of that for MG for equivalent weight (ng) injected. What is the probable cause for failure to meet this requirement?** Loss of LMG signal is an indicator that the PbO_2 has been exhausted and the reactor column should be repacked.

9. **A chromatogram for an injection of a LMG/MG standard shows two peaks, but they are misshapen: broad, with shoulders, or "fronting". What could be causing this?** The use of the post column increases the chromatographic volume and will cause some band broadening. An excessive amount of broadening or fronting may indicate a void in the post column, possibly formed because the column was not packed tightly enough.

9.3.2 Chloramphenicol

1. **Why is it OK to use plastic centrifuge tubes plus aspiration for liquid-liquid extraction in the Rupp and Stuart methods (see below) versus traditional separatory funnels, such as in the Neuhaus method? What are the advantages?** It is OK because only the manner of physical manipulation has changed, not the chemistry of the separation. The use of plastic centrifuge tubes is generally faster and more convenient for the analyst. Also, it allows the analyst to use centrifugation to break any emulsions that form between the solvent layers, avoiding traditional chemical means for breaking emulsions (the serendipitous use of which by the analyst would be considered an unvalidated chemical modification of the method).

2. **What is the advantage of performing shrimp homogenization using dry ice? What precautions should be taken?** When ground in a food processor, raw shrimp becomes quite gummy and it is difficult to achieve uniform homogenization. Using excess dry ice in the processor bowl along with the shrimp meat freezes the meat and prevents it from gumming up, facilitates the grinding of the meat to a small and even crumb, and facilitates an even distribution of particles. The frozen shrimp crumbs can easily be transferred to a storage container (whose lid should be left ajar over night in a freezer to allow the CO_2 to sublimate). Safety precautions include the use of a sturdy metal food processor bowl, appropriate personal protective gear, and the adequate identification of the CO_2 breathing hazard to other personnel in the area (especially for multiple storage containers placed in a walk-in freezer to sublimate overnight which can overwhelm the oxygen in a small enclosed space).

3. **Why are the standards made up in a blank matrix extract?** A small amount of endogenous shellfish chemicals make it through the extraction process (no matter how extensive) and end up in the final solution that is injected into the LC/MS. In this case, the presence of these chemicals effects the ionization and fragmentation to a small but measurable extent. Making standards in matrix extract provides a "level playing field" for the comparison of MS data between standards and food samples.

4. **What are criteria for successful confirmation of the presence of CAP in crab meat:**

    - A retention time agreement between sample and (matrix) standard of within ±0.3 minutes.
    - Parent ion of *m/z* 321.
    - Daughter ions of *m/z* 152, 176, 194 and 257; no other significant peaks present.
    - Daughter ion *m/z* 152 is the base peak.
    - When comparing the numbers for a sample versus a standard, the ratios of *m/z* 176, 194, and 257 versus *m/z* 152 are within 10% (relative).
    - The signal to noise ratio for the weakest daughter ion (*m/z* 176) should be ≥ 5 X.

5. **How does one best use mass spectral data to mathematically confirm the presence of a target analyte?** We want to compare peak areas of the chosen significant ions in a spectrum, to the one peak that is the largest in that spectrum (the "base peak" – typically m/z 152 for CAP). From this we get a series of ratios, which can be converted to simple percent of the base peak. We then compare these ratios of a standard injection to those of a sample (or spike) injection. We calculate a relative percent difference, and we may consider standard and sample spectra to match if this relative percent difference is less than 10% for each ion. Of course the ratios for any given ion may change from day to day, especially at low levels of analyte in matrix.

9.3.3 Oxolinic Acid

1. **State the general difference between oxolinic acid and oxalic acid. In which part of the method is oxalic acid used and what is its function?** Oxolinic acid is an antibacterial agent, whereas oxalic acid is a strong organic acid (found in spinach and rhubarb, incidentally, and capable of chelating calcium which one's body needs). Close, but not quite the same in spelling and pronunciation – but close enough to be confusing. Therefore, pay close attention when reading the method to *use the correct compound when specified*. Oxalic acid is used during clean-up to facilitate holding the oxolinic acid in the aqueous phase during a lipophilic extraction. It is also used in the mobile phase to again modify the pH, and to reduce possible chromatographic tailing.

9.5 Document/Change History

Version 1.3 Changes:
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"

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