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.

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