Sections in This Document

1. Introduction

The Food, Drug, and Cosmetic Act protects the public from the presence of filth, putrid or decomposed material in food products, and those products that may have been exposed to insanitary conditions that may contaminate the product with filth or render it injurious to health. The terms filth, foreign material, or extraneous material are used interchangeably. The courts define filth in a common-sense manner; filth does not have any specialized or technical definition. Filth is any type of matter that obviously does not belong in a food product. Representative examples of filth in food products include but are not limited to rodent excreta, insects, parasites, and extraneous materials such as metal and glass shards.

Filth can enter a product through many forms and sources; and is often invisible to the consumer. Filth may be present in food naturally and unavoidably, or as the result of an intentional or unintentional controlled bad practice. The identification, confirmation and quantitation of the type of filth can help determine how the material was found in the product, if it was a natural and unavoidable event, an accidental event, a controllable event, an unintentional event and/or a deliberate intentional event.

More importantly, analysts can assess the real or potential health risk involved with these adulterants. Given today's food production operations and storage facilities, when the presence of a health hazard or vector potential is confirmed, the findings may place added emphasis on certain problems and the surrounding circumstances. The objectives of this chapter are to introduce the visual/macroscopic and microscopic techniques, practices, and procedures used to identify and confirm the presence of adulterants in various commodities and provide practice in reporting such findings in a clear and concise manner.

Given the nature of the work and the broad definition of filth, analysts with a strong background in the biological/agricultural sciences with special emphasis in entomology, pest control, botany, agricultural and food production have an advantage in this course work. However, FDA filth analysts come from diverse disciplines. This chapter is not intended to be all inclusive but will provide FDA

analysts the basic six to nine-month orientation program for beginner filth analyses.

1.1. Reference Books and Materials

The trainer provides, as a minimum, the following instructional materials for the trainee's general use:

- Gorham, J. R. (Ed.). (1991). *Ecology and management of food-industry pests* [Formerly released as FDA Technical Bulletin No. 4]. Gaithersburg, MD: AOAC International.
- Vail, D. J. (n.d.). *Micro-analytical biology workbook for food sanitation control analysts* (out of print, Vol. 1). Atlanta, GA: FDA, Southeast Regional Laboratory.
- *AOAC official methods of analysis* (current Edition). Gaithersburg, MD: AOAC International.
- U.S. Food & Drug Administration, Center for Food Safety and Applied Nutrition. (1998). *Macroanalytical Procedures Manual* (FDA Technical Bulletin No. 5). [Originally released in print by FDA in 1984 by Olsen, A. R. (Ed.), Knight, S. A. (Tech. Ed.), Ziobro, G. C., Ph.D. (Assoc. Ed.)]
- Harris and Reynolds (Eds.) *Microscopic-analytical methods in food and drug control* (out of print, FDA Technical Bulletin No. 1).
- Gorham, J. R. (Ed.) (1981). *Principles of food analysis for filth, decomposition and foreign matter* (FDA Technical Bulletin No. 1, 2nd Ed.). Gaithersburg, MD: AOAC International.
- Gorham, J. R. (Ed.). (1978). *Training manual for analytical entomology in the food industry* (out of print, FDA Technical Bulletin No. 2, HHS Publication No. (FDA) 77-2086).
- Gentry, J. W., Harris, K., Gentry, Jr., J. W. (1991). *Microanalytical entomology for food sanitation control* (Vols. I and II, revised reprint of O.D. Kurtz and K.L. Harris AOAC publication by the same name). Gaithersburg, MD: AOAC International.
- Olsen, A. R., Sidebottom, T. H. and Knight, S. A. (1996). *Fundamentals of Microanalytical Entomology, a practical guide to detecting and identifying filth in foods*. Boca Raton, FL: CRC Press.
- Hui, Y. H., Bruinsma, B. L., Gorham, J. R., Nip, W., Tong, P. S., Ventresca, P. (2002). *Food plant sanitation.* New York: Marcel Dekker,

Inc.

- Bennett, G. W., (2010). *Truman's Scientific Guide to Pest Control Operations (7 ed.)* Questex Media Group LLC & Purd.
- Arnold Mallis; Stoy A Hedges and Dan Moreland, Mallis Handbook of Pest Control, 10th Ed., GIE Media, Inc.

1.2. Sample Collections

The trainer arranges for the collection of these 22 food samples for Section 4.4 training assignments:

- Wheat or other whole grain (Section 4.4.2)
- Green coffee beans or cocoa beans (Section 4.4.2)
- Flour (Collect enough to satisfy Section 4.4.2 and 4.4.3.1)
- Whole or crude spice: capsicum pods (Section 4.4.2)
- Whole or crude spice: peppercorns (Section 4.4.2)
- Whole figs or dates (Section 4.4.2)
- Shell nuts (Canned/Mixed) (Section 4.4.2)
- Blueberries, raspberries or cherries (Fresh/Frozen) (Section 4.4.2)
- Whole tamarind pods (Section 4.4.2)
- Fig paste or other fruit paste (Section 4.4.3.1)
- Chocolate (Section 4.4.3.1)
- High bran content bakery goods (Section 4.4.3.1)
- Dried ground spice (Collect enough to satisfy Section 4.4.3.1, 4.4.3.2 and 4.4.4.1)
- Peanut butter (Section 4.4.3.2)
- Canned tomato juice (Collect enough to satisfy Section 4.4.3.2 and 4.4.4.2)
- Canned sliced button mushroom or dried wild mushroom (Section 4.4.3.2)
- Whole fruit or vegetable (Canned or Fresh) (Section 4.4.4.1)
- Cinnamon or Cassia sticks (Section 4.4.4.1)
- Tomato concentrate product (puree, sauce or paste) (Section 4.4.4.2)
- Berry or citrus juice product (Section 4.4.4.2)

- Rodent, insect, floor sweepings, exhibits (Section 4.4.5 and 4.5.1)
- Rodent contaminated food and packaging (Section 4.4.5 and 4.5.1)

1.3. Investigations Training

The trainer makes arrangements for the trainee to accompany a district investigator on the following types of inspections:

- Establishment Inspection (EI) of a food processing plant and a food warehouse.
- Wharf examination of import foods.

General Information

The trainees will receive Basic Orientation Training through ORA University web-based modules, discussions, exercises, and videos; the trainees are expected to complete the ORA University Analyst Bingo card within six months of employment.

Before beginning to work in a district laboratory, the trainee should be given a thorough safety orientation that includes familiarization with the laboratory's safety features and regulations, and the local laboratory's Standard Operating Procedures (SOP's) and Quality Assurance Procedures. Emphasis is placed towards the policies and procedures inherent in the filth analysis program. Additional information can be found in the ORA Lab Manual, Volume III, Section 2, "Environmental Health and Safety."

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2. Procedure

2.1. General Information

2.1.1. FDA Law and Filth

Objective

This exercise describes the legal basis for the agency's regulatory activity in foods, drugs, and cosmetics contaminated (adulterated) with filth.

In order to analyze a filth sample, the analyst is to be intimately familiar with the requirements and prohibitions of the Food, Drug, and Cosmetic (FD&C) Act. The filth analyst demonstrates compliance or non-compliance with sections of the Act. Filth Analyst work primarily with evidence collected to

show non-compliance with Sections 402(a)(3), 402(a)(4), 501(a)(1), 501(a)(2)(A), 601(b), 601(c), 801(a)(1), and 801(a)(3) of the FD&C Act.

It is very important to have a clear understanding of the difference between sections 402(a)(3) and 402(a)(4). Section 402(a)(3) deals with a condition of the food that is objectionable in itself, whereas section 402(a)(4) deals with an objectionable practice.

Sections 501(a)(1), 501(a)(2)(A) deal with adulterated drugs and devices 601(b), 601(c), deal with adulterated cosmetics, and 801(a)(1) and 801(a)(3) deals with import and exports for the same types of offenses as described above.

A careful reading and discussion of the assigned literature is essential towards a more complete understanding of these and other sections of the FD&C Act.

Assignment

Complete: Introduction to the FD & C Act, web-based training module.

Read and discuss starred (*) items with the trainer from the following sections of the FD&C Act:

201(f)*, (g), and (i) Definitions

301(a)*, (b)*, (c)*, (g), and (k)* Prohibited Acts

402(a)(3)* and (a)(4)* Adulteration (Also, review 21 U.S.C. 342(a)(3), (a)(4), and (b)(2))

501(a)(1) and (a)(2)(A)

601(b) and (c)

702(b)* Reserve Sample

704(d)* Letter

801(a)(1) and (a)(3)

Read and discuss with the trainer:

1. Gorham, J. R. (Ed.) (1981). *Principles of food analysis for filth, decomposition and foreign matter* (FDA Technical Bulletin No. 1, 2nd ed., pp. 3-9). Gaithersburg, MD: AOAC International.
2. Gentry, J. W., Harris, K., Gentry, Jr., J. W. (1991). *Microanalytical entomology for food sanitation control* (Vols. I and II, pp. 3-11). Gaithersburg, MD: AOAC International.

3. Olsen, A.R., Gecan, J.S., Ziobro, G.C., Bryce, J.R. (2001). *Regulatory Action Criteria for Filth and Other Extraneous Materials V. Strategy for Evaluating Hazardous and Nonhazardous Filth*. Regulatory Toxicology and Pharmacology, 33, 363-392.
4. Olsen, A. R., Sidebottom, T. H., Knight, S. A. (1996). *Fundamentals of Microanalytical Entomology, a practical guide to detecting and identifying filth in foods* (pp. 1-9). Boca Raton, FL: CRC Press.
5. U.S. Food & Drug Administration, Office of Regulatory Affairs, Office of Enforcement. Filth from Insects, Rodents, and Other Pests in Foods. *Compliance policy guides*, Sec. 555.600 (CPG 7120.).
6. U.S. Food & Drug Administration, Office of Regulatory Affairs, Office of Enforcement. Tree Nuts - Adulteration Involving Rejects (Insect Infestation, Moldy, Rancid, Otherwise Decomposed, Blanks, and Shriveled). *Compliance policy guides*, Sec. 570.425 (CPG 7112.05).
7. U.S. Food & Drug Administration, Office of Regulatory Affairs, Office of Enforcement. Foods, Adulteration Involving Hard or Sharp Foreign Objects. *Compliance policy guides*, Sec. 555.425.
8. U.S. Food & Drug Administration, Office of Regulatory Affairs, Office of Enforcement. Foods, Rail Car Sanitation – Adulteration. *Compliance policy guides*, Sec. 545.300 (CPG 7117.08).
9. U. S. Food and Drug Administration, Center for Food Safety and Applied Nutrition (May 1998 revision). *The food defect action levels* (read the Introduction).
10. U. S. Food & Drug Administration, Office of Regulatory Affairs, Office of Enforcement. Apricots - Canned - Adulteration with Insects. *Compliance policy guides*, Sec. 550.150 (CPG 7110.02).

2.1.2. Microscopic Examination and Microscope Accessories

Objective

The trainee receives instruction towards the use and care of laboratory microscopes and their accessories, and learns proper terminology and definitions of the various forms of microscopic examination.

Discussion

Types of Examinations and Microscopes

Visual/Macroscopic Examination

This type of product examination generally depends on the direct sensory input of the analyst. Examination is typically conducted with the naked eye but also may include the use of a hand lens (generally in the range of 3-10X), magnifying ring lamps (3X), or a pair of jeweler's loupes (3-10X).

Widefield Stereomicroscope or Dissecting Microscope

Results from a macroscopic examination may not be conclusive because the adulterant/defect cannot be completely identified without the assistance of a stereo microscope. This instrument, the most frequently used in filth analysis, has a wide variety of applications. The main application is for examining gross filth and for reading (examining) extraction papers for microscopic filth. The most commonly used magnifications are from 6-30X; higher magnifications (50-75X) are used to confirm the identity of small objects. The microscopes exhibit a large field of view and have large working distances (from the lens to the focal point) in order to move and manipulate objects under the lens.

Microscopic Examination

Filth/adulterants in the product are usually not visible to the naked eye and therefore are examined microscopically. A widefield stereo is often used first to manipulate and separate the contaminated product and then a compound microscope is used to positively identify the material and to see fine microscopic detail, morphology and sculpture. Compound microscopes optimize lighting techniques and are engineered to manipulate light to see details not observable in stereoscopic examination, thus they have a very limited field of view and they have short working distances. The typical working magnifications are 100-400X, but may be used at 1000X with proper technique and special oil that helps capture and retain the light.

<u>Fundamental Microscopic Techniques and Procedures</u>

Lighting and Ocular (eyepiece) Optimization Techniques

Whether using a widefield or compound microscope, eye fatigue is usually the most important limiting factor when working with microscopes for any period of time. To help reduce headaches and eyestrain, the analyst needs to learn the following:

Lighting: Adjustment begins with the light source, which should be daylight, blue, or white (not frosted). Light strength should be adjusted with the transformer or iris diaphragm, so that details can be seen clearly with a minimum of glare and a minimum of intensity or brightness. For a stereomicroscope, the light source should be positioned slightly to one side

and at approximately a 70° angle from horizontal, or overhead, as with fiberoptic ring lights. The light field should be centered so that both eyes receive the same intensity of light (i.e. balanced).

Oculars (eyepieces): are adjusted to the individual analyst's eyes and facial features- while the eyes are in their most relaxed state. This procedure should be done before each magnification. Note: Leave both eyes open while adjusting the oculars. Wearing eyeglasses is a matter of individual preference. People with astigmatism may find wearing their eyeglasses will reduce eye fatigue. If eyeglasses are to be worn, high eyepoint eyepieces may be ordered and should be used, and the ocular adjustments should be made with the eyeglasses on.

Procedure:

Adjust the interpupillary distance of the oculars so that the oculars are centered on the respective pupils.

Determine which ocular is independently focusable.

Place a specimen on the stage of the instrument, focus to the clearest image, and center it in the field of view.

Hold a black or white index card between the focusable ocular and the eye, or simply remove the eyepiece, blocking vision on that side. *Do not close the eye or squint; the eye remains in a relaxed eye position.*

Using the main focusing adjustment knob, clearly focus the image of the specimen for the eye that is not blocked.

Remove the index card from the first position and use it to block the vision of the other eye (or remove the opposite eyepiece.) Using the focusing ring mechanism of the focusable ocular (not the main adjustment knob), clearly focus the image of the specimen.

Remove the card and using both eyes, view the specimen. The image should now be clearly in focus for both eyes in their most relaxed state

Resolution (compound microscopes)

For compound microscopes, the trainee should learn how to achieve optimal or *Köehler illumination*. The Köehler Principle focuses the field iris in the same plane as the specimen, thereby obtaining maximum resolution. Step-by-step instructions for achieving Köehler illumination are found in "Training Manual for Analytical Entomology in the Food Industry," Chapter 2 Part III D; FDA Technical Bulletin No. 2 and/or in the microscope manuals. The trainer

demonstrates how to accomplish Koehler illumination and the trainee repeats the work on their unit.

General Microscope Maintenance

The analyst is responsible for simple maintenance procedures when using the instrument e.g. tightening loose focusing mechanisms, properly cleaning the lenses, and changing the light bulbs. These and other maintenance operations are described in the instruction manuals accompanying each instrument and should be addressed in the laboratory's Standard Operating Procedures. Maintenance records should be kept in the instrument logbook, and more complicated maintenance is directed to the assigned microscope monitor's attention and should not be attempted without prior approval.

Special Types of Microscopy and Accessories

Phase-Contrast Microscope

The phase-contrast microscope is a compound microscope that has special sub-stage accessories and objective lenses designed to produce optical contrast between the specimen and the mounting medium. Using phase-contrast optics, the analyst can observe many details that are obscure or indiscernible under a conventional compound microscope. This type of microscope is widely used for examining mites and small, somewhat transparent insects, like maggots, and in glass identification.

Success with the phase-contrast microscope begins with and is highly dependent on the refractive indices of the specimen and the mounting medium. In order to get good contrast, the mounting medium should have the greatest possible difference in refractive index from the specimen. The difference between the refractive indices creates variations in light intensity. To the observer, it appears that a halo of intense light is surrounding the very dark edges and surface structures of the specimen, starkly silhouetting otherwise vague details.

The instrument is adjusted in the same manner as a conventional compound microscope. Once optimal illumination is achieved, the phase-contrast optics are aligned in the following manner:

Each phase objective lens has a metal ring of a defined size imbedded between two of the lens elements. The ring size is indicated by a number (usually "Ph 1," "Ph 2," or "Ph 3") printed on the lens casing.

Similarly, the condenser has annular rings glued to a rotating clear plate that rotates into set position They are marked "1," "2," "3," and "clear (or J)" This

latter position is the “normal” position for transmitted light microscopy, or the “starting" position for phase microscopy.

In phase microscopy, the analyst properly selects the matching objective and condenser ring numbers, positioning the ring of the condenser in the light path, so that it will just encircle the ring in the objective. That is, the analyst pairs the objective ring to the condenser ring.

Initially, the adjustment of the instrument proceeds in the same manner as conventional transmitted light microscopy, using the “J” condenser position. Establish Köehler illumination first, and then focus on a mounted slide object (like a mite’s hairy leg). Finally, select the low power objective and begin aligning the phase-contrast optics, i.e. each objective with its corresponding condenser ring. Phase-contrast microscopes vary significantly by manufacturer and the proper alignment techniques are unique to each (some use focusing eyepieces, while others use swing-in focusing lenses). Therefore, consult the manual for the particular microscope, or have the trainer demonstrate how to critically align the rings.

Polarized Light Microscopy (PLM)

A polarizing light microscope is a compound microscope fitted with polarizing prisms, called "Nicols," below and above a rotating circular stage. When two Nicols are placed in the optical train, the first acts as a polarizer and the second as an analyzer. The vibration direction of the plane-polarized light produced by the polarizer is conventionally the north-south direction. If the analyzer, which can be rotated in most instruments, is set in the same relative position as the polarizer (parallel Nicols), then through light is transmitted, producing a light field of view. But, if the analyzer is rotated through 90 degrees so that its plane of vibration is at right angles to the polarizer (crossed Nicols), no light will pass, except that refracted into the analyzer’s plane, producing a dark field of view.

Optically active substances show interference colors when placed between crossed Nicols. Observations of optical activity can be useful to the analyst for identifying to some extent such diverse things as glass fragments, plastics, synthetic fibers, crystals, starches, and mites. This segment of training gives the trainee the basic principles of PLM techniques. Additional descriptions of PLM techniques will be found in Advance techniques Chapter 4.6.2 Optical Crystallography.

Comparison Microscopy (Forensic microscopy)

Comparison Microscopy involves “bridging” the optics of two microscopes, so

that they may be viewed independently, side by side, or overlapped for direct comparison purposes. In this manner, filth analysts can compare known specimens, with unknown specimens, and confirm similarities or identify differences.

Lightfield/Darkfield Stereomicroscopes

Lightfield/Darkfield stereomicroscopes are conventional stereomicroscopes mounted on a special light base, the base of which produces either a white background (fully illuminated field of view) or a dark background (with incident light coming in at an angle). The technique is very useful in increasing contrast (similar in a way to phase contrast) and in viewing light subjects against a dark background. Examples include using darkfield illumination when trying to count mites, versus counting white mites or maggots against a white background.

Scanning Electron Microscopes (SEM)

Scanning electron microscopy (SEM) is used to visualize samples at higher magnifications (10X - 500,000X) and can provide morphological information about a sample. SEMs have particular applications in particle analysis and detailed micro-structural analysis. Several publications describe the use of electron microscopes in the examination of mites and stored product beetle mandibles, antennae, animal hair and related structures. Tabletop/Benchtop SEMs can be configured to easily image an object *in situ* or without many modifications. This may include using a low vacuum or atmospheric mode to allow for the imaging of biological samples. An Energy Dispersive X-ray Spectrometer (EDS) can be added to an SEM and is used to obtain qualitative elemental information about the sample. In the area of particle analysis, SEM/EDS can help to identify particles using not only their structure and morphological form, but also their elemental composition. Follow your local laboratory's SOP or SEM operation manual for the particular unit in your lab.

Microscope Accessories

Eyepiece Micrometer (or graticule)

The eyepiece micrometer consists of a clear disc with a graduated scale or pattern printed on it, which is inserted in the eyepiece of the microscope for use in measuring specimens. In order to obtain meaningful measurements, the graticule is calibrated for each magnification, by using a stage micrometer with a graduated scale of known increments (usually 0.1 mm for stereomicroscopes or 0.01 mm for compound microscopes).

Calibration Procedure

Place the stage micrometer on the microscope stage and focus on the stage micrometer scale. (The eyepiece graticule should always be in focus; if not, the disc is probably improperly inserted or out of adjustment.) Move the stage micrometer so that the zero end of its scale coincides with the zero end of the eyepiece scale and the two scales are superimposed on each other over their entire lengths. Reading from the eyepiece scale, find the farthest division from zero that coincides with a division on the stage micrometer.

Record the following information:

Magnification

The number of eyepiece scale divisions between the zero coincident and the farthest eyepiece scale coincident. This value is designated EMD (eyepiece micrometer divisions).

The number of stage micrometer scale divisions between the zero coincident and the far stage scale coincident. This value is designated SMD (stage micrometer divisions).

The millimeter value of one division of the stage near scale. This value is usually found printed on the micrometer. This value is called mm/SMD (millimeters per stage micrometer division).

Calculate the millimeters per eyepiece micrometer division (mm/EMD)

mm/EMD = (mm/SMD) X (SMD)/(EMD)

The mm/EMD is the number of millimeters per eyepiece micrometer division for that particular magnification. This value is used to convert eyepiece micrometer divisions to millimeters by multiplication. (*Note:* These calculations need to be determined for each set of eyepiece and objective combinations.)

Example: At 10X magnification, 12 EMD coincide with 18 SMD on a stage micrometer in which 1 SMD = 0.1mm

(0.1mm/SMD) X (18 SMD)/(12 EMD) = 0.15 mm/EMD

If an object is observed to be 5 EMD long at 10X magnification, then

(0.15 mm/EMD) X (5 EMD) = 0.75 mm

The object's length is calculated as 0.75 mm, the last digit being only an approximation. The significance of calculated numbers should be carefully considered in light of the mathematical rules concerning significant figures.

Mechanical Stage Micrometer

This “accessory” consists of two graduated scales that are engraved or inscribed on the mechanical stage. One scale is on the moving portion and the other scale is directly parallel to the first on the stationary portion of the stage. As the length of the specimen's image is moved through a fixed point in the field of view, the number of divisions on the stationary stage scale can be counted by observing the starting and finishing positions of the sliding scale's end point. By substituting this number for the EMD in the equation found in the previous section, the trainee can calculate a conversion factor using a stage micrometer with a graduated scale of known increments. Quite often, mechanical stage micrometers will have a provision for interpolating the final digit of a reading, which adds to their accuracy.

Note: The eyepiece micrometer has more versatility than the mechanical stage micrometer; the eyepiece micrometer can be positioned over the image at exactly the angle desired for determining longest and shortest dimensions.

Camera Lucida (Abbé type)

The Camera lucida (Abbé type) consists of a set of prisms that can superimpose the image of a specimen onto a piece of paper lying on the bench top beside the microscope. With practice, while viewing the specimen through the scope, the trainee can see the paper and pencil at the same time as the specimen, thus producing accurate outlines or detailed drawings of the specimen under observation. (*Hint:* Illuminate the drawing paper with a strong light.)

This technique is particularly useful when photography does not show the desired details (poor depth of field or cluttered information), that line drawings can produce.

Assignment

Read the following:

Gorham, J. R. (Ed.) (1981). *Principles of food analysis for filth, decomposition and foreign matter* (FDA Technical Bulletin No. 1, 2nd ed. pp. 219-228). Gaithersburg, MD: AOAC International.

Gorham, J. R. (Ed.). (1978). *Training manual for analytical entomology in the food industry* (out of print, FDA Technical Bulletin No. 2, chap. 2).

Möllring, F. K. *Microscopy from the very beginning* (Brochure G41-100, p. 58). U.S. and Germany: Zeiss, Inc. Or, Möllring, F. K. (1973). *Beginning with the*

microscope. New York: Sterling Publishing. Both out of print, but useful if one can obtain a copy.

The microscope manufacturer's manuals for the scopes the analyst will be using.

Demonstrate the optimal set-up and illumination with specimens provided by the trainer using the following:

Widefield stereoscope

Compound microscope

Phase-contrast microscope

Prepare a table of measurements for an eyepiece micrometer on a compound or stereomicroscope in the laboratory.

2.1.3. Preparing Microscopic Slide Mounts

Objective

The trainer provides basic instructions for properly preparing permanent and semi-permanent microscopic slide mounts of hairs, insect fragments, minute whole insects, or other "filth" elements. Slide mounts may be prepared for use in reference collections or as teaching aids or, in regards to regulatory samples, as evidence in a court of law.

This training is applicable to all analysts involved in the identification of filth elements found in foods. General entomological knowledge and/or training are not a prerequisite for this section.

Discussion

Introduction

No amount of microscope alignment, focusing, or other manipulation can undo the damage done by the improper mounting of a specimen on a microscope slide. The mounted filth specimen prepared for regulatory work is an item of evidence, ultimately subject to the scrutiny of a court of law. Fine detail observations may provide significant clues and to the identity of the object. Additionally, the need for quality mounted materials for use in a reference collection is essential and is tantamount to good museum practice. As such, the analyst strives to become proficient at producing professional-quality slide mounts.

Equipment and Reagents

dissection microscope

dissection needles

fine (needle) point forceps

microscope slides

microscope slide cover slips (square and/or round, 1-1½ thickness)

adhesive labels

hot plate

alcohol lamp (IF the laboratory allows open flames)

slide warming plate (typically at 45-50ºC)

water bath

glycerin

gelatin

phenol

gum arabic (crystalline form)

chloral hydrate

distilled water

2% solution of aqueous acid fuchsin or lignin pink

commercially purchased "permanent" microscope mounting media (i.e. Permount, Euparal, and/or Canada Balsam)

ringing compounds- nail polish (clear preferred) or Glyptal® (electrical insulating varnish)

fume hood

standard safety equipment (laboratory coat, eye protection, gloves)

Specimens for Observation

General information

There are many text books and articles written on mounting specimens for observation under the scope. Many techniques date back to the early development of the microscope, as people worked with various formulas to accomplish the perfect slide mount for whatever material they were studying. Over time, people and disciplines developed preferences based on their

needs, ease of use, or understanding of the media. Selecting the media of choice varies significantly based on the following factors:

How easy is it to use?

What is the refractive index and how well does it work with certain specimens of higher or lower index?

What preparation steps are needed?

How long does it take to prepare the finished slide?

How long will the slides last - temporary, intermediate, or permanent, before they discolor, crack, or cloud over?

What effect does the media have on the specimen or stain specimens?

Does it need ringing?

Is it expensive?

Does it call for the use of noxious chemicals?

How valuable is the specimen - what type, an authentic, forensic value as evidence, or quick, non-permanent observation?

Based on these questions this next section offers some discussion of the media FDA analysts have found to be of the most value and the easiest to use.

Media

Wet Mount

A wet mount is a quick and temporary slide mounting technique. A drop of water or glycerin alcohol is placed on the microscope slide using a dropper or pipette. The specimen is then added to the drop and a cover slip is placed on top of the specimen. The edge of the cover slip is placed on the slide at a 45^{o} angle. It is then gently lowered over the specimen reducing the chance of air bubbles. Tapping the cover slip gently can release trapped air bubbles. To insure the specimen is covered a sufficient amount of liquid needs to be on the slide. If too much liquid is on the slide the cover slip will float off the specimen. This can be corrected by placing a tissue or paper towel along one edge of the cover slip to draw up the excess fluid. If the slide mount is too dry, add a small amount of fluid on the side of the slide that is lacking fluid.

One advantage with a wet mount, a liquid stain can be added to the specimen, while the specimen is still under the cover glass. Along one edge of the cover slip place a drop of the stain to be used. At the opposite edge of the

cover slip place a tissue or paper towel to absorb the water under the cover slip. The stain will get drawn under the cover slip and stain the specimen.

Glycerin Jelly (GJ) Media

The most commonly used and preferred medium for mounting hairs and insect fragments is glycerin jelly (GJ).

The formulation is 10 g gelatin, 70 ml glycerin, 60 ml H_2O and 1 g phenol. The gelatin is poured on cold water to soak, and then heated over a water bath to completely dissolve the gelatin. The glycerin and phenol are mixed while hot. When cooled, the mixture has the consistency of semi-hardened gelatin and it melts around 35-40ºC. Glycerin jelly can also be ordered from some chemical supply houses. The phenol can be substituted with an antiseptic mouthwash like Listerine, using 1-2ml of the mouthwash., thus creating an alternative mounting media called Kaiser's Glycerin Jelly.

To mount a specimen, a small piece of glycerin jelly is placed on a slide and warmed to the point where it becomes fluid. As an alternative, the analyst can use pre-melted material, from a glass rod dropping bottle, held on a slide warming plate. With practice, the analyst will have more control of the media droplet size using pre-melted media. The specimen is placed, then pushed into the media with a needle probe into the bottom/center of the liquid medium, then oriented to the desired position, and covered with a coverslip. Warming the slide again is sometimes needed for the jelly to engulf the specimen and fill the space under the coverslip. If trapped air is present near the specimen, the air can be removed by gently heating it over an alcohol lamp or low temperature hot plate.

Caution: This may cause the specimen to migrate to the edge of the coverslip. If this happens, the analyst may need to remount the specimens and make another preparation. It is important for the analyst to practice mounting specimens in order to get a "feel" for the peculiarities of glycerin jelly.

To mount hairs in glycerin jelly, the analyst uses a little extra heat to drive out the air in the center (medullary) portion of the hair. The characteristics of the hair cannot be observed until the air inside the hair has been replaced with glycerin jelly. One common method of removing air is to heat the mounted specimen carefully over an alcohol flame or hot plate until the glycerin jelly under the coverslip begins driving the air out of the hair (*Note:* just below the media's boiling point), then cool and observe the specimen at high magnification to see if the air is gone. Continue heating and observing until sufficient air is driven out to make definite identification. Again, practice is

needed, as too much heat will curl and distort the hair, warp the coverslip, and denature or discolor the medium. See also Reference: LIB 2243, “Improved Procedure for Liquid Replacement of Entrapped Air in Mammalian Hairs.”

Note: Some analysts make a slide by piling the specimen and coverslip on top of a solid piece of glycerin jelly and then warming the slide so that the glycerin jelly engulfs the specimen. Two problems may occur using this method. One, the specimen migrates with the melting media towards the edge; secondly, large air bubbles can be formed and trapped under the coverslip. These are considered permanent mounts when ringed (ringing is discussed below, Section C). They should be held flat, even when rung. They are stable for at least 5 years and longer if rung, and generally do not discolor or cloud over time. Specimens do not need special drying (water removal) processes as in other resin or Canada balsam mounts, however, mounting from dishes wet with glycerin-alcohol (50/50) or 70% alcohol does reduce some of the trapped air problems.

Hoyer's Solution

Hoyer's solution is a mounting medium that has been used by entomologists for decades and is now gaining popularity in some areas of food analysis. It is found commercially, but has many formulation variations, all principally gum-chloral hydrate derivatives. In addition to its excellent optical properties (~1.47), Hoyer’s solution renders muscular and visceral tissues transparent (clearing effect), allowing the analyst to observe cuticular structures on the intact specimen without interference. This medium is used primarily for mites and small insects, but it can also be used for insect fragments. It is not used for hairs, except as a temporary mount, as it will disintegrate a hair over time. When rung the slides will last several years, but eventually moisture will enter, and the slides will cloud over.

Hoyer's solution consists of 50 ml distilled H_2O, 30 g gum arabic, 200 g chloral hydrate, and 20 g glycerin. The gum arabic should be in crystalline form since the powdered form is difficult to wet. Ingredients are mixed in the given sequence; allowing time for one ingredient to be completely dissolved before adding the next. The final product is filtered through bolting cloth or glass wool. This medium has numerous modifications with names such as Berlese's fluid and de Faure's Fluid. (*Safety note*: Care is to be taken with chloral hydrate compounds; breathing the fumes and exposure to the chemical are not recommended. Use only in a hood. Additionally, chloral hydrate is a schedule IV controlled substance so has additional requirements for its use and acquisition.)

Specimens may be mounted in Hoyer's solution directly from aqueous solutions or may be mounted live. This medium has good optical properties for phase-contrast microscopy. To obtain a longer-lasting slide, the slide mount is cured for 48 hours to one week at 45ºC (113°F) and then held at room temperature for one week before sealing. Slides left undisturbed at relatively uniform room temperature will cure naturally in about three to four weeks. Temperatures above 45ºC will harm the medium. Whenever using this medium, care is taken to properly vent fumes.

Canada Balsam, Permount, Euparal

For permanent slide mounts, Canada balsam is the most commonly recognized medium. Other commercially found mountants include Permount® and Euparal. They are desirable for museum quality work and for extremely long-term storage of authentic materials. All of these are natural or synthetic resin based mountants. The major drawback to these materials is timeliness and almost all typically call for tedious and often difficult specialized water removal drying techniques to prevent clouding. The drying techniques employ a series of gradient alcohols, to xylene, to mixed xylene/mountant solutions, prior to mounting in the diluted resin. Euparal, an alcohol based mountant, is an exception to the full xylene based systems, but it still calls for gradient alcohol fixing stages. For purposes of this training, these mountants demand advanced techniques beyond the scope of this section, however students should be aware of their usefulness and need for long term storage. *Note*: Use xylene and toluene in vented areas only. Histological hoods are recommended if these compounds are used with any frequency.

Ringing (Preserving Slides)

Preservation of slide mounts is accomplished through a technique referred to as ringing. Glycerin jelly and Hoyer's solution are primarily media for nonpermanent slide mounts. However, with careful preparation and maintenance, they can be made to last many years. Once the medium has set (hardened), the coverslip edges should be sealed to prevent moisture exchange and to hold the coverslip in place. The most common sealants are nail polish or Glyptal, (a flexible sealant for electrical connections). The sealant is painted on with a small brush. Round cover slips slides are typically centrally mounted on a rotating table (like a Petri dish turntable). The table is spun, and while holding the brush steady overhead, the sealant is applied around the edge as the coverslip rotates underneath.

Clearing (Removal of Interfering Material)

Clearing is a process that clears, removes or dissolves excess proteinaceous, gut or optically interfering materials from specimens. Clearing renders a specimen more optically usable for mounting, as a cleared specimen often shows more detail and surface characteristics.

Some common clearing agents are dilute (10%) potassium hydroxide, dilute (10%) sodium hydroxide, (50-85%) lactic acid (the strength depending on sclerotization; stronger sclerotization needs stronger acidic concentration), and lacto-phenol, which consists of (85%) lactic acid, phenol and water in the ration Specimens cleared in any of these solutions can be rinsed in water and mounted directly in Hoyer's solution or other water-based media.

Since this is a destructive process, do not let the specimen stay too long in the solution. Consideration should be given to neutralizing the hydrolyzed specimens, to prevent undesirable continued hydrolysis compatibility problems with subsequent mounting media.

This technique is not to be confused with bleaching, which removes excess pigmentation and coloration. However, some bleaching action may still occur in cleared specimens.

Staining Specimens

After clearing, most small arthropods are more clearly observed with differential staining. Two percent solutions of aqueous acid fuchsin or aqueous lignin pink have been used successfully on aphids, mites and *Collembola*. Lactophenol cotton blue solution can be used to stain yeast and mold.

Specimen Orientation on Slides

Specimens being mounted for examination under a compound microscope should be mounted in the middle, oriented with the head or front end directed towards the bottom of the slide, and centered under the coverslip. Normally, mount one specimen per slide. However, there will be situations when it is acceptable to mount multiple specimens on one slide. Examples include similar items mounted next to each other to show similarities or differences, sex differentiation in the same species, or to demonstrate different orientations of the same species. When mounting multiple specimens, keep in mind labeling space and the added difficulties of orienting multiple objects without them moving from the desirable positions.

Labeling Slides

Promptly identify every slide. Label the right side (lot label) with the sample

number, sub number, date, analyst's name or initials. If space allows include the product, country of origin, lot or location. Label the left side with the specimen identification (to the correct taxonomy level) and include any additional information that is useful, e.g. sex, stage, size, and fragment. The identifier's name or initials and date, if different from the preparer, should be on this label. Either side may include the mounting medium or ringing material, to facilitate later remounting.

Storage of Slides

Slide mounts should be stored flat. The mounting media may retain a small amount of fluidity if excessive media is used, or if it is not properly cured or rung. Over time, gravity may cause the coverslip or the specimen to migrate downward if held vertically. Protect the slide from crushing or accidental inversion.

Exercise

Prepare the following:

Glycerin jelly

Hoyer's solution

Prepare a wet mount:

a) Make a slide mount of some mold

b) Stain the mold with lactophenol cotton blue solution
Properly mount and label specimens of:

1. Any whole insect which measures less than 3mm
2. Insect fragment(s) elytra, a pair of mandibles, antenna, and legs
3. A mouse hair
4. A mite

References and Supporting Documents

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4. Bullington, S. W. How to mount maggots on microscope slides. *Forensic Entomology* website: http://www.forensic-ent.com/.
5. Schauff, M. E. *Collecting and preserving insects and mites: techniques and tools.* Washington, D.C.: Systematic Entomology Laboratory, USDA, National Museum of Natural History.
6. Horobin, R. W., Kiernan, J. A. (Eds.). (2002). *Conn's biological stains: a handbook of dyes, stains and fluorochromes for use in biology and medicine* (1st ed.). Abingdon, UK: Bios Scientific Publishing, Ltd.

2.1.4. Collecting and Preserving Whole Insects and Arthropods

Objective

This section will provide basic instruction for collection and preservation techniques for whole insect and arthropod specimens found in regulatory samples and those specimens collected for the laboratory's authentic reference collection. A filth analyst does not routinely collect insects in the traditional sense (for example with a butterfly-net), and we rarely preserve them as a pinned specimen. Although the majority of the laboratory's work will deal with insect fragments, whole specimens are first discussed. Analysts do find whole insects, (and in some cases, whole live insects) in regulatory samples. These insects are evidence that are properly preserved for courtroom presentation. If the specimens are collected for use as authentics, as reference materials and/or direct comparison with unknown specimens, only the best museum preservation techniques are applied.

Procedure

Recommended Equipment

Vials (1/2 dram, 1 dram, and/or 2 dram sizes, glass with screw-on cap with polyseal® cone insert recommended)

Sieves (U.S. Standard #8, #20, #40 and pan; a "collar" is also recommended)

Berlese Funnel and/or Tullgren Funnel

Aspirator (recommend "exhalation" style or "inhalation" style with in-line filter)

Artist's camel hair brush (recommend #2 or #3)

Jeweler's or needle point forceps

Flexible steel forceps

5X Magnifying lamp

White plasticized butcher paper

Various sized white or stainless-steel pans

Recommended Reagents

95% Ethanol/Isopropanol

Glacial acetic acid

Kerosene

Dioxane ("Triton X-100" or "Tween 80" may be substituted)

Formalin (40% formaldehyde)

10% KOH, 10% NaOH, 50-85% lactic acid, or 2:1:1 lacto-phenol

Distilled Water

Ethyl acetate

Note: There are commercial killing fluids, clearing agents, and preservatives. If these commercial products are used, the above list of reagents could be reduced.

Collecting Insects

Insects found in regulatory samples by macroscopic examination or microscopic examination are collected and preserved as evidence. In some cases, these insects may be alive. The method used to extract insects from a product will vary based on the composition of the product. Although a method may be dictated, there are some "traditional" entomological methods that may be of value.

Sieves

Insects may be removed from some products by placing the product in nested sieves (generally, a #8 is placed over a #20 or finer.) If the product being sieved needs to be contained, a collar or lid is added to the uppermost sieve, and a pan to the lower. Generally, the product is placed in the uppermost sieve and shaken, causing insects and other foreign material to fall onto the lower sieve and into the pan. Conversely, if the filth analysis is looking for larva in flour, the larva is retained in the uppermost sieve, and the flour passes through to the pan below.

Berlese/Tullgren Funnel

The Berlese Funnel, originally developed by Antonio Berlese to remove live mites from leaf litter, is an option for removing live insects and mites from reasonably dry leafy food materials (i.e. taro or palm leaf). The device is a large funnel with a coarse (U.S. STD #2, #4, or #8) woven metal screen inserted above the neck to hold back the product, yet allow insects to pass through into a jar of preserving fluid. Insects and mites will be driven down by a heat source (generally a 40-60 watt light bulb) in a lid that covers the mouth of the funnel. The Tullgren Funnel, a modification using a series of baffles rather than a screen, has been used to remove insects and mites from dry powders or granules that are too small for a typical Berlese. A workable Berlese apparatus is described in LIB 883 and some useful modifications in LIB 1816. See also *J. Assoc. Off. Anal. Chem., 55(1), 51-56, 1972.*

Aspiration

An aspirator may be used to vacuum up small insects and/or mites. One type of aspirator involves inhalation or a vacuum pump to draw the insects into the container. Another type uses exhalation or an air pump to collect the specimen. It is recommended if an inhalation type aspirator without a vacuum pump is used, insure that there is an in-line filter between the user and the collection chamber, in particular where the substrate, insects and/or mites are harmful (from microbes, spores, or chemicals). A simple aspirator is described in LIB 1637 and in *J. Assoc. Off. Anal. Chem., 55(1), 51-56, 1972.*

Killing Insects

Freezing

When dealing with live insects or where insects are in dry products, freezing is the easiest and safest technique, provided the analyst allows time for proper penetration of the cold temperatures to the center of the product or exhibit. This can be checked with a thermometer. During the freezing process, insects are driven away from the cold temperatures and into the center of the product or exhibit. Care is taken to maintain the specimens in a frozen condition, until they can be examined. This is needed because allowing them to come back to room temperature for extended periods of time (e.g. a day), will cause discoloration and damage to a specimen. Freezing does not stop enzymatic and gut microbes from continuing their actions inside the warmed specimen. Once frozen, the specimens can be manipulated and picked out of the exhibits for preservation as identified below.

Killing Fluids

Immersing a larva in hot (near boiling) water is the best killing fluid for larvae in

a laboratory situation. This treatment stops enzymatic and gut microbe action and distends the larva. The larva is then removed and put into cold water, or directly into 70% alcohol. Killing fluids such as KAAD (a mixture of kerosene (1 part), ethanol (7-10 parts), glacial acetic acid (2 parts), and dioxane (1 part)) may be used to kill and "fix" a larva to avoid discoloration or distortion. The killed larvae will need to be removed from the KAAD solution and transferred to a preservative within 24 hours.

Safety Note: Dioxane may become unstable if stored more than 12 months. Use of "Triton X-100" or "Tween" is recommended instead.

Fumigants

Generally used for adult insects, a fumigant is a substance that generates a poisonous gas.

Fumigants of choice in a filth laboratory are quick, easy to use and lethal to insects and mites, but relatively safe for the analyst.

Gas fumigants like CO_2, can be used to temporarily stun insects to slow them down for photography.

Liquid fumigants are more common, (e.g. ethyl acetate); a small amount of the liquid can be placed on an absorbent pad and placed in an airtight container or killing jar. *Note*: Cotton balls are NOT recommended as the absorbent pad, as insects may become entangled in the fibers.

Solid form fumigants (e.g. paradichlorobenzene) may be used; the solid form is held inside a screened chamber built into the lid of the killing jar. (*Note*: Paradichlorobenzene is a slow acting fumigant.)

In both cases, the insect is placed into the airtight container with fumigant and asphyxiated.

Safety Note: Numerous fumigants are used, but all generally have higher human health risks. Regardless of the fumigant used, the analyst works in a well-ventilated area or fume hood; care should be used to avoid breathing the fumes.

Caution: Ethyl acetate and other fumigants may have solvent actions that may dissolve the container, lid, or seals. Always check compatibility first, before using solvents.

<u>Preserving Insects</u>

Preservatives

Most specimens are stored in poly-cone capped glass vials of 70% ethanol, others (soft bodied maggots) in a 1:1 of 70% ethanol and glycerin, some (insect eggs) in pure glycerin, and still others (pigmented soft bodied) in commercially prepared solutions such as Pampel's or Kahle's. Mites are generally stored in 70% ethanol, lactic acid, or in AGA (87 parts 70% ethanol, 8 parts glacial acetic acid and 5 parts glycerin).

The analyst is reminded that some preservation agents, like formaldehyde, are also fixatives. They may actually cause damage to the specimens through subtle color loss or by tissue shrinkage from the denaturing of the proteins. Analysts are encouraged to ask the senior analyst questions as the need arises, or consult the references cited below or in the Reference Appendix.

Pinning and Air Drying

Pinning, spreading, and air drying are techniques commonly used for whole adult insects that are generally placed in museum boxes. Smaller insects (too small to be properly pinned) can be placed (glued) onto triangular shaped paper points, the points of which are then mounted on pins. This technique is commonly used for authentic specimens, where handling is minimized and easy access to dry specimens is needed. Normally, given the size of most stored product insects and their brittle nature when pinned, we do not preserve regulatory sample specimens by pinning them, as the specimens are too fragile to stand much handling, especially where handling is out of the analyst's control. However, some flies and thin cuticle specimens are better preserved on pins and careful packaging measures would be needed to assure that the pinned regulatory specimens are not damaged from dropping, crushing, or shaking of the finished exhibits.

See Schauff's document for details on pinning specimens for the best results.

Labeling Preserved Specimens

Every specimen (or group of like specimens) collected are to be labeled immediately. The value of a specimen is seriously diminished if the specimen is left unlabeled or incompletely labeled.

Depending upon the type of preservation method used, labels should be on acid-free 28-60# index-weight paper using indelible ink or laser printing. Inkjet printing is not usable for wet vialed specimens. The labels can consist of one or more (broken up into sample collector's or lot label(s) and identification label as grouped below) labels and they are placed in the vial or dish or on the pin with the specimen(s). The complete label should include the following information:

Group 1

Sample number (Lot)

Sub number

Date collected (or date extracted by analyst)

Collector's or Analyst's name or initials

Type of preservation fluid (if applicable)

Group 2

Product

Country of Origin

Group 3

Identification of specimen

Who identified specimen and date

Supporting Documents

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6. Olsen, A. R. (1973) Apparatus and Technique: Insect Filth Exhibits. FDA *Laboratory Information Bulletin,* No. 1637.

7. Stehr, F. S. (1987). *Immature insects* (Vol. I). Dubuque, IA: Kendall/Hunt Publishing Co.
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9. Vazquez, A. W. Examination of bulk samples of food products for infestations with living insect and mites using the Berlese funnel technique. FDA *Laboratory Information Bulletin*, No. 883.
10. DeCamp, P. W. Modifications of the Berlese funnel for filth work. FDA *Laboratory Information Bulletin*, No.1816.
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Glossary

Collar - An old sieve with the weave removed; extends the height of a sieve.

Fix - Chemical process that prevents or minimizes pigment discoloration, loss and/or tissue distortion, to preserve in place.

Overs/Throughs - After a sieving operation, anything retained on top of the screen is referred to as overs, those that pass through, as throughs.

Exercises

Prepare the following solutions:

60% ethanol

Glycerin-Alcohol (1:1)

A.G.A.

10% KOH

KAAD

Kill and preserve the following, including a label:

adult stored product beetle (for regulatory sample, for authentic use)

larval stored product insect (for regulatory sample)

a mite

a cockroach

a spider

2.1.5. Taxonomy

Taxonomy Principles

Objective

This exercise will provide background in taxonomic principles and zoological nomenclature.

Discussion

Taxonomy is the science of giving names to organisms in order to classify them. The system used to name organisms was designed by the 18th century botanist, Linnaeus. It consists of a basic name for each kind of organism and a hierarchy of categories for grouping similar kinds of organisms together. Identifying an organism, then, is simply finding the proper name to call it.

The basic name or scientific name of an insect or any other biological entity consists of two Latin or Greek based words. This two-name system is often referred to as binomial nomenclature system. Each name combination is unique; there is no duplication of names under the system of Linnaeus. Scientific names also have a strict format. To check the validity of a scientific name and see the phylogenetic relationship, see the Integrated Taxonomic Identification Service at: http://www.itis.gov. The format is as follows:

a) The scientific name is always underlined or italicized.

b) The first letter of the first word of the scientific name (the genus) is capitalized.

c) The second word (the species) is not capitalized.

d) Immediately following the scientific name is the name of the scientist who originally named the species.

The basic unit of the system is the species. Next is genus, a group of species that is closely related phylogenetically (by ancestry). The system continues building larger and larger categories, each indicating a more remote phylogenetic relationship. The general progression of categories is given below with examples of each category.

a) Genus: Apis = honey bees

b) Family: Apidae = bees in general

c) Order: Hymenoptera = bees, wasps, and ants

d) Class: Insecta = all insects

e) Phylum: Arthropoda = insects, crustaceans, spiders, etc.

f) Kingdom: Animalia = animals

Apis mellifera (Linnaeus) is the scientific name of the insect commonly called the "honeybee." In order to be sure however, that this is the correct name for the insect in question, a test is applied. For insects, and most other organisms, the test compares a specimen to either a validated (or authenticated) specimen whose identity is assured, or to compare the specimen to a written description of the validated specimen. In FDA work, both methods are used to confirm the identity of an insect.

In entomology, "validated" specimens are called "type" specimens. Type specimens are specimens that have been designated as examples of a particular species by the scientist who originally named the species (the author). In FDA, "authentic" specimens are similar to type specimens in that they have been verified by experts to be good examples of a particular species of insect.

Technical descriptions are written, detailed descriptions of a type specimen. Since most type specimens are housed in large museum collections and not provided for casual examination, published technical descriptions are needed and useful. Although we may not have the original author's description, most district laboratories will have literature containing technical descriptions of common food-infesting insects, which we use to make identifications.

Assignment

1. Examine the laboratory collection of authentic whole insect specimens.
2. Determine the books or journals that contain technical descriptions of stored-product (food-infesting) insects.

Questions

1. Give a literature reference for a technical description of the coffee bean weevil, *Araecerus fasiculatus* (DeGeer).
2. What is the full scientific name, including author, of the confused flour beetle?
3. Based on the scientific names of the insects in "a" and "b," would one expect them to be similar to each other or dissimilar?

<u>Identification Keys</u>

Objective

The trainee will learn how to use identification keys.

Discussion

A key is a guide to the identification of some specimen. Although there are various kinds of keys, for each kind of key attempt to arrange the characteristics of a particular group of specimens into an orderly format with the intent of guiding the analyst through a series of observations until every species but one is eliminated. Keys are not infallible, final, or all-inclusive. Keys provide a tentative answer; the final identification of a specimen depends on direct comparisons with technical descriptions and authentic specimens. The value of a key tells the analyst which technical descriptions and authentic specimens to look at first, and helps narrow down the search in a structured fashion. Keys can be grouped into two broad categories: single access keys and multiaccess keys.

Single access keys are keys with a fixed number of sequenced identification steps, each with multiple alternatives. By making a single choice you determine the direction of your next step. The advantages to these types of keys is that they use many reliable characteristics which can be used in specific groupings. Some of the disadvantages to these types of keys: you can get 'lost' in the terminology if you not familiar with it, if a characteristic is missing it may become difficult to identify your specimen, and finally the key is only as good as it is designed for the specimens or species in question. Common single access keys are dichotomous keys and pictorial keys.

Dichotomous keys: the most common entomological identification keys. They can be of two types: Diagnostic (artificial) or Synoptic (natural). *Diagnostic keys* are convenient and reliable, they can incorporate geographic distribution or even habitat preferences, and contain artificial groupings. *Synoptic* keys follow taxonomic classifications, often incorporate difficult characteristics to observe, and difficult terminology. The basic composition of both keys are a series of pairs of mutually exclusive statements about the specimen being identified, called couplets. By choosing the statement that best satisfies the specimen being observed, the analyst is directed to another couplet. This process continues until a couplet is reached that indicates a name (typically to the species level). That species name is the most likely identification of the specimen. However, FDA demands the analyst confirm this name identification with authenticated material, or at least to a literature description.

There are variations on the basic dichotomous key format. Sometimes a key

will include a triplet (three mutually exclusive statements) or even a quadruplet. In these cases, the analyst still chooses only one statement. As a kindness to identifiers who occasionally backtrack, lengthy keys will often provide parenthetical reference to the previous couplet immediately following each couplet number.

Pictorial keys: although dichotomous keys are often illustrated, pictorial keys rely on illustrations to guide the analyst towards an identification. Using a series of illustrations with terse legends, the pictorial key guides the analyst by directional arrows, much like an agency personnel table of organization.

Multiaccess keys give the user the freedom to pick multiple characteristics which are convenient to evaluate. These keys can be printed in a tabular or matrix type format. They can even be designed as computer generated interactive keys. The advantages of these types of keys: they can be used on damaged specimens, not limited to a few characteristics, easily updated when new specimens are added to the key and can have computer aided identifications with hyperlinks to authoritative text and images. The disadvantages to these types of keys are that they can become cumbersome in selecting the multiple characteristics and takes practice in knowing how to select the various characteristics. Examples of multiaccess keys are tabular keys and interactive keys.

Tabular keys are useful for distinguishing members of a small group of similar appearing specimens. A table compares the distinguishing characteristics of each specimen. Frequently, a single characteristic may be duplicated, or characteristics may be overlapping, but each species will have a unique total set of characteristics that will distinguish it from others.

Interactive key with the aid of a computer, the analyst will select a series of characteristics, then the computer gives the best choice (s) based on the characteristics which were selected.

Hint: Experience shows that the analyst doesn't always get the answer expected when using keys, especially if the keys are complicated, or some of the characteristics are ambiguous. To help remedy mistakes, and keep from going back to the beginning, on a separate piece of paper, try keeping a running list of choices, i.e. 1, 3, 4, 17, 18, 23, 30 etc., and circle the couplets that are major breaking or grouping points in the key. Also, put a question mark (?) above the ambiguous or questionable couplet choices. This will allow one to retrace steps and review decisions. This will save a lot of time,

especially in unfamiliar territory. Other techniques which can be helpful to confirm the specimen is keyed out correctly: send the specimen through a second key, compare the specimen to the authoritative literature and authentic specimens or have a second analyst send the specimen through a second key.

Assignment

1. Examine one or more examples of each of the four types of keys: dichotomous, pictorial, tabular and interactive.
2. The trainer presents a set of objects. Construct a simple dichotomous key.
3. The trainer provides an unknown specimen(s). (Beginner Level). Identify the specimen(s), using each of the four types of keys.

2.1.6. Digital Photography and Photomicrography

Objective

This procedure applies to all analysts using digital cameras or scanners for photodocumentation of evidence/sample casework. This procedure is written for film-less photography with images stored on magnetic or optical (CD) media.

Definitions and Acronyms

Aperture - Circular hole in the camera that controls the amount of light reaching the sensor or film emulsion.

Blooming - The bleeding of signal charge from extremely bright pixels resulting in over-saturated pixels. “Blooming” in digital photography compares with over-exposure in film photography.

BMP - Bitmap (.BMP file extension): this is a standard image file format for Windows®.

GIF - Graphic Image Format (.GIF file extension): gif format is what is termed as a "lossless" compression format. This format is referred to as a "paletted" image or a 256-color image. It is limited to 256 colors.

JPEG - Joint Photographic Experts Group or jpeg (.JPG file extension) this format is a lossy compression format. The higher the compression ratio the more the pixelization or "blockiness" occurs.

Cropping - The act of cutting out a portion of a digital image for blow-up/display

as a separate image.

Photodocumentation - The process of recording images representative or demonstrative of a sample or object.

PNG - Portable Network Graphics (.PNG file extension): this file format is an alternative to the GIF (Graphic Image Format) format.

TIFF - Tagged-Image File Format (.TIF file extension): a lossless bitmap image format supported by virtually all paint, image-editing, and page-layout applications.

Discussion

The replacement of film emulsions with digital sensors for imaging brings many conveniences to laboratory photodocumentation. Digital imaging provides an instant review of composition for quality and facilitates archival through modern digital storage techniques.

Due to variations in equipment from laboratory to laboratory, the trainee learns to use equipment with the guidance of an analyst familiar with its use. As with conventional film photography, it is important to pay attention to the composition, lighting, and overall contrast of a scene when photographing samples. A well-composed picture has the following qualities:

- The subject is positioned and contrasted to attract the viewer's attention.
- Important details are highlighted.
- The subject is magnified so it is not lost as an inconspicuous speck in the photographic field.
- Sample identification is included in the scene, if possible
- When possible, a scale of known distance intervals is included in all images. If not possible, all photographic conditions, including magnification and lenses used, are recorded and a calibrated bar marker inserted in the photomicrograph.

For digital photographs or scans, the following equipment (if used) should be documented on the worksheet or a separate attachment:

- Brand and Model of the Digital/Video camera
- Image Capturing device

- Scanner type
- Printer Brand and Model
- Microscope(s)
- Illuminator(s)
- Lens Brand and Model
- Focal Length and Aperture Site
- Exposure time (if applicable)
- Filters (if applicable)
- Image Storage (location, file names, etc.)

Digital cameras often have numerous settings that can be set to automatic or manual modes of operation. Such features will usually call for an SOP, tailored to the equipment, that outlines which settings should be left as automatic and which should be set manually so as to maximize quality, repeatability, and to facilitate in archival.

Original images are not to be modified. When an image is modified (contrast/color adjustments, sharpening, or cropping for instance) a copy of the original file as obtained from the camera is kept. The new, modified file should be named by appending the word “modified” to the original file name. It is vitally important to document what adjustments were made to the original file in creating the modified one. A print of the original image alongside or below the modified image may not be needed if the analyst has safely stored the original file.

Both the modified and unmodified files should be written to media for submission with the analyst report. Each print of an image should be identified with the image name, description, and variables recorded at the time of capture, as previously described.

<u>Considerations</u>

A. Printer

Only paper recognized by the printer manufacturer as fade resistant and fade resistant ink (cartridges) are used. Printer paper stack should be covered when not in use. Printer heads cleaning is conducted as needed. (See instructions with printer manual).

B. Camera

Mount the camera on a tripod and set the exposure manually by adjusting the aperture so as to maximize depth of field. Set the image quality to maximum and turn off (or manually set) as many automatic features as possible so as to increase repeatability/consistency. In particular, in-camera sharpening, white balance, and use of a flash (as well as flash intensity) should be fixed. Choose a lossless file format if possible; an option to produce a TIFF file is often provided and is desirable seeing as how the TIFF format uses a lossless compression scheme (or no compression at all).

C. Scanner

Output Resolution should be set to a minimum 150DPI*. Sharpening level should be set to low or none (some scanners automatically sharpen a scan). Output Dimensions: the file format chosen should use lossless compression, or none, as described in step (a).

* The concept of DPI (dots per inch) is often confused with camera, or scanner, resolution. In reality, the two terms are not interchangeable. Resolution as it pertains to digital imaging refers to the number of pixels captured by the sensor while DPI is a display (i.e. print) characteristic which indicates how many of those pixels are displayed per inch. Thus, the DPI of an image may be adjusted to no end without affecting the amount of data (pixels) in an image. The human eye is incapable of perceiving more than 340 pixels per inch (approximate) at a viewing distance of 10 inches. In practice, a print with a DPI of 150 pixels (dots) per inch or greater will appear acceptable. As an example, the analyst is to use the maximum resolution of a Nikon Coolpix 4500 digital camera to determine what the DPI of an uncropped image should be set to for printing on an 8.5x11inch sheet of Photo Quality Inkjet Paper:

The resolution of an uncropped Coolpix 4500 image is 1704 pixels wide x 2272 pixels high (using a portrait aspect ratio). Due to printer margins, we could assume the maximum printable height of the image on the paper will be 10 inches. The equation describing the relationship between resolution and DPI in the vertical dimension is thus 2272/n = 10. Solving for n, we see that approximately 228 DPI is needed. If we carry this same DPI over to the width, we see that the image will be 1704/228 = 7.47 inches wide. Thus, the solution is to adjust the DPI of the image to 228 before printing. This is greater than 150DPI so, for most subjects, this print should appear acceptable. This 150DPI rule of thumb is common in the graphics profession.

Adjusting the DPI as in this example does not change the amount of data in the image since no pixels are being added or removed. By default, some

image editing applications will inappropriately "resample" the image (i.e. interpolate to increase or reduce the number of pixels) when given the command to change the DPI. Ensure that this is not occurring by checking the number of pixels in both dimensions before and after the DPI adjustment.

Acquiring and Saving Images

1. A scale (ruler) should be positioned in the field of view when a camera or scanner is used to capture an image. A proportional scale may then be used in the worksheet to provide distance and size information.
2. Avoid "blooming" an image by checking the exposure prior to capture. For many scanners this entails running a test scan so as to allow an automatic calibration. The reflectance properties of some surfaces may need manual exposure adjustments. For digital cameras, ideal exposures are often a result of experience or trial-and-error. The LCD preview screen on the rear of most digital cameras can provide some indication of the effectiveness of an exposure, but such displays are un-calibrated and are highly dependent on ambient lighting or brightness/contrast controls. As a result, apart from providing a check for sharpness they may be far less useful than at first imagined. One exception is the display of tonal or color information in the form of histograms by some cameras. Such histograms can provide detailed exposure information when interpreted correctly.
3. Images are saved on removable media. Image storage on the local hard disk should only be temporary; there is no need to retain copies of images on a local computer or server once they have been written to the media to be submitted with the worksheet (multiple analysts doing so would quickly overwhelm the storage capacity of the server or perhaps even a central computer designated for managing digital photography). A preferred medium for storing images is the CD-R disk. Such disks can be "closed" after being written to and thus offer an unalterable means of storage with the added benefit of tolerance to environmental conditions that would otherwise destroy data on common magnetically-based media.

Printing

Images are to be printed on photographic quality paper using the printer's highest quality and resolution. Often the best paper for a given situation will be branded by the printer manufacturer itself. Specialty papers should be covered when not in use. Printer head cleaning is conducted as needed with periodic checks to counter nozzle clogging.

A photograph does not replace written results and descriptions. Normally FDA

does not require the photographic documentation of negative results. In short, use discretion. A photograph can be particularly useful, for example, when it can demonstrate the lack of an item that should be present in a product.

Assignment

Read a suitable introductory text; Low, A. (1991). *Introductory computer vision and image processing*. New York: McGraw-Hill Book Co. Ltd.

2.1.7. Analytical Filth Worksheet

Objective

To acquaint the trainee with the analytical worksheet (form FD-431) and other standard form worksheets, emphasizing proper presentation of analytical results.

Assignment

1. Read the AOAC Official Methods of Analysis, current edition, Chapter 16 on "Light and Heavy Filth;" and see the reporting format described in Chapter 5 of the Macroanalytical Procedures Manual for various products (in particular the 10 sub dried peas and beans, sequential sampling plan for nut products, and others selected by the trainer).
2. Learn the procedures for completing filth analytical worksheets.
3. Examine recent filth food sample worksheets and review the laboratory's various forms and formats used to report filth results.

2.2. Basic Techniques

The next stage of the training program is the dissection of insect specimens in order to learn insect morphology and recognize insect fragments. Practice is needed for the development of dissection skills, and the following guidance will enable the trainee to begin properly.

2.2.1. Dissection Equipment

Dissection equipment is described as follows:

- *Dissecting microscope.* Dissecting widefield microscopes are the best choice.
- *Probes*. Standard dissecting probes, either straight or bent tip, are purchasable from most biological supply companies and are acceptable for general use. Microprobes are probes made by inserting a minuten or

#2-3 insect pin into the end of a thin wood dowel, such as a cotton swab stick. A spatula-type specimen lifter may be fashioned by flattening the tip of a microprobe, and a micro-scapula can be fashioned from a flattened # 2 pin which is then sharpened on a wet stone or fine grinding wheel.

- *Forceps*. In addition to tweezers, a pair of fine-tipped jeweler's forceps (Dumont #3 or 5, or equivalent) are needed for handling small objects, and a pair of coverslip forceps. The tips of the jeweler's forceps should be protected against damage when not in use by a sleeve of small diameter rubber or plastic tubing. Always keep a spare pair on hand.
- *Additional equipment*. Insect pins, a fine (#11) scalpel, and a pair of fine tip surgical or iris scissors will be useful. Small hotplate (for boiling solutions), an alcohol lamp (if open flames are allowed), and a slide warming plate (50° C.). Disposable petri dishes of various sizes (100X10, 100X15, and tight-fitting lid 65X10) are needed and lined (S&S # 8 ruled filter paper) and unlined filter papers are needed. A No. 2 or No. 3 artist's camel hair paint brush is an excellent tool for picking up insects without damaging them.
- *Glassware.* A wax bottom dissecting dish is useful as an arena (an alcohol insoluble pinning board) for the dissections. To make one, melt paraffin/bees wax in the bottom of a small glass petri dish to the depth of approximately one-half of the height of the dish, and cool. After a period of use, the paraffin/bees wax surface may become rough and full of holes. Simply remelt the paraffin/bees wax and cool again to obtain a smooth surface.
- *Other useful items*. Syracuse watch glasses (2 - 5/8" diameter), Coors porcelain casseroles (size 00) or 10-25 ml beakers with glass watch glass covers for operations that call for heating specimens in liquid, and various shallow watch glasses for use as lids.

Note: Clean white beach sand can be wetted in a petri dish and used as a formable orientation dish for wet specimens (to orient specimens for different views under the stereomicroscope).

2.2.2. Dissection Techniques

Most dissections are performed in liquid (usually alcohol) to prevent the specimen from drying and to better control the movement of small pieces of the specimen. Dry dissection can be performed in shallow petri dishes with

filter papers wetted with 50/50 glycerin alcohol; however, the specimens should be softened by gentle boiling in pure water prior to the dissection.

Dissecting a specimen essentially dismantles it. Pulling off or teasing apart is the easiest technique and works well for most large appendages such as antennae, legs, elytra, wings, or even smaller exposed appendages such as labrum and labia (top and bottom mouth parts). The insect's body is firmly held at a point near the appendage while the appendage itself is pulled off or severed at its base using forceps and the microprobes. Leg and antennal segments may be separated in the same manner.

Main body plates or structures (sclerites) can be broken free by judicial application of pressure from a probe or forceps. Ventral abdominal plates may crack however, so it is advisable to cut them free along the sides and then separate them. Cutting the plates free, especially if only a few straight cuts are needed gives more control than breaking the plates.

Mouthparts and other small structures like genitalia can usually be teased free using probes. Adhering excess muscle and visceral tissue should also be teased away. Tissue that cannot be teased free can be macerated with a caustic solution (5-10% potassium hydroxide) but the specimen is thoroughly rinsed in distilled water before mounting. Gentle heat will speed this maceration process.

Slide mounting media are described in Section 4.2.3. If a specimen is very thick, it will cause the coverslip to rest unevenly. This can be remedied by propping up the coverslip with bits of glass (broken coverslip fragments), nylon fishing line, or other material strategically placed in the medium before laying on the coverslip.

Finished slides are labeled, ringed, and then stored flat, as the specimen is not fixed to the slide surface. Remember to mount only one specimen/fragment/hair per slide.

2.2.3. Insect Morphology

Objective

The trainee should review and become familiar with the "Micro-Analytical Biology Workbook for Food Sanitation Control Analysts," Volume I (1981) by Don J. Vail, Jr., FDA Atlanta Regional Office, Atlanta, GA. or materials provided by the trainer.

An excellent, additional approach to learning insect morphology is described in the exercise

below.

Discussion

In order to learn basic insect morphology, the analyst needs to recognize the various parts, which make up a particular insect. The analyst will need to use their dissection techniques they learned in section 4.3.2 to aid in this process.

Assignment

This exercise is geared towards learning insect morphology and serves as a useful tool for later analyses. The trainee sets up a series of petri dishes, each petri dish representing a particular insect or series of insect fragments from a group of insects. For example, the analyst will label a dish for *Tribolium confusum,* and another larger dish for stored product beetle mandibles, with adults on one side and larva on the other.

The trainee will perform the following two step process:

Step One:

The trainee performs a series of dissections on various stored product beetles. These are referred to as Known Species Plates. Using the air tight lid small petri dishes with the bottom lined with glycerin/alcohol wetted filter papers, the trainee labels the lid in indelible ink with the species name and source information or lot number (one species per plate.) The trainee places 3-4 intact whole adult insects of that species on the wetted filter paper and begins their dissections, one insect at a time. The dissection serves as a learning tool for morphological terminology and how the insect comes apart. This information will prove useful towards understanding what the fragments look like when separated from the body. (*Note:* this exercise will also provide some insight into what may happen in a milling, flour making operation when the insect is crushed or broken up). Complete the dissections of a group of 2-4 identical specimens; some species may show some size variation or will call for several dissections to get intact fragments representing all body exoskeleton parts. For example, some beetles (like the rice weevil) have closed coxal cavities which prevent the analyst from getting either a complete prothoracic fragment or complete coxae without sacrificing one fragment for the other. When this segment is completed, then dissect the mature larva on the same plate, keeping the fragments segregated.

Upon completion, these plates can be used throughout one's career for additional dissections or for reference.

Step Two:

Step Two consists of a series of Fragment Plates, where the trainee uses the 100 X 10 petri dishes and labels the lids describing the morphological fragments, e.g. all stored product beetle mandibles on one plate. Once Step One dissections are completed, the trainee will code in India ink each line of a S&S #8 ruled filter paper with numbers or the actual species name of the insects dissected. When dry the labeled filter papers are placed in the petri dishes and wetted with glycerin/alcohol. Then species by species, the trainee places the corresponding fragment on the species line with a representative number of fragments present to show size variations, or larval/adult conditions if possible.

When the analyst completes the plates, the plates serve as a rapid identification tool for unknown species, but clearly identifiable morphological fragments. The analyst can pick the fragment up on the end of a probe, and move the fragment next to the known fragments on the plate, comparing size and gross shape or character. Narrowing down the final identification without having to do a slide by slide analysis reduces analytical time.

Note: The trainer identifies the common stored product insects the trainee should work with in preparing the initial plates. The trainee can and should set the plates up for a life time of learning; the plate collections will take considerable time to develop and may never be completely done throughout the employee's career.

The object of this section is simply to set up the system in which the trainee can systematically learn insects. It is difficult and tedious work, and has a very steep learning curve relying heavily on memorization skills and the ability to perform minute dissections under the stereomicroscope. The initial training time should be limited to one or two weeks to learn the approach alone, with additional time granted as needed. The time for these exercises needs to be granted to the employee as continuing education and quality assurance throughout their career. For initial training, the trainees should not attempt to memorize the fragment to species as Section 4.3.4 covers this aspect. However, if needed, and if the trainee already possesses a strong entomological background, these two sections can be combined and taught as one section. If done effectively, the trainer/trainee may also spend some time learning more about the particular insect's biology. Descriptive literature accompanies each dissection.

Assignment

This exercise is geared specifically towards learning how to dissect genitalia from an insect. The genitalia of adult Coleoptera and Lepidoptera are

important characteristics used in their identification. It takes lots of practice in order to tease out genitalia and mount them on a microscope slide for identification.

1. The trainer supplies similar beetles to the trainee. The trainee will dissect out the genitalia from the beetles. Then mount the genitalia on a microscope slide and observe the differences in the genitalia. Some examples to use: *Oryzaephilus mercator and O. surinamensis; Stegobium paniceum and Lasioderma serricorne; Cryptolestes sp. and Ahasverus advena*
2. The trainer supplies the trainee with at least two different species of moths. The trainee will dissect out the genitalia from the moths. Then mount the genitalia on a microscope slide and observe the differences in the genitalia. Examples of some moths to use: *Plodia interpunctella, Sitotroga cerealella, Ephestia figulilella.*

2.2.4. Fragment Recognition

Objective

The purpose of this exercise is to learn how to distinguish microscopic fragments of insects, and how to segregate and identify them from other plant tissues on the plate.

Discussion

In order to accurately distinguish microscopic fragments, the analyst needs a thorough knowledge of insect morphology, and access to a reference collection of authenticated fragments. This knowledge is a prerequisite (see Chapter 16 of the AOAC) before completion of any filth analysis.

Assignment

The previous insect morphology exercises (Section 4.3.3) were the starting point for learning and accumulating reference material. The learning process is never truly finished; the analyst should never pass up a new dissection opportunity in order to accumulate further knowledge and additional reference material.

There are certain qualities peculiar to cuticular fragments of insects that serve as proof of insect origin. Even though a suspect fragment may have a genuine insect appearance, it cannot be reported as an insect fragment unless there is proof of insect origin. As outlined in the AOAC, the following diagnostic characteristics are the proofs by which a positive identification of insect

fragments can be accomplished.

Diagnostic Characteristics of Insect Fragments

- *Shape*. The shape of a fragment is diagnostic if it is recognizable as an entire or particular portion of, an appendage, body segment, or specialized structure of the insect body.
- *Setae*. The presence of one or more non-cellular setae with associated setal pit (papilla) is diagnostic. Should the setae have become separated from the fragment, the presence of setal pits is sufficient for identification.
- *Sculpture*. Surface pattern (sculpture) that is typical of a particular part of an insect is diagnostic, quite often to the family or genus level.
- *Sutures*. Fragments that actually consist of portions of interlocking plates (sclerites) are, by the complex form of the joining interface (suture), proof of insect origin.

Certain qualities of insect cuticle may alert the analyst to look closely for one of the above diagnostic characters. Even though these secondary qualities are not unique to insect fragments, they are useful to the analyst in that their absence casts doubt on the insect origin of a fragment.

Secondary Characteristics of Insect Fragments

- *Texture*. This is thinness combined with flexibility or toughness.
- *Luster*. Insect cuticle often has a distinctive sheen that the trainee soon comes to recognize.
- *Lack of cellularity*. This negative aspect separates insect fragments from many types of plant material. Some types of sculpture may give a superficial impression of cellularity, but close examination finds that plant cells exhibit much individual variability of size and configuration while insect cuticular sculpture tends toward repetitious uniformity of cell-typed units.

Comparison with authentic reference material is the final, irrefutable proof of insect origin and should be employed as often as possible. Identification of fragments to family and genus is routinely possible; occasionally species determinations are accomplished when reference materials (authentics or literature) are provided.

Assignment

1. Systematically arrange the fragment reference material from section 4.3.3 so that it is most useful to the individual analyst preparing the material, OR, if the trainer prefers, integrate the reference material into the laboratory reference collection.
2. Using the laboratory library, compile a personal bibliography of literature concerned with insect fragment recognition, especially from AOAC and FDA publications.
3. Review and discuss with the trainer what has been learned thus far on fragment identification. Additionally, discuss the difference between whole, whole/equivalent (w/e), and large body parts of insects. Maggot mouth hooks: fragment or (w/e)?
4. Practice identifying unknown specimens supplied by the trainer until the analyst is familiar with the literature and confident in their own ability to identify insect fragments.
5. Using any literature resources and reference material, identify at least 10 unknown fragment specimens supplied by the trainer. Identifications are to the lowest taxonomic level supportable by literature resources and reference materials found in the laboratory.

2.2.5. Mites

Objectives

The purpose of this exercise is to learn the basic morphology of mites.

Discussion

1. Mite Features

Mites are chiefly recognizable by their small size (usually 0.5mm or less), general lack of body segmentation, and four pairs of legs. In order to understand mite taxonomy, analysts learn basic mite morphology, which differs considerably from insect morphology.

Body regions are defined in relation to the positions of the legs and mouth. Anteriorly, the *gnathosoma* bears the mouth and oral appendages. Following this is the *propodosoma*, whose area is defined by the first and second pairs of legs. Collectively, these two regions, gnathosoma and propodosoma, comprise the proterosoma**.** The *metapodosoma* bears the third and fourth pairs of legs. The remainder of the body behind the last pair of legs is called the *opisthosoma*, and collectively the last two regions are called the *hysterosoma.* The term *idiosoma* refers to the entire body exclusive of the gnathosoma.

Appendages of mites are of three basic kinds, each of which may exhibit varying degrees of modification. The *chelicerae* are the front-most pair of oral appendages. They are basically pincer-type appendages, although in some groups they may be highly modified for specialized feeding while in other groups they may be greatly reduced. In addition to chelicerae, the gnathosoma may bear a pair of leg-like segmented appendages called *pedipalps*. Although generally very small, the pedipalps sometimes have the proportion of true legs, which can be distinguished by position, segmentation, and lack of claw-type structures or pretarsi. Legs are usually eight in number for adult mites, although certain immature stages may have only six legs. Like other arachnids, the mite has a six-segmented leg consisting of a proximal coxa, trochanter, femur, genu, tibia, and distal tarsus. The latter exhibits no secondary segmentation as found in insect tarsi. The tip of the tarsus bears a pretarsus that is often fleshy or membranous and may bear one or more claw-type structures. Mite pretarsi exhibit literally hundreds of variations among the different mite groups.

Mite setae are basically similar in general appearance to insect setae. The base of a mite seta is slightly swollen and a papilla is usually evident. Due to a central cytoplasmic core, mite setae exhibit optical activity between crossed Nicols on a polarizing microscope. As with insect setae, mite setae may be variously modified.

Solenidia are hair-like structures found on mite legs that differ from setae as there is no basal swelling, no optical activity, and very little, if any, modification of the basic hair-like form.

Other mite features include a postero-ventral anus, genital structures whose position varies among species, leg and anal suckers, various sclerotized body areas called *shields***,** and simplified respiratory structures roughly analogous to tracheae (*peritremes*) and spiracles (*stigmata*). These structures may each be modified or absent in any given group of mites.

2. Preparing Mite Microscope Slide Mounts

Mites are mounted on a microscope slide and observed under a compound microscope for identification. Prior clearing (See Section 4.2.3 *Preparing Microscope Slide Mounts)* may be needed. Because of its desirable optical qualities, the mounting medium of choice is Hoyer's solution or one of its variants. For general work, the specimen is mounted venter up with the gnathosoma pointed towards the bottom (south) edge of the slide. (This is so the compound microscope image will appear with the gnathosoma at top). The specimen is centered, pushed to the bottom of the drop of medium, and the

legs spread as much as possible, before placing the coverslip. The weight of the coverslip may produce further leg spreading, but a coverslip that is too heavy will burst the bodies of delicate specimens. The smallest sized, lightest weight coverslip found should be used.

Small amounts of heat may be applied to the mount to help spread the legs and aid penetration of the body by the mounting medium. Hoyer's-type solutions are not to be boiled as this affects the storage life and may release harmful fumes. (*Safety note:* Work with proper ventilation to avoid breathing fumes.)

3. Effects on Human Health

The effects on human health of mites in foods have not been completely documented, but some deleterious attributes of mites are becoming evident.

- Mites can cause considerable physical damage to stored foods.
- Mites can impart a distinctive disagreeable, sweetish musty odor to foods they infest.
- Mites can produce a sugary, white, encrusted coating on dried fruits.
- Some kinds of mites can induce allergic reactions, including asthma-type symptoms, in sensitive individuals.
- Mites can transport spores of molds that will grow on and spoil food products.
- Certain mites are potential intermediate hosts for parasitic organisms that infect mammals.

Assignment

1. Read pp. 63-82 of: Gorham, J. R. (Ed.) (1981). *Principles of food analysis for filth, decomposition and foreign matter* (FDA Technical Bulletin No. 1, 2nd ed.). Gaithersburg, MD: AOAC International.
2. Review mite slides and discuss with the trainer.
3. Compile a list of references concerning mites found in the laboratory. Check intra-agency documents such as the Laboratory Information Bulletins and FDA By-lines.
4. Under the direction of an experienced trainer, practice mounting mite specimens until good quality whole mounts for microscopic examination can be produced.

5. With the specimens mounted in "4," practice using the phase-contrast microscope.

2.2.6. Hair Identification

Objective

The trainee will gain experience in the identification of mammalian hairs, especially rodent hairs.

Glossary

Commensal - one who eats at the same table with others; an organism, not truly parasitic, that lives in, with or on another.

Discussion

The regulatory analyst is able to identify hairs or hair fragments from murine rodents or commensal rodents. The commensal relationship is between certain murine rodents and man, and not some other commensal relationship they might have with other animals.

Murine rodents are so termed because they are placed taxonomically in the family Muridae. Rodents are all those animals placed taxonomically in the order Rodentia. Examples of rodents familiar to us include squirrels, ground squirrels, chipmunks, various field mice, cotton rats, muskrats, beavers, and porcupines. These are placed taxonomically in families other than Muridae but within the larger taxonomic unit, the Rodentia. This very general description serves us simply by pointing out that the term "rodent hair," used in general by microanalysts, is simply too general to use for describing the hair of commensal rodents. The hairs of most concern to the microanalyst are those from the commensal rodents, but not necessarily limited to these. The commensal rodents of most concern are the Norway rat *(Rattus norvegicus*), the roof rat (*Rattus rattus*), and the house mouse (*Mus musculus*). These animals are not native to North America but were introduced by commerce. Because of their close relationship to man and documented evidence of their role in disease transmission, they are considered probable health hazards, and evidence of contamination by these animals is weighed heavily by regulatory and health officials. Other commensal rodents of concern is the lesser bandicoot rat (*Bandicota bengalensis*). This rat is found in the area surrounding India, Pakistan, Burma and some of the islands in the area. The Polynesian rat (*Rattus exulans*) is widespread throughout the Pacific islands and Southeast Asia.

Another important commensal animal is the Asian musk shrew (*Suncus*

murinus). An insectivore, found in Southern Asia and eastern Africa, this animal belongs in the shrew family Soricidae. The hairs of this animal are found on a regular basis and they have a distinctive appearance. The Asian musk shrew has been known to be a reservoir for *Salmonella* and plague.

Other animals, many of them native rodents, often establish a temporary commensal relationship with man. These include various squirrels, muskrats, etc. Contamination by these animals, domestic animals, pets, and human hair are also be considered and recognized by the analyst.

It is not assumed that an analyst can learn to identify hairs by reading about their various characteristics. This ability can only be acquired by careful study of authentic specimens. Suspect material should always be compared to authentic specimens.

The basic structure of most hairs consists of an external layer of scales underlaid by a cortex of generally amorphous tissue. In the center of the hair is the central core, called the medulla. Striated hairs have discontinuous medullae that give these hairs their characteristic banded appearance. Striated hairs cause the most concern since the mammals that pose the greatest threat to world food supplies, the commercial rodents, all have striated hairs. The primary task of the trainee is to learn to identify hairs of the commensal rodents. This knowledge can then be applied to learning the identification of other types of hairs.

Hairs are examined under the compound microscope for identification. Most striated hairs contain considerable amounts of air trapped in the medulla. This air is removed by heating to prevent interference with microscopic observation by diffracting light away from the objective lens. As heating procedures vary widely, the trainee chooses a personal technique under the guidance of the trainer. The simplest techniques involve heating the hair in the mounting medium, glycerin jelly, so that the air is replaced entirely by medium (See section 4.2.3, Preparing Microscope Slide Mounts).

1. General Microscopic Characteristics of Rodent Hairs

   - *Prominent scales*. Under the compound microscope the edges of rodent hairs have a serrated appearance due to the projecting tips of the external scales.
   - *Clear cortex*. The usual color with unfiltered light is bright hyaline green with virtually no dark spots. The cortex is also typically very thin.
   - *Discontinuous medulla*. This type of medulla is thought to be composed

of cell remnants embedded in a solid matrix. Each cell or segment contains numerous pigment granules packed tightly in one end leaving the other end clear. An intervening clear air space separates each cell from the next. This contrasting alternation of dark pigment and clear areas in the medulla gives the hair its striated appearance.

2. Guard Hair Characteristics

Guard hairs are the long, coarse hairs of the rodent pelt. Microscopically, the medulla is seen to consist of several rows of segments or cells, each with pigmented and clear areas as well as separating air spaces.

3. Fur Hair Characteristics

The most striking feature of rodent fur hair is the zig-zag configuration of the hair itself. This is evident even at low magnification and is a result of bending of the hair at the internodes. A single row of medullary cells is typical of rodent fur hairs. These hairs are thinner than guard hairs.

- *Internodes*. Fur hairs exhibit this rapid constriction of the hair diameter at one or more points along the length of the hair. The area where an internode occurs shows proportionate size reduction of the medulla.
- *Air spaces*. The most singular characteristic of rodent fur hair medullae is the shape of the air space between each cell. This is typically the shape of a capital "I" with the stem of the "I" at right angles to the hair's length.
- *Cortical pegs*. This small extension of cortical material into the medulla appears as a single indentation on each side of the medullary cell, usually in the pigmented area.

A hair possessing the above characteristics is probably a rat or mouse hair and should be identified by comparison with authentic specimens. The analyst compares the sizes and configurations of all structures, including scales, cortex, air spaces, medullary cells, pigment granules, and internodes.

Mammalian hairs can be deceptive look-alikes. Shrew hairs are virtually identical to some mouse hairs except for the tips, which are more elongated, and the scale pattern, which is asymmetrical, the scales on one side projecting more prominently than those on the other and the internodes tend to be narrower. Squirrel and rabbit hairs are similar to rat or mouse hairs in general, but differences in the shapes of the air spaces and medullary cells can be used to differentiate them.

Hairs of mammals are sufficiently different between families and genera to permit identification to these levels in most cases. The analyst pursues this expertise through the study of authentic specimens with guidance from experienced analysts and from the literature.

Note: As noted with insects and fragment identification, only time and experience, or specialized study will improve the analyst's proficiency in this area of expertise. The initial objective is to distinguish commensal rodents from non-commensal, but analysts are encouraged to continually study these materials throughout their careers through continuing education and QA programs.

Assignment

1. Read pp. 125-170 of: Gorham, J. R. (Ed.) (1981). Principles of food analysis for filth, decomposition and foreign matter (FDA Technical Bulletin No. 1, 2nd ed.). Gaithersburg, MD: AOAC International.

2. Read pp. 157-216: Olsen, A. R., Sidebottom, T. H. and Knight, S. A. (1996). Fundamentals of Microanalytical Entomology, a practical guide to detecting and identifying filth in foods. Boca Raton, FL: CRC Press.

3. Prepare acceptable slide mounts of authentic hairs supplied by trainer.

4. Practice identifying rodent hairs until one feels confident in their own ability.

5. Examine the following types of hairs and discuss how each can be distinguished from rat or mouse hairs:

- rabbit
- shrew
- bat
- dog
- cat
- human

2.2.7. Feather Identification

Objective

The trainee will gain experience in bird feather identification, especially recognizing the difference between feather, feather barbs and barbules. The trainee will also learn how to recognize the difference between feather

fragments, hairs and gill fragments. They will also learn how to identify the feather barbules to commonly encountered bird Orders.

Glossary

Feather-the primary covering on a bird and aids in flight.

Feather Barb-the individual units which grow from the feather shaft or *rachis.*

Feather Barbule-are like mini barbs growing from the shaft of the barb.

Node: the swollen structure spaced at regular intervals along the feather barbule. The shape of the node can vary in shape from having spines, prongs, round points or even flared.

Rachis-the central shaft of the feather.

Discussion

The regulatory analyst is able to identify feather, feather barbs and feather barbules found in samples and with experience can identify those fragments down to order. There are numerous commensal birds including English sparrow (*Passer domesticus*), feral pigeons (*Columba livia domestica*) and crow (*Corvus sp.*). Food can become contaminated by birds from direct contact of the bird, feather material or their excreta. Feather barbules being the most common bird contaminate found in samples. Feather barbules often can be confused with animal hairs like bat and gill tissue from shrimp and lobster. Birds are common carries for diseases like histoplasmosis, salmonella and E.coli, just to name a few.

The birds most commonly found contaminating samples can be broken down into 4 main orders: pigeons/doves (Columbiformes), chickens/turkeys (Galliformes), ducks/geese (Anseriformes) and songbirds/passerines (Passeriformes). The Passeriformes is the largest group encountered in samples.

Some of the general characteristics to separate the barbules from the four orders. Columbiformes: the nodes are flared on the proximal barbules. The flaring is so distinctive that they resemble small flowers and are often called crocus-shaped. Galliformes: the proximal nodes often are slightly expanded with small spines. Typically, the distal nodes will be encircled with rings. These rings can sometimes slip at the node juncture and slide along the barbule. Anserifromes: the node can have an expanded triangular shape or more prong shaped. The nodes typically are isolated toward the distal end of barbule or proximally on the barb. Passeriformes: the nodes are variable but

typically are very slightly expanded with flared or rounded transparent like projections. Nodes normally are not prolonged or spined.

Assignment

Read pp.15-61: Dove, C. J., Koch, S. L. (2010) Microscopy of Feathers: A Practical Guide for Forensic Feather Identification. Journal of the American Society of Trace Evidence Examiners Vol. 1 (1),

http://www.unitedstatesbd.com/images/unitedstatesbdcom/bizcategories/2961/files/JASTEE_2010_1_1_3.pdf

1. Prepare acceptable slide mounts of authentic feather barbs and barbules supplied by the trainer.
2. Prepare acceptable slide mounts of feather barbules supplied by the trainer and compare with mammalian hairs especially, bat. Also compare the feather mounts to gill material from either shrimp or lobster. Discuss with the trainer on how to recognize the differences.
3. Practice identifying barbs and barbules until one feels confident in their own ability.
4. Examine feather barbs and barbules from each of the four bird orders until one feels confident in their own ability to recognize the four orders.

Columbiformes

Galliformes

Anseriformes

Passeriformes

2.2.8. Excrement, Urine, Uric Acid (Morphological and Chemical)

Objective

The trainee will become familiar with the major types of animal (including insect) excrement that may be found in foods, and the potential health hazards presented, such as Hantavirus, coccidiosis, and related diseases, carried by vermin pests.

Discussion

Excrement is a term that may be applied to feces as well as other excretory products such as urine and various glandular substances, including sweat.

1. Feces

Feces is the word that is commonly used for the material ejected from the intestine through the anus. Fecal pellets are feces ejected in discrete units, as in the case of rodents and many insects. Feces consist mainly of undigested food remnants. Alternate terms are "dung" or "manure."

Fecal pellets are identifiable by visual examination under a widefield microscope with comparison to authentic material. The salient characteristics of fecal pellets are size, shape, color, and, in the case of rodents, surface coating and embedded hairs.

Rodent fecal pellets are elongated with pointed or tapered ends. The color ranges from tan to dark brown to black under dry conditions and is also dependent upon what the animals were feeding. Interesting color variations may occur in rodents that have fed at bait stations, with blood inclusions observed. One of these color variants can be greenish blue, depending upon the coloration of the bait. Immature rodents undergoing weaning may, for a short period, produce pellets of a light brown color. Color variations of these sorts are not routinely encountered alone, but are mixed with other, more typical, pellets. Size range is 5-20 mm for rats and mice, with mouse fecal pellets rarely exceeding 10 mm. When moistened, rodent fecal pellets exhibit a surface coating of grayish-white mucous. Mice do not need free water to survive, therefore typically exhibit dry pellets with heavy mucous coatings, while rats need a source of free water to survive and have thinner mucous coating and moister pellets. To confirm the mucous coating, a small drop of water is placed on the surface, usually softening the pellet and producing a mucous like characteristic. In addition, as the animals are constantly preening themselves, embedded hairs may often be seen protruding from a rodent fecal pellet, or they may be disclosed by crushing the pellet. These hairs offer vital information and are essential in determining the kind of rodent involved, especially commensal rodents (rats or mice, as opposed to muskrats, squirrels, etc.).

Bat fecal pellets have a similar size range to mouse excreta pellets. They are slightly spindle-shaped with a mucous coating. The pellets will contain bat hairs and normally contain insect fragments.

Fecal pellets of deer, sheep, goats, or rabbits are rounded, without an intact mucous surface coating. They are usually less dense than rodent fecal pellets.

Insect fecal pellets are generally small, although some grasshopper and cockroach pellets may approach the size of rodent pellets. Pellets of the orthopterans and larval lepidopterans are characteristically barrel-shaped, having truncated ends and longitudinal ribs. Coleopterans and some other

insects pass small, elongated, irregularly shaped pellets. Insect fecal pellets are often the same color as the food substrate. This is especially true of stored-product beetles; these pellets do not have a mucous surface coating. Sowbugs and pillbugs (Crustacean: Isopoda) often found in damp areas in manufacturing plants. They normally feed on detritus including excreta pellets. The fecal pellets produced by Isopods are rectangular in shape and relatively thin and flat. They can contain animal hairs, due to their behavior of feeding on excreta pellets.

Due to the dietary habits and digestive processes of cockroaches, their fecal pellets may resemble mouse pellets in color. Generally of smaller size (1/8 inch or less), cockroach pellets exhibit longitudinal ridges and often have a somewhat six-sided appearance caused by pressure from the internal rectal glands prior to expulsion through the anus. Cockroach pellets do not have a mucous coating. Since cockroaches habitually eat their own cast skins, the presence of these fragments in a pellet is an additional clue to the pellet's origin.

Caution: The contents or components of a particular pellet (mammalian or insect) should not be the sole basis for the pellet's identification but rather one of many observations, the sum total of which constitutes the basis for identification. For example, rodents living in the same environment as cockroaches may feed on dead cockroaches, resulting in rodent fecal pellets that contain cockroach fragments. Conversely, cockroaches may feed on rodent pellets, with the result that a rodent hair may occasionally be found in a cockroach fecal pellet. Bats are insectivores and their pellets consist almost strictly of insect fragments and exhibit no mucous coating. Therefore, the analyst carefully weighs all of the characteristics observed (size, shape, color, surface coating, and embedded components) in order to identify the source of the fecal pellet.

2. Bird Excrement

This term is applicable to bird droppings, which consist of a mixture of glandular excretions and feces. Bird excrement exhibits a texture varying from liquid to semi-solid. Drops of bird excrement usually take the familiar form of a chalky white amorphous material mixed with darker food and watery residues. Morphologically suspect material is chemically tested for uric acid to confirm the identification as bird excrement. Important: only test the white amorphous material from the excreta pellet for uric acid.

3. Reptile Excreta

The excreta pellets of the common house gecko (*Hemidactylus sp.*) range in color from a light to dark brown, often with a whitish circular mass at one end. This whitish mass easily breaks off from the pellet and will test positive for uric acid. The excreta pellets are normally tapered at both ends and have a similar size range to rat and mouse excreta pellets. The surface of the excreta pellets is relatively smooth to wrinkled without a mucous coating. The matrix of the pellets is densely packed with insect fragments. Besides the common house gecko, the day gecko (*Phelsuma sp.*) can be found in dwellings as well. The excreta pellets of the house gecko have been known to contain various bacteria including; *E. coli, Salmonella, Staphylococcus and Shigella*, while the day gecko can contain at least *Salmonella*.

4. Urine

Urine is a term describing the fluid excretions of a mammalian kidney. This term also has applications, for birds and reptiles, which are not usually encountered in FDA work.

Defilement of food with rodent urine is usually detected initially by observing urine-stained packaging. These stains exhibit a typical greenish fluorescence under long-wave ultraviolet light. Rodent urine stains often exhibit "streaking" configurations caused by the rodent urinating while running or by dragging its tail through a wet urine spot. Many times, even with dry stains, a urine odor is evident. Suspect stains are confirmed chemically under many circumstances, as defined by agency policy.

Safety Note: Handle these materials as biohazards; practice universal precautions. Discuss with the trainer safety practices needed for the handling of these materials. Rodents and other animals serve as potent carriers for numerous diseases. Diseases such as Hantavirus, Histoplasmosis, some tapeworm, and related organisms are the principle concerns. Exhibits should be prepared for safe presentation in the courtroom, while retaining recognizable characteristics.

Assignment

1. Read pp. 201-216 of: Gorham, J. R. (Ed.) (1981). *Principles of food analysis for filth, decomposition and foreign matter* (FDA Technical Bulletin No. 1, 2nd ed.). Gaithersburg, MD: AOAC International.
2. Examine authentic specimens of rodent and insect fecal pellets, rodent urine stains, and bird excrement. Review and discuss with trainer.
3. Under the direction of the trainer, learn to perform the various AOAC

methods for the xanthydrol chemical confirmation of mammalian urine, identify fecal materials and find the citations for confirmation of fecal material, and perform the chemical identification of bird excrement for Uric acid following AOAC method 970.13.

2.3. Basic Analysis

2.3.1. Sample Analysis

A. Sample Analysis

Guidance

Before beginning each analysis, the analyst should ask their supervisor if any administrative guidance has been issued concerning the product about to be analyzed. Analysts should be aware and familiar with the guidance or program for which the product was collected under, shown on the Collection Report as the “PAC/PAF” number. The analyst may also want to study and gain insight into the history of the product or the firm, pest problems, processing, and production as it relates to the agency’s regulatory policy and the philosophical/economic impact on the regulated industry.

Sources of information range from the Sample Collection Report (C/R, OASIS and/or FACTS), the supervisor and senior analysts, the sample collector, the EIRs in the firm’s jacket, the FD484, and photographs taken by the Investigator. The analyst can request additional information not commonly found with the sample.

While reading the documentation provided with the sample, the analyst asks the following questions: (who, what, when, where)

- What does the analyst need to determine (problem area) for this sample? (i.e. insect, rodent, mold, and/or particulates- like glass or metal adulteration).
- What is the scale of the analysis? (i.e. visual, macro and/or microscopic?)
- What methods, preferably official, are found for this kind of analysis?
- What equipment and reagents will be needed, and how much of each is needed?
- What steps/timeline will be followed before, during, and after the analysis?
- Is there enough material/sample/time to do the analysis? E.g., can the

analysis be modified such that one can accommodate the situation?

- Does this analysis require a 702(b) portion and is there one provided?
- Will help be needed, and when will it be needed?
- What kind of problems/interferences can one expect to see, and what can be done to avoid them? E.g., does the ingredient label show any unexpected, unusual ingredients that the analyst will have to deal with by method modification?
- Is there any additional analysis needed, if so, what, when, where, and with whom does one coordinate?
- What kind of results does one expect to see, how are the results going to be reported and what kind of format is to be used?
- Is the sample fit for use? (i.e. accountability, storage, seal, damage or integrity issues, lack of 702(b) portion).

If these questions cannot be answered, seek help and discuss the issues before proceeding with the analysis. Information from the investigations branch can be obtained through the supervisor; sample investigators may have valuable information not always present on the C/R, (e.g. photographs).

The Observant Analyst: If the above questions can be answered, then, the analyst is ready to begin the analysis, with this added note of caution. Regardless of the stated or implied objectives of the C/R or the analysis chosen, the FDA analyst is always to be alert for unexpected developments. The purpose of regulatory sample analysis is to discover evidence of a violation of one of the various laws enforced by the agency. Many routine analyses have taken a sharp change of direction due to a chance observation by an alert analyst. Each analysis represents a new and potentially provocative situation that challenges the analyst's powers of observation and scientific curiosity. An alert, inquisitive approach to sample analysis is every bit as valuable to the agency as any scientific expertise the analyst may have.

B. Scale of the Analysis

Macroscopic and microscopic procedures for characterizing defects in foods tend to supplement each other, and together provide a comprehensive evaluation of defects in the product. It is important that the analyst realize the close association of the macroscopic and microscopic methods for use as a joint approach in solving analytical problems

1. Macroscopic Methods of Analysis

To consumers, "macroscopic" analysis of a product refers to an evaluation of the substance through the use of their unaided senses (primarily sight, smell, or taste). Every consumer in our society who exercises some judgment in the purchase of foods and other consumer goods, knowingly or unknowingly conducts some form of macroscopic examination to detect apparent or obvious defects. The examination may range from a cursory, perhaps unconscious visual check of the product to confirm that everything "looks right", to a much more detailed scrutiny for defects. Regulatory authorities, in fulfilling responsibilities for protecting the public health and safety, conduct systematic examinations to disclose not only apparent defects, but also hidden defects. Over the years, standardized methods of macroscopic examination have evolved for determining filth, decomposition, and foreign matter. These methods of analysis have evolved with the input of producers and consumers as well as regulatory authorities.

In general, "macroscopic" or macroanalytical methods for food examinations primarily depend upon the direct sensory input of the analyst. For example, visual examinations are typically conducted with the naked eye. These exams are occasionally supplemented by low power magnification to confirm defects observed initially with the naked eye, or to describe the defects in greater detail.

There are several major advantages to the use of macroanalytical procedures. They are inexpensive and call for little specialized equipment. They generally permit the analysis of a large quantity of product in a relatively short period of time, thus allowing the analyst to assess the overall condition of the lot quite rapidly. The analyst can quickly identify and isolate those portions of the lot which may contain defects and thus limit the amount of material which may need a more detailed, microscopic evaluation.

Although macroscopic methods have many positive aspects, they may not be the method of choice for every analytical situation. In fact, the very features which add to their usefulness may also limit their application in some situations. Because macroscopic procedures deal with defects which are discernible to the unaided senses, they are not usable for defects hidden from the senses such as those defects too small to be visible to the eye, or those obscured through processing or other factors. In such cases, microscopic methods are essential for characterizing and evaluating the defects in the sample.

2. Microscopic Methods of Analysis

Microscopic methods of analysis involve the detailed examination of a very

small portion of the sample; these procedures provide a different type of information than macroscopic methods. They are used to describe and quantify defects on a different scale than macroscopic methods, and to identify "hidden" defects that cannot be detected through a gross evaluation of the sample. However, microscopic methods also have limitations; they tend to be more time-consuming and more expensive, and they need more specialized equipment. Also, because they are limited to the analysis of a very small sample, the results are not always representative of the overall condition of the lot, thus representative sampling plays a more critical role in this type of analysis.

C. Method Selection for Filth Analysis

See ORA Lab Manual, Volume II, Section 5.4, on Test Methods and Validation.

1. General information

Filth methods commonly used have been published in the AOAC Official Methods of Analysis or the Macroanalytical Procedures Manual. AOAC methods employed by FDA analysts have been proven to give reliable, consistent results; these methods have been validated through collaborative study. If possible, the analyst should use official methods whenever they are found, as written and without modifications.

a. Association of Official Analytical Chemists (AOAC)

Many of the analyses in the following sections will be found in the Official Methods of Analysis published by AOAC INTERNATIONAL (AOAC). The analyst should become familiar with the editorial conventions of this text. All editorial conventions are described in the front of the manual. Boldface reference numbers in the text of a method refer to safety precautions, apparatus, or other critical information. The entire method should be read and each reference looked up before beginning the analysis. A star after the title of a method indicates that the method will be dropped from the next edition, as “Surplus”. “Surplus” methods can be used, but if one of these methods is used, the analyst should inform the AOAC Section Editor, so the surplus decision can be reconsidered. Journal references at the end of each method refer to the article that reported the results of the collaborative study for that method. These journal articles often contain useful additional information, such as problems encountered and expected recoveries. Formulae, such as (2+1), indicate volume ratios for mixing the reagents being discussed.

The AOAC’s chapter, "Extraneous Materials: Isolation," initially discusses

general techniques, apparatus and reagents. It also cites how to record and report results and gives counting/identification instructions. This information is important and should be read by the trainee before the first attempt to use the manual; use the most current edition found.

b. Macroanalytical Procedures Manual

Also known as the FDA Technical Bulletin Number 5, originally published by the AOAC, this publication is now out of print, but has been placed on the FDA intranet. The web version does include some minor changes (error corrections) that are not reflected in the printed versions. The web version also includes web citations to the Compliance Policy Guides for products, making it easier to find related guidance.

This one volume manual compiles and organizes the standardized methods of macroscopic analysis which are useful in determining defects in various types of foods. Although in a general sense, the term "macroscopic" is not as broad as the term "macroanalytical," for the purposes of this manual, the terms are used interchangeably.

This manual compiles standardized macroanalytical procedures for identifying defects in food products. However, macroscopic procedures are frequently interrelated with and supplemented by microscopic ones, each providing the analyst with different types of information. For this reason, the Macroanalytical Procedures Manual will refer to microscopic procedures in some situations.

These microscopic procedures may be grouped into three categories:

- Microscopic methods which have been published by the AOAC in Chapter 16 ("Extraneous Materials") of the Official Methods of Analysis. Where needed, this manual simply refers the analyst to the applicable section of the AOAC volume for the correct method.
- Microscopic methods which have been published in the AOAC volumes, but which are adapted by the analyst for a particular situation. In these cases, special instructions are provided in this manual so that the analyst can modify the microscopic procedure as needed. Reference is made to the correct section of the AOAC.
- Microscopic procedures which have been developed and are in use, but have not been subjected to collaborative study and thus are not yet published by the AOAC. These procedures are included in full in this manual so that they are not lost to the analyst.

Thus, when using this manual, the analyst may be instructed to combine both

macroscopic and microscopic techniques. Examples of this can be seen in the method for determining decomposition in frozen strawberries, which utilizes macroscopic "pick-out" of defects (see Chapter V, Section 9.N.(4)b.) supplemented by the microscopic mold count technique (Chapter V, Section 9. N.(4)c.). Information provided by the microscopic techniques will aid the analyst in interpreting and evaluating the macroscopic findings and in determining the overall quality of the food.

D. Sample Contamination

Read and follow the local laboratory SOP’s dealing with cleanliness and quality control in operation.

The filth analyst exercises constant vigilance against the inadvertent introduction of any type of outside contamination into the sample(s) under the analyst's care. This vigilance extends to those who work in the laboratory if their work can contaminate the sample.

Techniques to Prevent Sample Contamination

- Glassware and other equipment are kept scrupulously clean and stored in clean, enclosed areas when not in use. Equipment should be given a preliminary "extra" cleaning prior to analysis by rinsing with clean water or wiping with clean towels. Purity of reagents are assured by proper storage, and filtering.
- Steps are taken to assure that the analyst's person or clothing do not contribute anything to the sample. A clean laboratory coat is essential. The analyst develops personal techniques of handling samples and equipment that avoid bodily contact with the sample or sample contact surfaces. Spoons, scoops, tongs, and rubber gloves are useful for this purpose. Hair (both human and pet) and lint are the primary contaminants to be guarded against. Precautions such as these combined with personal hygiene will preclude any possibility of analyst-related sample contamination.
- During analysis, the sample is protected from airborne contamination as much as possible without interfering in the analytical procedure. Containers such as trap flasks, beakers, and percolators that are to be left standing for a period of time should be covered. Petri dishes are employed in the conventional manner, with the larger diameter part used as the top or lid for storage of filth extraction papers. Bench tops and other surfaces should be cleaned to avoid dust buildup, and reagents should always be stored in capped or stoppered vessels.

- Guard against cross-contamination between two samples or two sub-units of the same sample. The same piece of equipment is never used twice without thorough washing. Unused portions of reagent are never returned to the original container, but disposed of in a proper manner. Spatulas, tweezers, and other implements are cleaned before they are used to manipulate samples or reagents. Any reagent whose purity is in doubt is disposed. Aerators and wash bottle nozzles should never come in contact with any sample or piece of equipment. If this happens, a thorough cleaning is needed. These and other common-sense rules are followed to protect the integrity of the results of analyses.

E. Use of Quantitative Transfer

The concept of quantitative transfer applies to each sample portion from the moment the sample container is opened until the final results are reported. This demands that every vessel, implement, and operation be considered a possible cause of sample loss.

Each time an analytical portion is transferred from one vessel to another, every effort is taken to assure that no amount of material, however small, is inadvertently left behind in the old vessel. While some small loss appears inevitable in every transfer operation, the analyst can take steps to keep this loss to a minimum.

- For methods using wet chemistry, the wash bottle is the analyst's most versatile tool for accomplishing quantitative transfer. Many methods specify wash bottle liquids for optimal recovery of filth, and care is taken to use the correct wash to avoid interference with the extraction process.
- The analyst also avoids losing small amounts of sample through dripping, splashing, or leakage. Once a loss has occurred, there can be no replacement or compensation, thus the results of the analysis may be compromised.
- Chipped or cracked vessels and overzealous heating (bumping) and sloppy or overpressure sieving operations should be avoided, as these have the greatest potential for sample loss. Sieves are also inspected for rips or deformities that could result in losses.

F. Apparatus Selection

For each analysis, the analyst chooses the proper apparatus. Often the analytical method will call for explicitly described apparatus. Regardless, the

analyst is to be aware of a few general restrictions on apparatus.

1. Avoid sample contact with plastic vessels and implements. Hairs and insect fragments will stubbornly adhere by static force or surface texture to plastic surfaces resulting in reduced filth recovery.
2. Do not use chipped, scarred or broken equipment. Irregular surfaces can snag material, causing problems.
3. Do not use glass equipment when analyzing a product for glass contamination.
4. Use plain weave (not twill weave) sieves. After each use, sieves should be backwashed and inspected for tears or deformities. Fine mesh sieves should never be touched by spoons, scrapers, etc. The most drastic cleaning allowable for fine mesh sieves is soaking in mild cleaning solutions such as bleach, pancreatin or soap. "Clogged" sieves should be discarded if they cannot be cleaned, or the fabric should be replaced.

G. Measurements

A complete description of the filth found during an analysis always includes the sizes, in metric units, of the filth elements. This important factual evidence should never be omitted. The analyst should use the correct number of significant figures indicated by the method in the measurement of filth elements, reagent amounts, net contents, etc. This will help avoid overstatement or understatement, rounding errors, and will increase precision.

See ORA Lab Manual, Volume III, Section 7, on Statistics.

H. Reporting

A complete, accurate report of the analyst's work (not just results) is essential. Agency and court decisions may be based, in part, on the analyst's written report of the analysis and reported results. The results should be reproducible, and the analyst's report contains an account of every operation performed and by whom, every result, and evidence of sample continuity. During the next phase of training, the trainee will be evaluated not only on performance of analytical methods, but on reporting of analyses as well. Reports should be clean, well-organized, and neat.

I. Laboratory Safety

Before working in the laboratory, the trainee should have laboratory safety training. Prior to starting an analysis, the analyst carefully considers the

hazards that could be presented by the equipment and reagents in the analysis, and plan measures to avoid or minimize these hazards. The analyst should have read the MSDS sheets and be aware of the location and use of the emergency showers, eyewash stations, first aid kits, safety hoods, personal protection equipment, and other safety or emergency equipment in the laboratory. Constant caution and care will assure a safe, accident-free working environment.

During the next phases of training, the trainee should always discuss beforehand with the trainer the potential hazards involved in the proposed analysis and the precautions needed for a safe analysis.

See ORA Lab Manual, Volume III, Section 2, on Environmental Health and Safety.

J. Assignments

Read the following:

1. Chapter 16, Section 16.1.1 and 16.1.2 of the current edition of the AOAC Official Methods of Analysis and look at the general organization of the methods.
2. Preface and Introduction in the MPM and look at the general organization of the manual.
3. The lab's Standard Operating Procedures that relate to the filth lab.

2.3.2. Visual and Macroscopic Methods

Objective

The trainee learns and performs various common food analyses for gross contamination, and reports analytical results on the official forms, especially the analytical worksheet.

Discussion

Visual and Macroscopic methods examine relatively large amounts of product for contaminants easily detected with the unaided eye. A portable magnifying lens or magnifying lamp may be used, but the magnification range should not exceed 5X for general purposes. Lenses of higher power have restrictive fields of view and impractical, short focal working distances. If the analyst suspects the contaminants are likely to be much smaller than a poppyseed, or otherwise difficult to detect, then reliance should not be placed solely in a visual examination. A stereomicroscopic method should also be used to evaluate the

contamination.

Sample Preparation

a. Sieves

Dry sifting is a common way of separating macroscopic contaminants from a large bulk of product. The material retained on a particular sieve is referred to as "overs" and the material passing through the sieve as "throughs." One versatile feature of analytical sieves is that they can be nested in order of increasing fineness so various sizes of particles can be separated out in one operation. Sieve mesh sizes are indicated by a standardized number scale, with the larger numbers denoting successively finer meshes. In general, standard Number 8 mesh sieves are used to retain larger rodent fecal pellets, gross contaminants such as sweepings, or product units the size of a small pea or larger. Standard Number 20 mesh sieves will retain most adult insects, many larvae, and smaller rodent pellets. Sieves that are finer than Number 40 mesh are usually too fine for macroscopic applications. Between operations, these sieves can be cleaned using an air current and a dry, clean towel, or they can be washed. Also note that for some products, like peppercorns, special sieves have been designed to sift and grade the product and to determine the “fines” in the finished product.

b. Jones or Riffle Divider

This device can be used to thoroughly mix products such as wheat kernels or coffee beans. Passing the sample six times through a Jones divider produces a totally random distribution of product units. Similarly, it can be used as a sample-halving device; the device halves a sample on each pass through the divider. A portion of product can be isolated from the main bulk by passing through the Jones divider and discarding successive halves until the desired portion size is approximated.

c. Seedburo Grain Inspection (Picking) Tray and Cover

Commonly used in USDA Grain Inspection Services, the lower unit consists of a metal tray with linear groves in which the product rests. The top is also a tray, but with a compressible foam pad. Spread and examine the product on the bottom tray, then when finished, add the top tray, compress, then invert the two trays together, and remove the (now, top) grooved tray. This allows one to see the opposite side of the materials. It helps insure that all sides of the individual grains are examined without having to turn each piece individually.

d. 20” Wide Roll of White Butcher Paper

Paper on one side, plastic coated on the other—this inexpensive paper is both strong and large enough to examine almost any sample. Used plastic side down, it can be cut to whatever length needed, and when replaced, (i.e. between each subsample analysis), it insures subsample integrity and a new clean working surface. Samples can be shaken into a singular flat layer, and then the items can be manipulated as individuals, or grouped together. The paper can be folded, or closed, to accommodate easy transfers back to the original containers. Wet samples can also be examined by using the plastic coated side.

e. Pharmacist's Tablet or Counting Tray

This small 6" X 8" tray has a flat surface with a round grove on one side of the tray. The grooved side has a matching cover that flips into place to cover the grove. Tablets (or in our case, beans, peas, nuts) can be counted (in groups of five or ten) and slid into the groove until the desired number is reached, then the lid closed, extra product removed, and the counted units poured out for closer examination or weighing. It can be used for estimating product weight versus count, and for sequential sampling purposes.

f. Examination Area

It is recommended that a 2' deep by 4' wide area be set aside that is clear of obstructions and superfluous equipment. It should be easy to clean, preferably with a white or wheat colored matte surface. The area is to be well lit, at 150-200 foot candles, preferably with natural colored, shadow and glare free adjustable lamps. Ideally, the tabletop height can be adjusted to the analyst's need and will be open underneath for knees, stool or chair work.

g. Interpretive Line System

This is a series of photographic slides or photographs originally developed by USDA for their GIPSA grain inspection service. These materials are purchasable from Seedburo, and provide exemplars of the types of damage that may be seen in various products including barley, beans, corn, lentils, peas, rye, safflower, sorghum, soybeans, sunflower, wheat and other products. It should be used in combination with FDA authentics, toxic and weed seed identifiers, and the FDA slide series.

h. X-ray machine

X-ray radiography can be used for rapid examination of grains, coffee beans, spices and legumes for internal insect damage. It can also be used for other types of contamination like metal and bone particles in processed foods.

Modern bench top x-ray machines are rapid and relatively easy to use. They do not require radiographic film and developing, like their predecessors.

i. Other Tools

Various spoons, spatulas, knives, flour slick, small brushes, pans, trays, fine and course forceps, needles, and various sized petri dishes.

Assignment

1. Perform the indicated visual and macroscopic analyses under the direction of the trainer. (Substitutions may be made based on product availability, preserving the type of analyses presented)
2. Report the results of each analysis using the correct forms and formats.
3. For each analysis, discuss with the trainer:

- the method used and difficulties encountered
- results and their significance
- the quality of reporting

Analyses

1. *Whole cereal grains* (Macroanalytical Procedures Manual (MPM), Chapter V.3.A.). The analyst is to pick out whole insects, webbing, fecal pellets, extraneous material, and kernels damaged by heat, mold, and/or pests. Good lighting and a light-colored, non-reflective background are needed. Insects cause damage to kernels either by surface feeding or by tunneling. Rodent-gnawed kernels can be recognized by the scalloped edges, a result of the rodents' paired gnawing teeth. Use of a Jones divider may be needed.
2. *Green coffee beans, dried beans, or lentils* (MPM, Chapter V.1.A, V.11.G.). For bulk sampled product, a Jones divider is useful for mixing or compositing as well as for obtaining the analytical portion. The trainee looks for insects, insect damage, and mold. Mold is confirmed microscopically, by observing hyphae and/or fruiting bodies. If possible, the grading of coffee can be demonstrated by a trainer. (FDA By-lines 7:285-91, May, 1977.) Lygus bug damage to beans should be demonstrated.
3. *Flour* (MPM, Chapter V.2.B). The flour is sifted portion wise through a standard # 20 mesh sieve. Examine sieve "overs" for filth. The portion size is generally the entire contents of a consumer package. Sieve

"throughs" may be saved for additional analysis in the next section.

4. *Whole or crude spices* (MPM, Chapter V.8.). Analysis of spices often entails breaking or cracking open a large amount of product in search of insects or signs of insect activity. Rodent defilement may also be encountered. A special sieve is used for peppercorns, over which the product is passed a specified number of times. The analyst should check beforehand whether the product, especially peppercorns, has been fumigated for bacterial contamination. Cinnamon or Cassia sticks are cracked for internal mold. The exterior and interior of *Capsicum sp.* pods need to be examined for insect damage and mold.
   Safety Note: Precautions and clean-up for *Salmonella*-type organisms may be indicated if there was no fumigation. Mite infestations appear as “moving” dust or surface feeding on inner or protected surfaces. Feeding surfaces are sometimes discolored, with adhering, fluffy, granular material (cast skins) often encountered in surprisingly large quantities.
5. *Whole figs or dates* (MPM, Chapter V.9.F). This type of analysis may call for a statistically-derived sequential examination plan that is faithfully executed for valid results. A sharp knife is needed to cut the fruit since the insect contamination usually occurs near the center or pit. An occasional small wasp may be found in some types of figs. These insects are tolerated since they are essential to develop and pollinate the fruit and they enter into the fig to complete their life cycle.
6. *Whole tamarind pods* (MPM, Chapter V.9.F). The tamarind pods need to be broken open to look for the presence of insect contamination and mold. Whole insects like the tamarind weevil (*Sitophilus linearis*) and mites can be found in tamarind pods. Evidence of Lepidoptera larvae often can be seen by the presence of webbing material and excreta pellets.
7. *Shell nuts* (MPM, Chapter V.10.A). For this analysis, a sturdy hammer and pounding block are helpful. The various types of reject nuts are described in the method. Mites can sometimes enter pecans or walnuts through breaches between shell halves and can build up impressive populations inside the nut, causing considerable damage.
8. *Cocoa beans* (MPM, Chapter V.4.A.). Mold should be confirmed microscopically. The trainee should discuss with the trainer the best technique for mixing the sample, considering all factors including size of sample, size and number of subsamples, administrative guidance, etc.

9. *Blueberries or cherries for maggots* (AOAC Official Methods of Analysis, current edition, Chapter 16 on "Extraneous Materials: Isolation;" or MPM, Chapter V.9.C or D). Preliminary manipulation of the product is needed to free the maggots from the fruit tissue. Other insects may be encountered, as well as decomposed berries. If substantial decomposition is suspected, additional analysis may be indicated. Maggots are preserved in dilute (60-75%) ethanol.

2.3.3. Microscopic Methods

Objective

The trainee will learn how to perform the most common types of analyses for “light filth” in foods using various flotation techniques, examine the recovered material for “microscopic filth,” and report the analytical results on the worksheet.

2.3.3.1. Flotation Techniques

Flotation methods are designed to isolate microscopic filth by floating the filth upwards, typically in an oil/water-phased system. Insect fragments, mites, and hairs are lipophilic and like to be in the oil phase, thus they float to the surface with the oils, (hence the term “light filth”). Plant tissues and most related tissues are hydrophilic, and they prefer to stay in the water phase. Common gravity further helps this process, and larger particles sink. To accomplish the separation of filth from food, use a number of solution systems to insure that the majority of the product sinks, while the oils with trapped filth, floats. Often, the analytical portion undergoes a pretreatment, to enhance this effect. The analyte (filth) portion is usually very small, both in a weight to weight relationship to the food (parts per million) and in size or scale. Typically, the recovered filth contaminants are examined under a widefield stereomicroscope. Once found, the filth items may have to be mounted for microscopic identification, thus the term “Microscopic examination”.

Most microscopic methods are found in Chapter 16 of the AOAC "Official Methods of Analysis," consisting of a compilation of validated methods. The trainee should be familiar with the initial information concerning reagents, apparatus, and techniques, and the safety precautions referenced parenthetically in the text of each method.

All microscopic methods begin with the analyst weighing out a prescribed amount of material to be analyzed. This step is critical because the guidelines for how much filth can be in a violative sample, is based on a set amount of

product being analyzed.

From this point on, the exact steps followed may vary significantly from product to product and are prescribed in the method; the method is to be followed precisely.

Variations in methodology based on product

- There may or may not be a pretreatment step to remove excess fats and oils found in some foods (e.g. spicy sauces).
- A digestion step is often used to hydrolyze or digest the product into small particles (e.g. - bread). Some foods, once digested, are wet sieved, that is washed with very hot water over a fine sieve, to flush away excess product and oil.
- Some products may need a hydration, to swell, add water, or saturate the product (e.g. raisins)
- Alcohol may be added to the water, to help saturate the product, penetrate the skins, and dissolve or solubilize excess fats or oils.
- Detergents may be added to loosen the filth, to saturate the food, to emulsify or trap the oils.
- Compounds such as salt or EDTA may be added to help products sink or make them heavier, or to make the water phase even heavier.
- Light hydrocarbon products may be added or the choice of flotation oil changed to help capture the lipophilic filth.

There are basically two physical systems to use in extracting light filth from foods, the Wildman trap flask and the corning percolator. The Wildman trap flask is a closed system, that is, the volume and composition of the aqueous phase remains constant. The filth laden oil interface layers are captured in the neck of the flask. Utilizing a rubber disk on a rod, the oil and interface layers are cleanly separated from the aqueous phase and removed (poured off) from the trapping system. The corning percolator is an open system which allows for repeated drain and refill cycles further isolating the oil filth interface layer. The final drain of the percolator results in a totally isolated oil phase with just a very small amount of the aqueous layer left.

The final step of most flotation methods is to transfer the oil and extracted filth to a ruled fast draining filter paper for microscopic examination. For this we use a Buchner funnel- fitted into a large side arm flask attached to a vacuum pump. A Buchner funnel is an open top funnel, with a porcelain screen about 1 cm

below the opening. A 60 mesh brass or stainless steel fine screen is cut to fit over the porcelain openings. This screen serves to evenly distribute the vacuum under the filter paper and to help distribute the product over the filter paper. The filter paper is wetted, and then centered over the opening, and the vacuum draws the paper down into the funnel forming a small cup-shaped filter. The trapped filth and oil is poured into the filter paper cup and the recovered tissues and filth are evenly distributed over the filter paper. Each extraction paper is then placed in a petri dish bottom plate, and glycerin alcohol, a (1 + 1) mixture of glycerin and 95% ethanol, is added to partially wet the material on the paper, but not to the point debris can float freely over the paper. The edges of the filter paper cup are laid down, and the lid is placed on the dish to keep out dust and minimize evaporation until the extraction paper can be examined. Each petri dish is immediately labeled with sample, sub number, date, and the analyst's initials. Extraction papers become part of the sample reserve and should be carefully preserved. Refrigeration or the addition of a few drops of formaldehyde are effective preservatives after the papers have been examined.

Note: The petri dish should not be inverted, with the filter paper in the larger diameter top, covered by the small diameter piece. This allows airborne contaminants to contact the filter paper and causes rapid evaporation of the glycerin alcohol. It may also cause loss of some filth by adherence to the parts of the dish that contact the filter paper.

Assignment

1. Read pp.173-180 in: Gorham, J. R. (Ed.) (1981). *Principles of food analysis for filth, decomposition and foreign matter* (FDA Technical Bulletin No. 1, 2nd ed.). Gaithersburg, MD: AOAC International.
2. Perform the indicated analyses under the direction of the trainer.
3. Report the results of each analysis using the forms and formats.
4. For each analysis, discuss with the trainer:

- the method used and difficulties encountered
- results and their significance
- the quality of reporting

Analyses

1. *Flour* (AOAC Official Methods of Analysis (OMA), Method 972.32 "Light Filth (Pre and Post Milling) in Flour (White)"). Acid hydrolysis is

employed to digest the flour, and the filth is isolated by flotation in a percolator. Due to the uniformity of flour milling processes, pre-milling and post-milling contaminants can be roughly recognized by size. It is, therefore, important to record the size ranges of contaminants so that compliance personnel can accurately interpret the analytical results.

2. *Fig or fruit paste* (AOAC OMA, Method 964.23 for "Filth in Fig and Fruit Paste"). Filth flotation is accomplished using a Wildman trap flask. The trainee practices quantitative transfer from the flask to a beaker prior to attempting the analysis. A smooth motion is needed to pour the trapped material into the beaker while holding the trap flask rod out to exclude the aqueous phase of the liquid system. About 1/4"-1/2" of aqueous phase is trapped off along with the oil phase to ensure that no filth is left below the stopper. While holding the stopper in the "up" position, the flask neck is rinsed with the same aqueous solution and the rinses are poured into the beaker.
3. *Chocolate* (AOAC OMA, Method 965. 38 B (b) “Filth in chocolate”). This method uses a detergent to defat the product prior to the flotation. The extraction papers (filter papers containing the extracted filth) will give the trainee a challenging exercise in distinguishing between very similar-appearing insect and plant fragments, and introduce bleaching techniques that may prove useful in other situations.
4. *High bran content bakery goods* (AOAC OMA, Method 972.36 for "Light Filth in High Bran Content Breads"). This method employs a defatting step to enhance the separation of filth from product. The defatting should be performed in a hood to avoid fumes.

2.3.3.2. Sedimentation and other Specialized Techniques

Objective

The trainee will learn how to perform some common types of food analyses for various types of filth not easily recovered by flotation, and how to record the results.

Discussion

Three very different problems that cannot be resolved by using any of the techniques described thus far will be demonstrated in this section:

- The first problem deals with “heavy” filth. As the name implies, these methods rely on a combination of gravity and density in a liquid system that allows the material to sink to the bottom of the container, while the

lighter generally organic materials are floated off.

- The second problem deals with thin-skinned insects, like maggots, insect eggs, and mites. Because of their thin exoskeletons or shells, they are less oleophilic than the more mature stages of the animal, and they too prefer to sink- even in oil/water phased systems.
- The third problem is how to count/detect maggots or mites if they are buried deep inside the plant tissues, and they are attached to tissue material?

1. Heavy Filth

In heavy filth methods, we are typically looking for heavy material contaminants such as sand, glass, metal fragments, and even feces. Basically, the analyst is going to make a liquid slurry of solid materials using different solutions, that, with stirring, act to both solubilize the material to loosen up the product and free the heavy materials, thus allowing the heavy materials to sink. In the past, carbon tetrachloride and chloroform were used, now solutions like chloroform alone or salts are used, which when added to water, solubilize and make the solutions much denser than water alone. The higher the density of the solution, the greater the chance that the light weight organic material will float and the heavy materials will sink.

Keep in mind, that usually hairs and insect fragments are not recovered by these methods. The analyst often will use the heavy filth method as a prelude or preliminary step to a light filth extraction. These methods are a very good way to defat or remove excess oils from the food product. The analyst usually retains the poured off organic materials for his next analysis, which is typically light filth. Hairs and insect fragments come off in the organic material, and that material should be examined for these and other types of filth (especially fecal pellet fragments).

Heavy sediments such as glass and metal shards, sand, minerals, bone and other materials may adulterate some products. The analysis should be tailored to the particular kind of analytes suspected in the product. For example, if glass contamination is suspected, the analyst avoids using all glass apparatus such as glass beakers and stirring rods. The product's original container is always examined to determine if it could have contributed to the contamination.

Once these materials are recovered, they are described. Pictures help, but a physical description of the recovered material is annotated on the worksheet. The descriptions should be precise, accurate and brief. Although it is not

possible to cover all of the characteristics or terms used in material identification, for training purposes, one or more of the following characteristics may help describe these materials (this is not an all-inclusive list; use a thesaurus if needed, and modifiers when needed).

Material Descriptions: Size, shape, color, finish (matte/gloss), variegation, surface texture, surface coating, lamination, presence of a parental (original) face or markings, scoring, density, fracture, fraying, melting point, density, inclusion, assemblage, distance, interval, parallelism, obliqueness, angularity, position, curvature, softness, elasticity, voids, optical character, etc.

For example, glass has many characteristics, but it can be differentiated from sand grains or other crystalline look-alikes by two principle characteristics. Broken glass exhibits acute angular, conchoidal fractures (resembling the markings of a clamshell) and it shows no color (isotropism- no optical activity) when viewed between crossed Nicols on a polarizing microscope. When combined with size, shape, hardness, density, solubility, parental face characteristics (if present), and refractive index, there is little doubt left towards the identity of the material. The analyst has to list the pertinent facts in the report and draw a conclusion based on those facts. Keep the report brief and to the point.

2. Maggots and Insect Eggs

Another problem area deals with the isolation of thin-skinned insects, like maggots, insect eggs, and mites. As stated earlier, because of their thin exoskeletons or shells, they are less oleophilic than more mature stages of the animal, and they prefer to sink even in oil/water phase systems. In the olden days, people did not want to go anywhere near tomato canneries because the canneries would dump the waste tomato skins in huge piles outside the plant. This created ideal breeding grounds for flies and maggots. Some of these flies ended up inside the plant and the eggs and newly hatched maggots would be in the product. One of the first things filth analysts learn is if a mixture of oil and tomato tissue floats, then the eggs and the larva sink to the bottom of the flask. In the method below, Analyses Section D.2., the maggots and eggs are drained off, the oil and tomato tissue remain in the separatory funnel. This method is exactly opposite of the extraction procedure in “light filth” methods.

3. Maggots and Mites in Mushrooms

Maggots and mites present the filth analyst with a real challenge, especially when embedded deep in the product’s tissues. To extract them, the maggots and mites are mechanically freed; this is done in a blender. A challenge still

exists when they are free; the maggots cannot float off, nor can they sink because of the extra tissues they are mixed with. This method demonstrates staining the maggots and mites so they are much easier to see. It is a unique method that works very well for all mushroom products, and incorporates a bleaching technique that illustrates how easy it is to clear tissues, yet not affect the filth one is looking for. It also teaches the analyst to be careful in how long to blend the products and how many subs can be done in a timely manner. Analysts should practice with training samples before receiving samples for regulatory analyses.

Assignment

1. Perform the indicated analyses under the direction of the trainer.
2. Report the results of each analysis using the correct formats.
3. For each analysis, discuss with the trainer:
   - the method used and difficulties encountered
   - results and their significance
   - the quality of reporting

Analyses

1. *Ground spices for heavy filth* (AOAC Official Methods of Analysis (OMA), Method 978.21 for “Light Filth in Capsicums (Ground), sedimentation”). The liquids selected for these methods are dense enough to float the spice material but not heavy filth such as feces, rocks, sand, and dirt. Decanting the spice material leaves the heavy filth behind to be examined microscopically before the weight is determined. The cautions indicated in the text are to be observed. Substitute Chloroform for all Carbon tetrachloride citations.
2. *Tomato products for fly eggs and maggots* (AOAC OMA, Method 955.46 “Filth in Tomato Products”). A large separatory funnel is used to separate product from maggots and fly eggs. The latter settle to the bottom and are drawn off through the stopcock. The analyst should become familiar with the appearance of fly eggs and maggots prior to attempting the analysis.
3. *Mushrooms for maggots and mites* (AOAC OMA, Method 967.24 "Filth in Mushrooms"). A dye is employed to make the filth highly visible (purple) against the background of bleached (white) product. The product is blended in order to free maggots and mites that are

embedded in the mushroom tissue or wedged tightly in the gills. The crystal violet dye is soluble in ethanol for clean-up purposes, but try not to get it on the hands.

2.3.4. Mold Detection

2.3.4.1. Gross Mold Contamination

Objective

The trainee will learn the general diagnostic characteristics of mold.

Discussion

Most people can recognize visible mold growth by its characteristic growth habit, colors, and musty odor, without the aid of magnification. Under a low-power hand lens, however, typical mold consists of a mass of thread-like, branched filaments. The mass is called a mycelium and the individual filaments are called hyphae. Spore-bearing fruiting bodies, whose shape varies between species of mold, may also be found.

In most cases, however, macroscopic observations are insufficient for FDA purposes. Some non-mold plant diseases or other conditions may superficially resemble mold. Also, decomposition can sometimes mask the presence of mold. For these reasons, the analyst confirms the presence of mold microscopically. This can be done by preparing an aqueous slide mount of a small portion of the suspected mold, examining it for the presence of hyphae. Microscopically, mold hyphae can be distinguished by one or more of the following characteristics:

1. *Parallel walls*. Although individual hyphae may vary in size, the diameter of any single hypha is constant. Mold hyphae are basically tubular, with parallel walls.
2. *Septation*. The presence of parallel cross walls (septa) in hyphae is a characteristic of many molds. They give the hyphae a segmented appearance. Some plant hairs may have cross walls but they are usually not parallel to each other.
3. *Granulation***.** The protoplasm of mold may exhibit a distinct granular appearance. This is an especially useful characteristic among larger species that may not exhibit septation.
4. *Branching habit*. When present, this is one of the most reliable characteristics. Mold hyphae typically have branches that are the same

diameter as the main trunk. Typical mold branches extend out at right angles to the main trunk.

5. *Rounded ends*. The natural tip of a hypha is normally rounded like a test tube bottom. Hyphae that are broken, however, typically break off squarely.

Assignment

1. Using moldy fruits or other foods, practice preparing aqueous slide mounts and distinguishing, under a compound microscope, the mold hyphae.
2. Examine at least two samples of different whole fruits, vegetables, or spices for macroscopic mold contamination, confirming the mold microscopically. Methods for some of these products can be found in the "Macroanalytical Procedures Manual".
3. Report the results of each analysis on the correct forms, discussing each report with the trainer.

2.3.4.2. Howard Mold Count

Objective

This section describes the Howard Mold Count technique.

Discussion

Howard Mold Count procedures are empirical methods that are to be precisely followed in every detail for each type of product in order to obtain satisfactory results. Experience has shown that mold counting cannot be reliably learned without the help of an experienced instructor who can give the trainee personalized instruction.

1**.** Microscope

A Howard Mold Count is performed on a compound microscope with certain features. The first requirement is a lens system that has a standard microscope field diameter of 1.382 mm. The eyepiece has a micrometer disk ruled in squares, each side of which is equal to one-sixth the diameter of the ocular lens opening. A Howard Mold Count cannot be performed using a microscope that does not meet these requirements.

2. Howard Mold Count Chamber

This is a specially constructed slide and cover glass unit that is used. It is

designed to contain 0.03 cc of material on a central platform with the cover glass in place. The platform is surrounded by a moat and flanked left and right, beyond the moat, by shoulders whose height is 0.1 mm taller than the platform. The combined exacting requirements of the microscope and Howard Mold Count chamber ensure that the analyst, at all times, views a precisely known amount of product. Each Howard Mold Count chamber has a scored calibration circle of 1.382 mm diameter, or has scored parallel calibration lines, 1.382 mm apart, to use to check the standard microscope field diameter.

3. Sample Preparation

The product is prepared exactly as stated in the method for that product. The sample is thoroughly mixed both before and after dilution. Immediately before each slide is prepared, the sample again is thoroughly mixed. This is important to assure uniform suspension of mold filaments.

4. Slide Preparation

For each preparation, the Howard Mold Count chamber is completely clean and dry. A clean scalpel is dipped into the well-mixed sample and then touched against the platform of the Howard Mold Count chamber so that just enough sample is transferred to fill the platform when the cover glass is in place. The drop of sample is evenly spread using the scalpel, and the special cover glass is lowered over the platform until it almost touches the product with the cover glass sides aligned with the shoulders. The cover glass is quickly pressed down, spreading the sample evenly over the platform and avoiding the trapping of air bubbles under the glass. A proper preparation has the platform entirely filled with the product, no air bubbles, and no spillage into the moat. If a proper preparation is not obtained, the entire chamber should be cleaned, dried, and another attempt made.

5. Newton's Rings

Newton's rings are a rainbow-type optical phenomenon produced between each shoulder and the cover glass when they are in contact without applied pressure. The rings are observed by holding the completed preparation at a slant so that light is reflected off the cover glass. The presence of Newton's rings assures that the depth of product on the platform is 0.1 mm. Their absence indicates that either the slide was not thoroughly cleaned and dried or that product solids thicker than 9.1 mm are holding the cover glass above the designated height. In either case, a new attempt is to be made with a clean, dry slide and cover glass.

6. Examination of the Slide

After the slide is placed under the microscope, it is brought into focus and the field examined. The fine adjustment is used to bring into view mold filaments that may be at different depths of the field. The method calls for a field be counted as positive when the aggregate lengths of not more than three filaments of mold exceed one-sixth the diameter of the field. One-sixth the diameter of the field is not enough to be counted as positive: the aggregate length exceed one-sixth the diameter of the field. The drop-in eyepiece micrometer disk, divided by etched lines so that 36 equal-sized squares are formed and any side of which measures one-sixth of the field of view, should be standard equipment for mold counting.

In the examination of a slide for mold, the field should be selected in a consistent manner. One method is to begin at what appears, through the microscope, to be the upper left portion of the counting area and go straight across, skipping every other field, then drop down approximately two fields and, reversing the direction, again cross the counting area, continuing this back and forth until 25 fields are examined. Fields to be counted should be selected at random. Under no circumstances should fields be selected for counting because they do or do not contain mold.

If it is readily observed that the field is positive, it should be so recorded and study of the next field begun. If not enough mold is observed at first glance, the field should be carefully examined and the fine focusing adjustment used to bring different depths of the field into focus. In some instances, the mold can be seen better by changing the light intensity. A systematic search of every part of the field is needed before it can be concluded that a field is negative. When branched filaments or clumps of mold are found, the length of one filament is considered as the sum of all the branches or the sum of all the filaments in the clump. The fruiting heads, such as those of *Alternaria*, with any attached mycelia are counted as mold filaments. If the examination of a field reveals a piece of suspect material extending into the field from the edge, the material should be traced back so that its true identity can be established.

However, only mold found within the field should be considered in determining whether the field is positive or negative. Should the identity of any filament be in doubt, it may be studied at a magnification of approximately 200X, although the length is determined at 100X. Unless the suspected filament is unquestionably mold, the field should be counted as negative. Small air bubbles, which in aggregate do not exceed one-sixth the diameter of the field, may be disregarded. Occasionally, a field is largely obscured by a mass of opaque material or air bubbles. In this case, if a count cannot be determined, the analyst should move on to the next field.

7. Calculation of Results

The results are calculated from the findings on examination of 25 fields from each of two or more slide preparations. Because comminuted fruit and vegetable products are mixtures rather than solutions, mold filaments are not always uniformly distributed among the plant fibers and tissues in the individual droplets used for slide counts. Therefore, the count of several slides of the same sample may vary, even though the slides are prepared and examined with the greatest care. Studies of deviation in mold counts indicate that the results are grouped about the average in the same way that other mixtures follow the rules of random distribution. This may account for the occasional wide variations of results.

A general rule is that two counts from the same sample should check within three positive fields; otherwise, two or more additional slides should be examined. For greater accuracy, more fields may be counted.

Assignment

1. Read pp. 191-200 of: Gorham, J. R. (Ed.) (1981). *Principles of food analysis for filth, decomposition and foreign matter* (FDA Technical Bulletin No. 1, 2nd ed.). Gaithersburg, MD: AOAC International.
2. Perform a Howard Mold Count on at least two types of tomato products and one type of berry product under the guidance of an experienced trainer. Use the correct AOAC method for each product.

Evaluation

With a sample provided by the trainer, perform a Howard Mold Count. The trainer performs an independent analysis to compare with the trainee's results for the evaluation of performance. The use of a compound microscope with a dual-viewing body is recommended so that the trainer and the trainee can view the sample at the same time.

2.3.5. Analysis of Factory Filth Samples and Filth Analytical Worksheets

Objective

This section describes the complete analysis of a factory filth sample.

Discussion

New Hire Analysts should have already reviewed the ORA U site on the preparation of Analysts Worksheets and should have discussed this with their trainers. Filth worksheets follow the same format and rules as all other

worksheets follow, differing only in the final content and formatting. To some extent, they are easier to complete, yet more complicated in content. This section provides guidance and practice in describing what initially appears to be very complicated samples, but in reality are relatively simple exhibits that need to be broken down into component parts. Also, submit photographs to help document findings and help others visualize in their minds what is being described.

For most exhibits, a generic method statement, similar to the following, covers all the analytical methods:

Methods: Visual, Macro and Microscopic Examination, with pick out, for Gross Filth. For the Confirmation of Rodent and Bird Adulteration, See FDA Bylines #3, Nov. 1970, pp. 153-164, with method up-dates in the AOAC 17th edition, Chapter 16, with Supplements. See also FDA Technical Bulletin #1 Chapters 9 and 13 and FDA Technical Bulletin #5 Macroanalytical Procedure Manual Chapter V Parts 2B and 3A(4)(B) and Chapter ∇II Part 4. See also Zimmerman, M.L. and S.L. Friedman, 2000, "Identification of Rodent Filth Exhibits", Journal of Food Science, Vol. 65 (8): 1391-1394, and “Insect Penetration through Packaging Material” (AOAC 16th 16.15.05 973.63). Exhibits placed in labeled petri dishes for examination. Insects examined as above, using visual and macroscopic exam. Where applicable, see Results below for method citations as they are used in the analysis.

This is followed with the Results headers shown below, with the Sub description being a direct quote from the C/R continuation sheet followed by the analytical recovery:

Results-

Sub # Sub Description and Filth Recovered

From C/R- “….”

Sub consists of …and analysis confirms….

For each subsample, the analyst identifies the subsample and the analysis called for (rodent, insect, or other filth, some or all of the above.). This is done by visual or stereoscopic examination and confirmation of the subsample's description versus the investigator's collection report. Begin by physically segregating the filth into general categories, being sure to capture loose elements first. The type(s) of analysis to be performed will be dependent on this information; do not limit the analyses to those requested on the C/R. Often, additional items of “filth” are found; include these items in the

description. For example, the investigator has identified rodent adulteration, but the investigator may not have seen or identified the mites or webbing from moth larvae also present. Also, keep in mind the differences between 402(a)(3) and (a)(4) evidence, and where needed highlighting (a)(3) evidence, as it could be a separate charge within the sample.

Consideration should be given to the preparation of the exhibits for use in the courtroom. These exhibits may be displayed or passed around; prepare these exhibits for optimum viewing, yet present no hazard to those handling the exhibits.

Safety Caution: Generally, these are grossly contaminated samples and the analyst should use caution and protect themselves in preparing the exhibits. Personal Protective Equipment, ventilation and preparation sites should be located and used to prevent aerosol release of potentially harmful agents or fumigants.

Whole insects

In addition to identifying the insects, the analyst should record the following:

- Counts or approximations- Count an area and estimate the number with multiplication factor - do not use “too numerous to count” (TNTC)
- Record if the insects were dead or alive.
- Record stages of growth present.
- Record evidence of fumigation or preservation by the inspector (This should be included on the C/R, but mistakes happen and they should be noted.)
- Pliability, moistness, presence or absence of body fluids if it adds information regarding the relative age of the contamination.
- Associated material (adulterated product, fecula or pellets, cast skins, etc.)
- Size ranges.

<u>Pellets and other excrement</u>

- Origin or source are identified, i.e. size and range, shape, presence of mucous coating and/or hairs, odors (weak, strong, fecal or urine-like), constituent make up. If applicable, confirm using chemical confirmatory tests. Size ranges are recorded.

- Age is difficult to determine but can be estimated, note if pliable, moist, insect damaged, bleached or discolored, or if brittleness is present.

Urine stains and bag cuttings

- For all layers, mark and identify the interior and exterior surfaces. Note approximate size, shape, layers and construction, e.g., "4 layer kraft paper bag cutting with inside 3rd layer plastic bag liner".
- Look for loosely adhering filth (hairs, mites, insects, etc) stereoscopically. Pick them off, prepare and identify. Note their presence.
- Look for visible stains. Note size, shape, characteristics.
- Switch from visible light to Long Wave Ultraviolet black light, and in pencil, accurately outline the fluorescing stains. Note if the stains penetrate and how far. Note size, shape, characteristics.
- Product beneath stains may also be contaminated, as evidenced by fluorescence, caking or lumping, or adherence to the packaging.
- Finally, select the most characteristic stains and perform the chemical tests needed to confirm the presence of mammalian urine and its source, human, mouse, or other mammal.

Gnawing

In addition to the gnawed hole itself, adhering hairs, pellets, or urine stains may be found at or near the gnawed site. Note and confirm these as above. Gnawed packaging or product should be examined macroscopically and characterized as rodent or insect gnawing. Rodent gnawing has a typical serrated appearance (i.e. paired crescent-shaped cuts, with double incisor tooth marks). Insect gnawing, exhibiting liner striations, may also exhibit webbing, pellets, cast skins, setae, etc.

- Record minimum-maximum diameter of each hole or gnawed area and location
- Record direction of penetration of gnawed packaging (terracing) AOAC 17th ed. 973.63.
- Record whether or not gnawing penetrated packaging completely
- Record adhering product

Dead Animals

Occasionally dead rodents or related materials may be collected as exhibits. As with insects, these items need to be identified following traditional mammalian taxonomy procedures. They may also yield additional forensic information such as the presence of parasites (CDC washing and combing procedures,) and decay, desiccation, or putrefaction stages can be estimated. The references cited below Section D can help in this work.

Floor sweepings, trash collections

These exhibits may contain all of the above, and more. The easiest procedure is to take a picture, then segregate out the important or significant items that may not be evident in the picture. A simple inventory of these items is typically sufficient, and unimportant items can be lumped together as “waste paper, product, and/or debris”

Product and packaging blanks

Product and packaging blanks (uncontaminated “control” portions) should have been included in the factory filth sample. These should be analyzed in the same manner as contaminated material. If no blanks are included in the sample, the analyst derives them from the uncontaminated portions of a subsample or by securing other credible blank materials.

702(b) portions

These should be set aside and not analyzed; the reserve samples are required by law. When returning the sample to the sample custodian, consider creating or segregating the 702(b) portion. It should be in a clearly identified sealed package and the contents fully described on the C/R. This will save a considerable amount of time should the firm request the 702(b) portion.

Reporting the Reserve sample

The reserve sample is described completely on the analyst worksheet. Quotations of all identifying labels prepared by the analyst are included. The analyst preserves as much of the sample as possible in its original condition.

Fumigation or preservation applied by the analyst is noted. Finally, the seal applied by the analyst is quoted and the disposition of the sealed sample is stated. Normal operating procedure is to return the sample to the sample custodian and the date need not be stated, however special storage instructions should be pointed out on the worksheet and made clear to the sample custodian.

See ORA Lab Manual, Volume II, Section 3, Chapter 5.8, on Handling of

Samples.

Assignment

1. Read Phillip DeCamps November 1970 article "A guide for the examination of rodent filth exhibits and related samples." *FDA By-lines* No. 3, pp.153-164.
2. Read Zimmerman, M.L. and Friedman, S. L. (2000). "Identification of rodent filth exhibits." *Journal of Food Science*, Vol. *65*(8): 1391-1394.
3. Under the guidance of an experienced analyst, analyze a factory filth sample. (*Note:* If the analyst has not received the training for chemical confirmation of rodent adulterants; the trainer will have to provide this training as needed.)

References and Additional Method Citations

Filth Exhibits: Urine

- LWUV light, Urine Stains on Food & Containers (AOAC Official Methods of Analysis (OMA), current ed., Method 945.88).
- Urease Test for Urea (AOAC OMA, current ed., Method 942.24).
- Xanthydrol test for Urea (AOAC OMA, current ed., Method 959.14).
- (AOAC OMA, current ed., Method 959.14, Xanthydrol test for Urea, modified to include 4 μg + urea response requirement from J. AOAC Intl. 81(6): 1155-1161).
- Magnesium Uranyl Acetate Test for Urea (AOAC OMA, 15th ed. (now surplused), Method 963.28).
- Urease Bromothymol Blue Agar Test for Urea (AOAC OMA, current ed., Method 972.41).
- TLC Method I (AOAC OMA, current ed., Method 980.28).
- TLC Method II- with potential interference material (AOAC OMA, current ed., Method 973.64).

Filth Exhibits: Fecal Material

- Alkaline Phosphatase Test for Mammalian Feces (AOAC OMA, current ed., Method 981.22).
- Alkaline Phosphatase Detection Method for Mammalian Feces in Corn Meal (AOAC OMA, current ed., Method 986.28 - See 990.10).

- Alkaline Phosphatase Detection Method for Mammalian Feces in Grain (AOAC OMA, current ed., Method 990.10).
- Alkaline Phosphatase Detection Method for Mammalian Feces in Ground Black Pepper (AOAC OMA, current ed., Method 993.27).
- TLC Coprostanol for Mammalian Feces (AOAC OMA, current ed., Method 988.17).
- Microchemical test for Uric Acid (AOAC OMA, current ed., Method 962.20).
- TLC Method for Uric Acid (AOAC OMA, current ed., Method 986.29).
- Spectrophotometric Method for Uric Acid (in Flour) (AOAC OMA, current ed., Method 969.46).

References for bone and skull confirmation:

1. Popesko, P., Rajtova, V. and Horak, J., (1990). *A color atlas of the anatomy of small laboratory animals* (Vol. 1, Rabbit, Guinea Pig; Translated 1992, ISBN 0 7234 1822 5). London: Wolfe Publishing, Ltd.
2. Popesko, P., Rajtova, V. and Horak, J., (1990). *A color atlas of the anatomy of small laboratory animals* (Vol. 2, Rat, Mouse, Hamster; Translated 1992, ISBN 0 7234 1823 3). London: Wolfe Publishing, Ltd.
3. Gottschang, J. L. (1981). *A guide to the mammals of Ohio* (ISBN 0-8142-0242-X). Columbus, OH: Ohio State University Press.
4. Nowak, R. M. (Ed.). (1991). *Walker's mammals of the world* (5th ed.). Baltimore: John Hopkins University Press.
5. Hamilton, W. J., Jr., Whitaker, J. O., Jr. (1979). *Mammals of the eastern United States* (2nd ed., ISBN 0-8014-1254-4, LC 79-12920). New York: Cornell University Press.

References for Forensic Entomology:

1. Smith, K. G. V. (1986). *A manual of forensic entomology*. London: British Museum (Natural History) and Ithaca, New York: Cornell University Press.
2. Hall, R. D. and Haskell, N. H., Wecht, C., (Ed. Forensic Sciences). (1995). *Forensic entomology; applications in medicolegal investigations*. New York: Mathew Bender.
3. Byrd, J. H. and Castner, J. L. (2001). *Forensic entomology; the utility of*

arthropods in legal investigations. Boca Raton, FL: CRC Press

2.3.6. Specialized Microscopy Techniques, Optical Crystallography (a.k.a. Polarized Light Microscopy), and ID Spot Tests

Objectives

Microscope discussions in an earlier section (Section 4.2.2), were intended only to introduce the variety, principles, set-up and maintenance of microscopes. It was not intended to teach the practical application of those tools. This chapter shows specialized microscopy methods and techniques, in particular those used in optical crystallography, as they apply to common problems often encountered in the filth laboratory.

Discussion

A thorough discussion of the principles of optical crystallography is beyond the scope of this manual, and in situations requiring advanced crystallographic techniques, e.g. measurement of more than one refractive index, measured structure, or precise optical characterization, these topics will be handled as a specialized course. An excellent discussion of this topic appears in the Food and Drug Administration Bulletin No. 1, Chapter X, reprinted in FDA By-Lines Vol. 6(1):20-53 (July 1975) and the instructor will identify the parts that should read be read. It will give the trainee more insight into the science of crystallography and the need for advanced study. This chapter will be discussing particular problem analytes and how they are analyzed in the lab, through polarized light microscopy or phase contrast microscopy, and the use of “spot testing” or micro-chemical tests to confirm visual findings.

In the past, the main thrust of the science of optical crystallography or PLM has been the identification of crystalline drug substances. Even today, in the hands of a skilled analyst, PLM provides a rapid and accurate identification of many substances and avoids elaborate and costly chemical analyses. But the work can be tricky; after learning, practice frequently for reliable results.

PLM has had a long history in FDA. The earlier work provided a reference catalog of known optical properties of crystalline substances. Most district laboratories have this index card catalog covering the thousands of substances (mostly drugs) studied. However, substances falling within the framework of filth analysis include glass, struvite, urea, dixanthylurea and the starches. Precise identification of these substances, and perhaps others, is most easily accomplished by optical crystallography, and/or with phase contrast microscopy; many are spot tests in and of themselves, or spot tests

can be used as confirmation of observations.

Assignment

Under the direction of an experienced analyst, study the principles, methods, and techniques of optical crystallography found in the Food and Drug Technical Bulletin No. 1, 2nd Ed. 1981. (*Note:* The trainee is to have the opportunity for first-hand observation of the optical phenomena described in that publication.)

Learn to apply the microscope and spot testing to the identification of the following substances:

- Glass
- struvite
- urea and dixanthylurea
- corn, wheat, potato, soy, and rice starch

Evaluation

1. Demonstrate an understanding of the following optical phenomena: Becke lines; birefringence; crystal habit; extinction; isotropism and anisotropism; Newton's rings; cross polarization; refractive index
2. Using an unknown crystalline substance provided by the trainer, correctly determine the refractive index of an isotropic substance.
3. Identify the unknown substance given by the instructor (#2 above) making additional observations.

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3. Glossary/Definitions

Commensal **-** one who eats at the same table with others; an organism, not truly parasitic, that lives in, with or on another.

Collar - An old sieve with the weave removed; extends the height of a sieve.

Fix - Chemical process that prevents or minimizes pigment discoloration, loss and/or tissue distortion, to preserve in place.

Overs/Throughs - After a sieving operation, anything retained on top of the screen is referred to as overs, those that pass through, as throughs.

Aperture - Circular hole in the camera that controls the amount of light

reaching the sensor or film emulsion.

Blooming - The bleeding of signal charge from extremely bright pixels resulting in over-saturated pixels. “Blooming” in digital photography compares with over-exposure in film photography.

BMP - Bitmap (.BMP file extension): this is a standard image file format for Windows®.

GIF - Graphic Image Format (.GIF file extension): gif format is what is termed as a "lossless" compression format. This format is referred to as a "paletted" image or a 256-color image. It is limited to 256 colors.

JPEG - Joint Photographic Experts Group or jpeg (.JPG file extension) this format is a lossy compression format. The higher the compression ratio the more the pixelization or "blockiness" occurs.

Cropping - The act of cutting out a portion of a digital image for blow-up/display as a separate image.

Photodocumentation - The process of recording images representative or demonstrative of a sample or object.

PNG - Portable Network Graphics (.PNG file extension): this file format is an alternative to the GIF (Graphic Image Format) format.

TIFF - Tagged-Image File Format (.TIF file extension): a lossless bitmap image format supported by virtually all paint, image-editing, and page-layout applications.

Feather-the primary covering on a bird and aids in flight.

Feather Barb-the individual units which grow from the feather shaft or *rachis.*

Feather Barbule-are like mini barbs growing from the shaft of the barb.

Node: the swollen structure spaced at regular intervals along the feather barbule. The shape of the node can vary in shape from having spines, prongs, round points or even flared.

Rachis-the central shaft of the feather.

4. Document History

Revision #	Status* (D, I, R)	Date	Author Name and Title	Approving Official Name and Title
1.2	R	09/01/2005	LMEB	LMEB
1.3	R	06/06/2008	LMEB	LMEB
1.4	R	02/02/2010	LMEB	LMEB
1.5	R	02/14/2013	LMEB	LMEB
02	R	06/15/2020	LMEB	LMEB

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

5. Change History

Revision #	Change
1.2	Table of Contents – deleted Sections 4.3.5, 4.3.7, 4.39, and 4.6-4.9; changed 4.5 to Document History Questions deleted from all sections.
1.3	4.2.3 D.- 4th Reference website updated
1.4	4.1.1 – bullet 4 web link updated 4.2.1 B. 3. – bullet 4-9 web links updated 4.2.4. C. 2 – web link updated 4.2.5.1 B. – revised second paragraph 4.4.1 C. 1. b – web link updated Footer – web link updated
1.5	Header – Division of Field Science changed to Office of Regulatory Science
02	Updated references, types of samples to be used for training assignments, and regulatory references in FD&C Act and USC. Removed broken/outdated web links. Significant updates to some sections, including scanning electron microscope, wet mount media, introduction to identification keys, insect morphology assignment for dissection of insect genitalia, discussion and assignment related to hair identification, entire feather identification section, excreta and fecal pellets, X-ray radiography, Machinery Mold: Geotrichum, rodent nesting materials. Reformatted document and moved to new template.