UNUSUAL ENTOMOLOGICAL SEPARATION TECHNIQUE IN USE AT THE FOOD AND DRUG ADMINISTRATION

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As the monitor of food wholesomeness, the Food and Drug Administration must be able to detect lapses in sanitation unrecognizable by the consumer. Frequently such mishandling is ascertained by objective analysis of food samples for arthropod residues. Such contaminants, when present beyond an irreducible minimum, are considered filth and are not permissible in food offered for sale. Because minute quantities of such residues must be isolated from foods, FDA scientists have developed sensitive flotation methods for their recovery. These have been published primarily in non-entomological journals. Except for one article by Harris[1] in 1943, this information has not been widely disseminated to entomologists. As a result, many of them lack even a key for access to this literature.

Detecting and isolating insect and rodent filth from food may vary from simply picking a rat dropping out of a handful of grain to extremely sophisticated methods. Since particulate matter is sought, separation techniques are usually physical rather than chemical. They must be sensitive. Because of dilution only minute traces of the original grossly insanitary situation may remain in a finished product. Our analyses must often be subject to judicial review; therefore they must have sufficient validity to "stand up" before minute dissection by a hostile adversary.

The flotation methods herein discussed depend on the preferential wettability of insect cuticle by oils, when solid food product, water or polar solvent, and oil are brought into intimate mixture. When the oil layer separates, most of the insect cuticle present in the food will adhere to its interface with the lower phase.

This discovery in the early 1930's by FDA analysts B. J. Howard and John D. Wildman was made while they were pulping raspberries by rubbing them over a sieve during a food plant inspection. The only convenient vessel available was an old kerosene tin. When the berries were placed in water to rinse away pulp and reveal insect larvae, the residual oil droplets floating on the surface each carried a tightly adhering larva. In 1935 Howard[2] discussed this phenomenon and described the Wildman trap flask, a simple piece of laboratory glassware which was to be the mainstay of sanitation analysis for three decades. It consists of a 2-liter Erlenmeyer flask with a rubber stopper on the end of a thin metal rod. The stopper is of such size that it can be lubricated and forced through the neck with difficulty. In normal use, it

resides in the body of the flask and can be raised into the area just below the neck to isolate any material present therein. Then, one can easily decant the oil and adhering arthropods present in the flask's neck. The plunger is also convenient for stirring the sample to disperse oils, etc.

In actual practice separations seldom occur cleanly. Possible interferences include: tendency of food to adhere to oil, tendency of insects to adhere to food particles, stabilization of emulsions by fine or colloidal matter, and insufficient oil adhesion to arthropods. Harris[3,4] furnished a good synopsis of early history of these separations.

The 6th Edition (1945) of the book, Official Methods of Analysis[5] of the Association of Official Agricultural Chemists (now Association of Official Analytical Chemists - AOAC) included several oleophilic extraction techniques. The Association requires that new methods be tested by several independent investigators prior to inclusion in their compendium, thereby assuring adequate and reproducible recoveries. Thus any techniques that are designated as Official are suitable for forensic purposes.

Rigorous "spike" recovery testing of methods for filth in food has shown that astonishing sensitivity is possible. Although the weight variation of insect fragments precludes gravimetric analysis, numerical recovery data for them are impressive, e.g., 70 to 100% recovery of added 2 mm rodent hair fragments from a large (50-200 g) sample of food is expected before a proposed technique is acceptable. Since each such fragment weighs 0.5 to 1 µg, each one recovered represents 0.01 part per million in a 100 g sample of food. Taken in this context, flotations are as sensitive as many chemical tests using modern instrumentation.

Strategies to cope with interference include removal of food lipids with a fat solvent or detergent to minimize food flotation. Similarly solubilization of food matrix by digestion, hydrolysis, or dispersion will remove interfering substances. Fortunately the tanned chitin-sclerotin exoskeleton of insects with its lipoprotein cuticle is highly refractory and resists chemical insult; thus isolation of fragments is possible after drastic treatment.

Preparatory manipulations to aid recovery of arthropod residues described in the 1945 Edition of Official Methods included defatting with chloroform and petroleum ether; use of sodium citrate or a detergent such as sodium oleate, for dispersing food material; digestion with pancreatin to release insects or their fragments in baked goods; and wet sieving to eliminate fine particles. The 11th Edition of Official Methods[6] included many more techniques and refinements.

Different oils adapted to a specific product were introduced when necessary, such as castor oil for flotations from canned tomatoes. Early studies had established that excess aromatic hydrocarbon content in gasoline was likely to cause inadequate recoveries, so it had to be tested for their presence before use. Later n-heptane was substituted for gasoline. The current use of

light mineral oil (U.S.P. laxative type) over hot aqueous phases has reduced flotation time to 2-10 minutes from the half hour or longer required for heptane. Different kerosene-mineral oil mixtures provide tailor-made properties for optimum separation of insects from products which used to severely foul extractions. Although Solomon[7] floated mites out of flour using the density of ethylene dichloride alone, oleophilic separations also adequately recover them. Currently the only density separation designated as Official by the AOAC is for *Curculio* larvae in pecan pieces using 50% isopropanol in water. An unpublished method for insect eggs in flours is under study; it too is a density separation.

Method improvements rapidly replaced the original #100 sieves with #140 and finally #230 sieves. The latter is capable of retaining on its 0.062 mm mesh any hair or insect particles recognizable by microscopic examination at the 30-70X magnification used for food analysis. Although overnight enzymatic digestion of food products minimized floating of food particles in unsieved samples, wet sieving permitted 2% acid hydrolysis which saved considerable time when digestion was necessary.

Sodium citrate used for dispersing cheese into a filterable slurry yielded first to sodium hexametaphosphate and then to sodium or ammonium EDTA, each producing better dispersion in less time. Similarly sodium oleate was replaced by several anionic and nonionic surface active agents which were selected for optimum usefulness in specific analyses. By wet sieving detergent-treated food slurry on a #230 sieve, one could adequately defat some products, thereby replacing costly, toxic, or inflammable organic solvents.

The first preferential suppression of hydrophilic material depended on lead acetate solution, which kept spinach or greens from floating during analysis for aphids. Later a mixture of ethanolic polysorbate-80 and sodium EDTA proved effective in preventing ground nut products from floating with the oil layer.

Originally the trap flask plunger was used to stir in the oil; more recently magnetic stirring improved oil dispersal and reproducibility of results. Efforts to improve recovery by using different aqueous phases kept pace with tests using different oils. Several concentrations of ethanol, isopropanol, and ethanol weighted with calcium chloride are now used. One hundred percent isopropanol is an effective water-soluble defatting agent. This relatively nontoxic and high flash-point solvent may supersede more hazardous materials before long. Acetone is unsuitable for this purpose; as it causes losses of arthropod material.

Even the Wildman trap flask is not immune to replacement. Large percolators used for oil flotations allow clean up of the oil layer by repeated changing of the bottom phase and replacing it with clean liquid. Insects and their fragments adhere tenaciously enough to the interface to allow this to be done without loss.

Extraction of the entomofauna of soils and litter is similar enough to food sanitation analysis that soil zoologists have developed similar techniques to isolate arthropods. Their task is

not as formidable since they usually do not seek fragmentary material. Although their methodology is published more often in entomological journals, much is published elsewhere. Therefore a tendency exists for this work to be overlooked by many of us. After perusing numerous papers dealing with both topics, I concluded that the two disciplines have evolved independently. Scrutiny by stored product entomologists of either literature has been minimal.

Emphasis on ethokinetic methods, which exploit the motility of the animals sought, has been common among pedozoologists ever since Berlese[8] published his milestone paper in 1905. His original side-heated funnel was modified by Tullgren[9] who used overhead illumination to give a more rational sequence of heating and desiccation. Careful study of the various modifications of Tullgren funnels has established that heating and desiccation rates greatly influence completeness of recovery. Attention is directed to Macfadyen[10,11] and Kevan[12] for discussion of optimum conditions. Kempson et al.[13] studied ways of insuring adequate relative humidity in the area of the collecting vessel. Vannier[14] coupled a Tullgren funnel to an automatic fraction collector, and also explored temperature programming. Bouché and Stawiecki[15] devised an ingenious means of using heat or chemical manipulations to drive organisms living in interstitial soil water into collecting vials. Kevan also describes a rather crude ethological method wherein net bags of soil or litter are hung between a light and a funnel with a collecting vial at its bottom.

Simple washing of substrates, with or without concomitant sieving, has been used to isolate thrips from rose flowers[16], or tabanid larvae from soils[17]. Unfortunately washing alone rarely gives clean separations and some form of flotation is required. Desiderata must be isolated by density separation, or entrainment with an oil.

In 1921 Berlese[18] described essentially a Wildman flask in miniature, developed to separate arthropods from clay particles which heavily contaminated some of his funnel residues. It was a constricted test tube with a captive cork in the lower portion. A stiff wire impaling it permitted closing off the bottom chamber thereby created, and selectively decanting material resting above the cork. Brine or glycerin was added until arthropods rose above the construction and could be decanted. Other investigators used dense liquids to float arthropods. Ladell[19] used magnesium sulfate at a sp. gr. of 1.11 in a complex laboratory-fabricated apparatus in which compressed air stirred the soil slurry. Sucrose at a sp. gr. of 1.12 gave longer floating times than $MgSO_4$ solutions of similar density when used by Anderson[20] and Pask and Costa[21]. The latter investigators found 33 -100% recoveries as measured by examining both floating and sinking fractions. Jeanson[22], confronted with clay-residue fouling in Tullgren funnel concentrates, used bromoform-ethanol mixtures to float organisms free. Hale[23] reported that, like food products, organic components of soils float at the 1.1 to 1.3 sp. gr. required for lifting arthropods. He satisfactorily recovered collembola by

boiling slurries at room temperature in vacuo to deaerate humus and litter particles and cause them to settle in $MgSO_4$ solutions. Block[24] tested the Hale technique against sophisticated Tullgren separations. He found that properly designed Tullgren apparatus gave better yields of mites from soils.

As an adjunct to the Ladell technique, Salt and Hollick [25] mixed benzene with the density-flotation concentrate in a flask. Selective wetting of arthropod cuticle concentrated it at the interface. The flask was placed in a beaker and enough aqueous phase was added to displace the arthropod layer into the outer beaker from which it was recovered by filtration. Raw[26] used similar methods, but froze the benzene and removed a plug containing the arthropods. Strickland[27] and Cockbill et al.[28] used kerosene as a flotation oil, and recovered arthropods by letting the vessel overflow. Cockbill used 1.18 sp. gr. brine as an aqueous phase. Davies[29] developed a bizarre technique using a hot gelatin solution which, on cooling, congealed below a kerosene layer, thereby holding vegetable debris while arthropods were removed with the kerosene.

Aucamp and Ryke[30] and Shaw[31] used a lanolin film on the walls of a rotating plastic box to extract arthropods from soil slurries. Shaw included an inner screened cylinder to retain large vegetable particles which interfered with clean separations. Speight[32] described a petrolatum coated bolting cloth belt which travelled through a soil slurry. High speed water jets then dislodged most insects and concentrated them on a fine screen. After the extraction was completed, the belt was washed with solvent to recover any remaining adhering animalcules.

An important caveat: Different extraction methods give different recoveries of different taxa. Similarly, different substrates cause variation. All these methods are physical separations, and the various environmental and experimental influences on them have not been determined. Also, any technique usually works best in the originator's laboratory. With Tullgren funnel separations, some sources of loss include heat coma onset, or death by desiccation, before animals can traverse the bulk of the sample, induction of dormancy, and sticking to the condensed moisture of funnel walls. Macfadyen[33] compared Salt and Hollick's flotation with funnel methods. He reported that mites were not adequately recovered by flotation and that the ethological technique was much faster. Satchell and Nelson[34], testing Raw's modification against a Tullgren funnel, showed no significant difference in clay soils, and found superior recoveries of mites from organic soils by the oleophilic flotation method.

Since flotations recover dead and fragmentary arthropods plus the living denizens of a substrate, their use provides additional information. While this may be overwhelming to someone doing a population study, a person looking for evidence of cantheriasis in an infant's stools might appreciate the added detail.

Several other techniques deserve at least passing mention. Nicholson et al.[35] used X-rays to find internal infestation

in wheat; Mills and Wilbur[36] used radiography to study the life history of *Sitotroga cerealella*. Stein et al. [37] used staining to differentiate animal residues from vegetable debris also extracted. Although helpful, it did not supplant recognition of arthropod residues by their morphology. Street[38] used sensitive microphones to monitor insect activity inside grain kernels. Since it is totally passive, this technique is useful for ecological studies. Atmar et al. [39] explored computer-based pattern recognition for automated counting of different insect species in large samples. This idea was suitable for making distinctions at the "common name" level.

The obvious question of how to identify isolated fragments can be largely answered by stressing the importance of comparison with known reference material. While some literature concerning the food sanitation field exists[40] [41] [42], most workers will have to establish their own needs and concentrate on heavily sclerotized components having good diagnostic features. Whitaker's [43] comments stressing identifiability of even the finely masticated fragments found in bat dung are both inspiring and instructive. Although Voth and Black[44] studied plant fragments, their article on mountain beaver food habits serves as a model for similar ways to quantitate arthropod residues.

In conclusion, it is hoped that this discussion of the general realm of entomological detective work has provided at least one item useful to other researchers.

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