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TECHNICAL NOTE

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Sub-sampling and Preparing Forensic Samples for Pollen Analysis*

ABSTRACT: The main forensic application of palynology is in providing associative evidence, assisting to prove or disprove a link between people and objects with places or with other people. Although identification and interpretation of pollen is a specialist job, sub-sampling and preparing pollen samples for analysis may be carried out by non-specialists. As few forensic laboratories have residing palynologists, laboratories may wish to reduce the cost of analysis or risk of contamination by doing their own sub-sampling and preparation. Presented is a practical guide for sub-sampling and preparing forensic samples for pollen analysis, providing a complete standard procedure for both the palynologist and non-specialist. Procedures for sub-sampling include a wide variety of materials commonly collected for forensic analysis (soil, clothing and other fabrics, footwear, twine and rope, firearms, granulated materials, plant and animal material, and illicit drugs), many of which palynologists will not be familiar with. Procedures for preparation of samples (pollen concentration) are presented as a detailed, step-by-step method. Minimizing the risks of laboratory and cross-sample contamination during sub-sampling and preparation is emphasized.

KEYWORDS: forensic science, forensic palynology, soil, clothing and other fabrics, footwear, twine and rope, firearms, granulated materials, plant and animal material, illicit drugs

Forensic palynology is the science of deriving evidence for court purposes from pollen (by convention, spores of plants such as ferns are included in the term "pollen"). Details of the theory of forensic palynology and case examples are given in Mildenhall (1–3), Bryant et al. (4) Stanley (5,6), Horrocks et al. (7–9), Bryant and Mildenhall (10), Horrocks and Walsh (11–14), and Brown et al. (15). Its main forensic application is in providing associative evidence, that is, assisting to prove or disprove a link between people and objects with places or with other people. For example, soil on a suspect's shoes or clothing can be analyzed for pollen and compared with control soil samples from the crime scene.

Materials and surfaces contain pollen that may be collected and analyzed. Wind-pollinated plants generally produce abundant pollen which may be dispersed long distances (up to 100's of km), whereas insect-pollinated plants produce much smaller amounts of pollen, most of which is deposited on the ground within a few meters of the parent plant. Spores from non-flowering plants also vary in dispersal distance. The difference between species' pollen production and dispersal results in pollen representations that may change considerably over just a few meters (12).

Identification and interpretation of pollen is a job for the specialist: the palynologist. However, sub-sampling and preparation of samples for analysis may be carried out by non-specialists (i.e., laboratory technicians). Preparation involves treating samples with a series of corrosive chemicals that removes almost everything except pollen and mounting the pollen concentrates on microscope slides. As few forensic laboratories have residing palynologists, laboratories may wish to reduce the cost of analysis by doing their own sub-sampling and preparation. Preparation in particular may take as long as the actual analysis. Also, because the risk of contamination is present until pollen samples are mounted on slides forensic laboratories may wish to confine preparation to their own premises.

Presented here is a practical guide for sub-sampling and preparing forensic samples for pollen analysis, with the intention of providing a complete standard procedure for both the palynologist and technician. Included are procedures for sub-sampling different types of materials, most of which are commonly collected for forensic analysis. Apart from soil, most palynologists will not be familiar with handling these materials. Also included is a step-by-step method for concentrating pollen in the samples.

Pollen Sub-sampling of Materials Collected for Forensic Analysis

Listed below are a variety of different materials, most of which are commonly collected for forensic analysis, that can be sub-sampled for pollen. However, note that if present pollen can be collected from virtually any material or surface using the procedures outlined below. Note also that pollen analysis is destructive, rendering samples unfit for any type of subsequent analysis, so should be carried out after non-destructive types of forensic analysis and only if other types of analysis are not going to be carried out.

Pollen on dry surfaces can be picked up and carried long distances by air currents, potentially contaminating forensic samples. Optimal laboratory conditions for pollen sub-sampling (and preparation) would be in the semi-sealed, air-filtered environment

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of air-conditioned buildings. If this is unrealistic, as is often the case, windows and doors should, if possible, be kept closed during sub-sampling (and preparation) to reduce the entry of airborne pollen from outside, and to reduce drafts that can pick up pollen already settled on surfaces inside.

To minimize the risk of cross-sample contamination during sub-sampling, open, sub-sample and reseal each sample bag one at a time. Consider the origin of the samples and decide on an optimal sub-sampling sequence. For example, all samples from the control scene should be sub-sampled, and then all from the suspect should be sub-sampled, or vice versa. Use a new pair of disposable, powder-free rubber gloves for each sample.

To obtain an approximation of airborne laboratory pollen contamination and sample cross-contamination during the pollen sub-sampling procedure, apply a thin layer of silicone oil to 2 microscope slides and place, oiled side up, near the samples for the duration of the procedure, commencing when sample bags are first opened. (Move the slides into the fume hood with the sub-samples for preparation.) When finished, place cover slips on oiled surfaces. The slides can then be scanned by the palynologist, for pollen that has settled on them during the procedures.

A. Soil

Soil includes silt, sand and clay, and mixtures of these. For bulk samples place 2–3 cm^3 in a 10 mL test tube. If low pollen concentrations are suspected (e.g., sand or sandy soil), use more material. Some soil samples, such as those scraped off items, may be much smaller in volume but will almost always contain pollen. Proceed from Step 1, Method for Preparing Forensic Pollen Samples for Analysis, below. For soil stains on clothing and other fabrics, footwear, and twine and rope, refer to B, C, and D, respectively.

B. Clothing and Other Fabrics

This category includes any woven materials such as clothes, bedding, curtains, wrappings, sacking, bags and baskets. Items made of animal hides, such as leather, suede and fur clothing, are also included (for other animal material, see I). Soil deposits can be scraped off and treated as for A. If soil deposits are insufficient but soil staining is apparent, cut out the stained areas and place in a glass beaker. Note that grass stains usually also contain pollen. Add 10% potassium hydroxide solution to cover. The less solution used, the less reducing by centrifuging required to concentrate to one 10 mL test tube at the end of this step. Warm (c. 70°C) for 10–15 min, periodically stirring. Dye may run from the item and discolor the potassium hydroxide solution but this does not adversely affect the procedure or subsequent analysis. Remove item and pour solution into a 10 mL test tube. If more than one tube, reduce down to one by centrifuging (3000 rpm for 3 min). Proceed from the end of Step 1, Method for Preparing Forensic Pollen Samples for Analysis, below (add distilled water to wash, stir, centrifuge and decant).

For large unstained items (e.g., plants may be brushed against and deposit pollen without leaving obvious signs), use a suitably sized stainless steel container (cooking pots are ideal). Immerse in 10% potassium hydroxide solution and warm (c. 70°C) for 20–30 min, periodically stirring. Lift out and wring into the pot. Pour potassium hydroxide solution through a 130–150 μm sieve to remove large particles of extraneous matter. Because there will be a large amount of potassium hydroxide solution do not reduce in test tubes, rather, pour through a 5 μm sieve to collect the pollen on top of the sieve (few pollen types are smaller than this). This may take considerable time, especially if fibers or debris loosened from the item are clogging the sieve, so consider using a device that can be left alone (e.g., a large volume sieve or a venturi to exert suction). Invert the sieve and wash off the pollen with as little distilled water as possible into a suitably sized container. Pour into test tube(s), centrifuge (3000 rpm for 3 min) and decant. If more than one tube, reduce down to one by centrifuging. Proceed from the end of Step 1, Method for Preparing Forensic Pollen Samples for Analysis, below (add distilled water to wash, stir, centrifuge and decant).

C. Footwear

Treads on soles of footwear often contain soil, which is easily removed with a dental pick, or similar tool. For leather, synthetic leather or rubber uppers soil deposits can be scraped off or washed off with warm (c. 70°C) 10% potassium hydroxide solution and treated as for A. If soil deposits are insufficient but soil or grass staining is apparent, cut out the stained areas and place in a glass beaker, as in B.

For uppers constructed of fabric, such as sports shoes, refer to B. If there are no obvious signs of staining, whole items can be immersed in potassium hydroxide solution in a large container, as in B.

D. Twine and Rope

Soil deposits on twine and rope can be scraped off and treated as for A. If soil deposits are insufficient but soil or grass staining is apparent, items can be cut to desired length and treated as for B. If there are no obvious signs of staining, whole items can be coiled and immersed in warm (c. 70°C) 10% potassium hydroxide solution in a large container, as in B.

E. Air Filters

Air filters are highly effective pollen concentrators. Although made from a variety of materials, including paper and foam rubber, they can generally be treated in the same manner. Immerse the whole or part of the filter in warm (c. 70°C) 10% potassium hydroxide solution in a large container, as in B.

F. Firearms and Tools

Soil deposits on firearms and tools can be scraped off and treated as for A. If there are no obvious soil deposits, whole items can be treated with potassium hydroxide solution in a large container, as in B. Stand oversize items, such as rifles, vertically in the container and wash down with a brush and warm (c. 70°C) 10% potassium hydroxide solution.

G. Powders, Crystals and Other Granulated Materials

Powders, crystals and similar materials, including illicit drugs in this form (e.g., heroin, cocaine), often contain pollen although often in low concentrations. Place 6–7 cm^3 of material into a test tube and proceed from Step 1, Method for Preparing Forensic Pollen Samples for Analysis, below. Steps 2–5 should not be necessary.

H. Plant Material

This category includes unprocessed plant material, and processed food and other material such as honey and tobacco. The above

ground parts of many species of plants (i.e., stems, leaves and inflorescences) trap the pollen of other species due to such characteristics as hairiness and stickiness. In the case of cannabis (*Cannabis sativa*), unprocessed inflorescences ("buds") or leaves/stems ("cabbage") and processed material (e.g., hash oil and buddha sticks) will contain abundant non-cannabis pollen if grown outdoors.

If plant material is abundant, select parts most likely to contain trapped pollen, such as leaves with dense fine hairs or sticky excretions. Cut up the plant material if necessary and place into a 10 mL test tube, filling no more than half full (or place into larger container, as in B) and proceed from Step 1, Method for Preparing Forensic Pollen Samples for Analysis, below. Steps 2–5 should not be necessary.

I. Human and Other Animal Material

This category includes hair, fur, feathers, mucus, stomach contents and fecal material. Soil-matted hair, fur or feathers can be cut into short lengths and treated as for A. For hair, fur or feathers with no obvious signs of soiling immerse in potassium hydroxide solution in a large container, as in B. For mucus, stomach contents and fecal material, place in a 10 mL test tube and proceed from Step 1, Method for Preparing Forensic Pollen Samples for Analysis, below. Steps 2–5 should not be necessary.

Method for Preparing Forensic Pollen Samples for Analysis

For trace samples, Bryant et al. (4) recommended that to reduce the risk of losing pollen in standard-size 10 mL test tubes, preparation should be carried out in very small tubes (1–5 mL) or in the depression of a hanging-drop glass slide. Because handling and some of the chemicals used (e.g., glacial acetic acid) remove ink, labels should be scratched onto the side of the tubes with a sharp metal point. The scratch marks can then be inked. Samples can be heated in water in a glass beaker on a hot plate, with beaker size determined by the number of tubes, which should be kept more or less upright (>45°). A swing-out rather than fixed-angle centrifuge is required otherwise the pellet is deposited at an angle up the side of the test tube, rather than all at the base. All acid work should be carried out in a fume hood with extractor fan.

Risks of sample cross-contamination during preparation can be minimized by using disposable:

- plastic test tubes, with permanently attached push-on/pull-off caps to prevent accidental cap mixup.
- plastic pipettes, replaced for each step for each sample.
- plastic stirring rods; plastic cocktail stirrers and plastic toothpicks are inexpensive and can be snapped to the desired length (avoid plant material such as wooden or bamboo cooking skewers and toothpicks in case these are contaminated with pollen).
- sieves; small squares of steel mesh cut to size are inexpensive and hold their shape (unlike nylon mesh) when indented. These may be reused for successive samples, but must be cleaned and decontaminated between samples by ultimately passing them through a flame until red-hot, in order to destroy pollen and spores. Indenting and heating steel mesh may change the pore size, but this will not adversely affect the procedure.

The following is based on the standard acetylation method (16). Add excess amounts of reagents to test tubes, so tubes are about half to two-thirds full, unless otherwise stated. Centrifuge at 3000 rpm for 3 min, unless otherwise stated. Dispose of the decanted fraction.

1. Potassium Hydroxide Digestion

This step breaks up the matrix (deflocculation) and dissolves humic materials, producing a dark brown solution. Place material into 10 mL test tube, add 10% potassium hydroxide and warm (c. 70°C) for 10–15 min, stirring occasionally. Then pour through a 130–150 μm sieve, resting on a 50 mL glass beaker, to remove large particles. Squirt distilled water through the sieve to wash in pollen trapped within the larger particles, ensuring that the sample volume does not exceed 10 mL. If necessary, break up larger particles with a stirring rod. Centrifuge and decant. Add distilled water to wash, stir, centrifuge and decant. Repeat wash if supernatant is not more or less clear.

2. Sodium Pyrophosphate Treatment

This step is usually required only for soil samples, removing clay (fine mineral particles, mainly silica). High concentrations of clay on prepared slides cause cloudiness, which obscures pollen identification. Add a 0.1 M solution of sodium pyrophosphate, stir and place in boiling (c.100°C) water bath for 10–20 min (17). Centrifuge for 5 min and decant. Repeat twice. Add distilled water, stir, centrifuge and decant.

3. Hydrochloric Acid Treatment

This step is required only if Step 4 is going to be carried out, and removes carbonates which can cause a reaction with the reagent used in that step potentially dangerous to the user. Add 10% hydrochloric acid until reaction (fizzing) ceases. Stir, centrifuge and decant. Add distilled water, stir, centrifuge and decant.

4. Hydrofluoric Acid Treatment

This step is usually required only for soil samples, and removes large silica particles. Treat with 40–80% hydrofluoric acid. The use of this highly corrosive reagent requires rigorous safety controls (e.g., see Muriale et al. (18)). Wear full visor, thick rubber gloves and boots, and protective apron. Do not use glass utensils as hydrofluoric acid corrodes glass. Add acid and place in boiling (100°C) water bath for 15–20 min, stirring occasionally. As silica is removed, the solution turns a greenish brown color. Centrifuge and decant. Add distilled water, stir, centrifuge and decant. This step may have to be repeated for samples with high concentrations of large silica particles.

5. Oxidation

To remove organic material including lignin, add oxidizing (bleaching) solution and leave for 1.5–2.0 h, stirring occasionally. Centrifuge and decant. To make 500 mL of solution (method used at Botany Division, DSIR, New Zealand), which will keep indefinitely, add 280 mL glacial acetic acid to 157 mL distilled water. Then add 75.5 g sodium chlorate. To this, add slowly and cautiously, 35 mL concentrated sulfuric acid. Stir until sodium chlorate is dissolved. Oxidation will also clear pollen that has become darkened.

6. Acetolysis

Acetolysis removes cellulose, a common plant polysaccharide. The reagents react vigorously with water so materials with which they come into contact must be dry. To dehydrate the sample, add glacial acetic acid, stir, centrifuge and decant. Then with test tubes

facing towards the back of the fume hood, add a 9:1 ratio of acetic anhydride and concentrated sulfuric acid. The solution must have been made up the same day. Place in boiling (100°C) water bath for 4.0–4.5 min. Avoid less than 4 min; longer than 4.5 min will darken pollen grains, making identification difficult. Centrifuge and decant. Add glacial acetic acid, stir, centrifuge and decant. Add distilled water, stir, centrifuge and decant. At this final stage of the concentration, if not before, samples that were initially very small, such as those comprising a few crumbs of soil may have been reduced to the point where they appear to the naked eye to have disappeared completely. However, usually there will be some pollen grains in such samples, often seen as a faint discoloration on the base of the tube after centrifuging if held up to the light. Pollen grains in this type of sample may also be seen with magnification (hand lens or stereomicroscope) as tiny specks when shaken and held up to the light. In these cases, take extra care when decanting after centrifuging.

7. Staining

For staining to make pollen grains more conspicuous, add a drop of 0.1% basic fuchsin solution. This concentration is usually adequate and should avoid overstaining, although samples with high concentrations of microscopic charcoal fragments, sometimes found in soil, may require a stronger solution (0.2%). To make 100 mL of 0.1% solution, add 0.1 g of basic fuchsin crystals to 100 mL of distilled water (add 0.2 g to 100 mL water for 0.2% solution). Leave for a few minutes, stirring or shaking vigorously several times, then pour through a 130–150 μm sieve to remove undissolved crystals.

8. Mounting

Mount slides for microscopy in glycerol jelly. Place a few cm^3 of jelly into a test tube in a warm water bath to melt. Place a drop of melted jelly onto a microscope slide. Then place a drop of the concentrated pollen sample onto the jelly and mix with a plastic stirring rod. Place slide on a hotplate to reduce the volume slightly, then affix a 22×40 mm cover slip. View for pollen and spores, which should appear shades of red, under 100× magnification. (One of the many illustrated pollen keys available, e.g., Moore et al. (16), will aid the non-specialist in differentiating pollen and spores from other material on slides.) Most of any extraneous material remaining will not have taken up the stain. Air bubbles under the cover slip will not affect analysis. If pollen concentration on the slide is very low (<1 grain per field of view at 100×), place more than one drop of sample on the slide and reduce volume by heating. The extent to which this can be done will be determined by the amount of extraneous material in the prepared sample, which will obscure pollen grains if too highly concentrated. If concentration is too high (abundant pollen grains or extraneous material obscuring grains) press down on the cover slip to squeeze out some of the material, or dilute the pollen concentrate with distilled water and make up another slide. More than one slide per sample may have to be made up, as palynologists usually count at least 100 pollen grains per sample, and may wish to scan slides looking for particular or rare pollen types. The glycerol jelly sets in a few minutes, and can be remelted if necessary. The edges of the cover slip do not need to be sealed and it does not matter if the set material extends beyond the edges. Note that glycerolielly is more suitable for temperate rather than tropical climates. For the latter, consider mounting in silicone oil (16).

9. Storage of Prepared Slides and Pollen Residues

To prevent gravitational pollen movement on prepared slides store horizontally in trays. House boxes on edge to ensure that slides are horizontal. Store away from direct sunlight as this may melt the glycerol jelly. Slides of pollen mounted in glycerol jelly can be stored for extended periods; however, grains usually deteriorate and swell after perhaps a decade or so. Pollen concentrates remaining after slides have been made up should be stored at 2–5°C for possible subsequent use. These can remain in their original test tubes with caps firmly on.

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