METHODS OF MELISSOPALYNOLOGY*

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An earlier edition of Methods of melissopalynology *was published in* Bee World 51(3): 125-138 (1970), *and has been widely used. It is now republished with minor corrections and updating, and with two significant additions. The acetolysis method is included, which has not previously been commonly used in melissopalynology; also the literature list is enlarged so that it provides an introduction to the extensive literature on palynology, which is scattered over many journals.*

1. SCOPE OF MICROSCOPICAL ANALYSIS

Microscopical analysis provides information:

1.1 about the geographical origin of honey,

1.2 about the botanical origin of honey.

Microscopical analysis allows additional statements:

1.3 about any contamination of honey with brood, dust, soot, etc.; about the yeast content (fermentation); and about other microscopic particles not usually present in honey.

2. FIELD OF APPLICATION

2.1 In theory, the geographical origin can be determined of any honey whose pollen has not been completely removed by filtering.

2.2 Determination of botanical origin is possible, provided that the honey was extracted centrifugally. The pollen spectra of pressed honey or heather *(Calluna)* honey extracted by a "loosening" device, and of honey which has been filtered through diatomaceous earth or a similar material, are changed by the admixture or removal of pollen. The botanical origin of such honeys cannot be determined.

With some samples, it may be necessary to measure the amount of sediment, and to determine the absolute number of plant constituents, in order to establish whether or not the honey has been extracted centrifugally (see **4).**

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* *Bee World* is now the official organ of ICBB (see page 13, 1977), and a financial contribution has made it possible to enlarge the present issue of *Bee World* to include the revised *Methods **of** melissopalynology.* Any communications to the authors may be sent to Dr. G. Vorwohl, Landesanstalt f*ür B*ienenkunde, Universität Hohenheim, August von Hartmann-Str. 13, 7000 Stuttart 70, German Federal Republic.

3. QUALITATIVE MICROSCOPICAL ANALYSIS

3.1 Principle

The microscopic elements are concentrated by centrifuging the honey dissolved in water, and examining the sediment and evaluating it under the microscope, either after acetolysis or without further treatment.

3.2 Reagents

These are:

Kaiser's glycerine gelatine
concentrated sulphuric acid (chemically pure)
acetic anhydride (chemically pure)
glycerine
glacial acetic acid (chemically pure).

3.3 Apparatus

This comprises:

Laboratory centrifuge 2500-3000 rpm
centrifuge tubes with conical ends, about 100, 50 and 10 ml capacity
microscope, magnification 320-450 X and 800-1000 X.
Water-operated vacuum pump.

3.4 Samples

3.41 A *laboratory sample* should consist of 100-200 g honey.

3.42 Transform the laboratory sample into the *test sample* by thorough stirring. If the sample is granulated hard, soften it by slight warming.

If the samp1e is dirty, liquefy at 40°C and strain through cheese-cloth or a fine sieve.

Comb honeys are carefully uncapped, and viewed against a strong source of light to identify cells which do not contain pollen. Withdraw honey from these cells only, using a pipette connected to a pump.

3.5 Procedure

3.51 *Preparation of slides*

An artificial fossilization of microscopic elements in honey by acetolysis seems unreasonable since the pollen grains in honey are recently produced and well preserved in the honey. Acetolysis destroys fungal hyphae, yeasts, algae, some thin-walled pollen grains and other particles which may be useful for evauating honey (see 1.3). But since most pollen descriptions in palynological literature are based on acetolysed material, the acetolysis technique may be helpful in special cases of microscopical examination of honey as a complementary method in addition to the routine technique without fossilization.

3.511 *Preparation of slides without acetolysis*

Weigh out 10.0 g of honey and dissolve it in 20 ml of hot water (distilled water or clean tap water) not above 40°C. Centrifuge the solution for 10 min (about 2500 r/min) and decant or draw off the supernatant liquid. The honey sugars will be more completely removed if the sediment is dispersed again with about 10 ml of distilled water. Pour the solution into a small tube, and centrifuge for 5 min. Put the entire sediment on a slide and spread it out over an area about 20 X 20 mm, using a thin glass or platinum rod. After drying, preferably by slight heating (not above 40°C), mount the sediment with glycerine gelatine, liquefied by heating in a water-bath at 40°C. Alternatively, mount the sediment with stained glycerine gelatine (see 5.2). The use of Pasteur-pipettes is also recommended for transferring the sediment from the centrifuge tube to the slide, the capillary end having been dosed by melting.

The pipette may be used to disperse the sediment; the point is then cut off, and the dispersed sediment drawn off and blown out on to the slide. Handle the pipette with care, to prevent the point splintering. Stir the sediment constituents remaining in the tube with a drop of distilled water and pipette again, then reject the pipette. The transfer of the sediment is almost quantitative, and contamination (e.g. of pollens from other honeys) is prevented since the pipette is used only once. If the honey sample is poor in pollen 20 g may be used (Vergeron, 1964); if it is rich in sediment, spread the residuum under two coverglasses. For honeys rich in colloidal matter dissolve the honey in dilute sulphuric acid or dilute potassium hydroxide instead of water (5 g H_2SO_4 or 100 **g** KOH to 1 litre of water). Most of the colloidal material is dissolved and remains in the supernatant fluid during centrifugation. Wash the sediment twice with distilled water to remove any remaining chemicals. Otherwise handle the sediment as described above. Another way to eliminate colloidal matter and small insoluble particles, which complicate examination of the pollen, is to fi1ter the suspended sediment through a Millipore fi1ter with pores from 3 to 5 p.m. The pollen grains remain on the filter; wash them out, centrifuge, and mount as described above. The filtration technique is described 4.25.

3.512 *Preparation of slides by acetolysis*

Based on the experience of A. Pons and Claude Gadbin, Marseille, J. Louveaux, Bures-sur- Yvette, and G. C. Ricciardelli D'Albore, Perugia, we recommend the following procedure. Dissolve 30 g of honey with 50 ml of hot water (distilled water or clean tap water) not above 40°C. Centrifuge the solution in equal parts in two tubes (50 ml capacity) for 10 min at 2500 r/min. Draw off the supernatant liquid with a fine pipette connected by a flexible tube to a filter pump. Not more than 1 ml should be left in the tubes. Disperse the sediment again and transfer it with several portions of water into two smaller centrifuge tubes (10-20 ml capacity). Centrifuge (5 min) and draw off the liquid phase. Place one of the tubes upside down on filter paper. The sediment should be as dry as possible. Set aside the second tube. Prepare 10 ml acetolysis mixture by adding 1 ml of sulphuric acid to 9 ml of acetic anhydride. All glassware must be absolutely dry. Add a drop of the acetolysis mixture to the sediment. If there is little water left, the reaction will not be too violent. Redisperse the sediment with a thin glass rod and add the rest of the mixture. Place the tube in a 70°C water- bath for 10 min. Take care to prevent contact between the water and the acetolysis

mixture. Centrifuge the tube after incubation for 5 *min.* Decant carefully into a dry beaker; the acetolysed sediment settles less tightly than an untreated one. Fill the centrifuge tube with distilled water and add a drop of a strong detergent (e.g. Teepol), shake vigorously, and centrifuge again (5 min). Draw off the supernatant liquid. If pollen grains adhere to the tube walls, shake again with distilled water and detergent and centrifuge at high speed (3500 r/min) for 5 min. Put the entire sediment on a slide and spread it out over an area about 20 X 20 mm. The addition of a drop of glycerine water mixture (1:1) to the sediment prevents the formation of air bubbles. Louveaux recommends the use of Pasteur-pipettes. Add a drop of liquefied glycerine gelatine (40°C) to the sediment and stir the mixture with the melted end of the pipette until it is homogenous. Cut the point off, draw the mixture up and blow it slowly on to the slide. On a hotplate (40-45°C) the surplus water will soon evaporate. A very small drop of glycerine gelatine in the centre of the cover glass prevents the enclosure of air bubbles when the cover glass is placed on the sediment-glycerine-gelatine mixture.

If there is little sediment in a honey sample, the pollen grains in the second tube which was set aside should be acetolysed and united with the sediment of the first tube. If there are sufficient pollen grains in one tube (15 g of honey) use the sediment. of the second tube for a slide as described under 3.5ll, or add it to the acetolysed sediment of the first tube and mount a mixed slide.

3.52 *Performance of microscopical examination*

The determination of geographical origin is based on the identification and counting of pollen grains and other particles in honey. Identification is made by reference to the literature and to comparative preparations (see 5).

3.52I The analysis may be either *orienting* or *complete.* An orienting analysis is limited to the identification of the most frequently occurring particles and to a search for certain significant characteristic elements in the sediment.

3.522 Complete analysis involves identifying, as far as possible, all pollen grains or other microscopic constituents in the sediment. Three degrees of accuracy in counting microscopic elements in the sediment are distinguished:

3.523 *Estimate:* Count 100 pollen grains and honeydew elements corresponding to 100 grains.

3.524 *Determination of frequency classes:* Count 200-300 pollen grains and the corresponding honeydew elements. If the pollen is from only a few species, it is sufficient to count 200 grains.

3.525 *Counts expressed in percentages:* Presentation of frequencies as percentages is permissible only if 1200 pollen grains are counted. Make counts on two slides, prepared independently, from the same honey.

3.526 In honeydew honeys poor in pollen grains, count 50, 100-150 or 600 grains, according to the degree of accuracy required.

3.527 Note separately any pollens of wind-pollinated or nectarless plants. Important wind-pollinated plants in this context are: Gramineae (wild grasses, cereals, maize), Cyperaceae (sedges), *Rumex* spp. (sorrel), *Cannabis* (hemp), *Quercus* (oaks), *Amaranthus* (pigweed). Pollens of conifers *(Abies, Pinus, Picea, Juniperus, Larix,*

Betula spp. (birch), *Fagus* spp (beech), *Carpinus* spp. (hornbean), *Populus* spp. (poplar), *Alnus* spp. (alder), and *Corylus* spp. (hazel), are not important in honeys, nor are pollens of *Urtica* spp. (nettles), *Typha* spp. (reedmace) *or Juncaceae* (rushes). The following plants are known to be nectar less but more or less entomophilous: *Papaver* spp. (poppies), *Plantago* spp. (plaintains), *Thalictrum* spp. (meadow-rues), *Chenopodiaceae* (goosefoot family), *Ambrosia* spp. (ragweeds), *Artemisia* spp. (worm- woods). The status of Cistaceae and *Filipendula* spp., which in general produce little or no nectar, is doubtful.

3.528 Count abortive and misshapen pollen grains so far as they can be identified,

3.529 Honeydew elements (HDE) consist of fungal spores and hyphae especially of sooty moulds), algae, and wax elements of honeydew-producing insects. A multi-cellular hypha or complexes or spores or algae are counted as one e1ement. Do not count wax elements. Because they are fragile, their number can be increased by breakage. Also, since their relative density is low, they do not necessarily settle in the sediment.

3.529.1 Plant-pathogenic fungal elements – e.g. spores of Uredinaceae (rusts), Ustilaginaceae (smuts) and Peronosporaceae – are noted separately from honeydew elements if they are present in appreciable quantities (Maurizio, 1959). Sometimes they are collected by bees with nectar, or occasionally bees pack them into pellets like pollen. They may also contaminate honeydew as a sediment from the air.

3.529.2 Finely granulated and microcrystalline matter often indicates honeydew, but it is also found in some flower honeys and should be noted under "other constituents of the sediment".

3.6 Expression of results

3.61 Many pollen grains cannot be identified as far as the genus or species, and such names should be used only if they have been reliably determined. Otherwise, a note should be added after the scientific name, to indicate that the term is used in a wider meaning, e.g. *Trifolium repens* s.l. *(sensu lato), or Trifolium repens* group (pollens which are identical with or very similar to *T. repens* in shape and morphological characteristics, but which may belong to another species e.g. *T. hybridum, T. resupinatum, T. arvense).* If detailed knowledge is not available, or if lack of time does not allow a closer identification, associate the pollens in larger groups (forms or types), e.g. *Teucrium-form* (a 3-colpate Labiatae pollen grain with opercula), or *Symphytum-type* (stephano- colporate Boraginaceae pollen grain); see Maurizio and Louveaux, 1967.

3.62 *Presentation of frequencies*

3.621 The following terms are used in estimates of pollen grain frequencies:
"very frequent" for grains constituting more than 45% of the total;
"frequent" for grains constituting 16-45% of the total;
"rare" for grains constituting 3-15% of the total;
"sporadic" for grains constituting less than 3%.

3.622 The following terms are used for frequency classes:
"predominant pollen" (more than 45% of the pollen grains counted);
"secondary pollen" (16-45%);
"important minor pollen" (3-15%);
“minor pollen" (less than 3%).

3.623 If 1200 or more pollen grains are counted the frequencies can be expressed in percentages with an accuracy of 1%**,** but the use of any digits after the decimal point is not justified. Pollens whose frequency is 1%, or less should be quoted as "present"

3.624 If the frequency of honeydew elements (HDE) is determined (see 3.529), the following terms are used (P = total frequency of pollen grains from nectar-production plants):

practically none	HDE/P	0.00-0.09
few	HDE/P	0.10-1. 49
medium quantity	HDE/P	1.50-2.99
numerous	HDE/P	3.00-4.49
very numerous	HDE/P	>4.50

3.625 The following terms are used in estimates of the frequency of pollen grains of anemophilous and other nectar less plants:

"sporadic" less than 3% of the total);
"rare" 3-15%
"frequent" 16-45%;
"Very frequent" more than 45%

Subtract the number of pollen grains of nectar less plants from the total number before calculating the frequencies of pollens of nectar-producing plants.

3.7 Interpretation

3.71 Geographical origin

Occasionally geographical origin can be established by the presence of characteristic pollens that are limited to a certain region. More often, the region in which the honey was produced can be determined from the presence of certain pollen combinations (honey types); details can be found in the literature. The pollen spectrum of a honey depends on the floral agricultural and forest conditions where it is produced. Political or administrative frontiers are rarely associated with sudden changes of these conditions, and microscopical data indicate the geographical provenance of honey rather than its country of origin.

3.72 *Botanical origin*

The extent to which a given honey sample is derived from different plant sources *can* be deduced from the frequencies of the pollens and honeydew elements in it. In general, honey has been produced mainly from one plant (unifloral honey) if the pollen of that plant is predominant: pollens of anemophilous and nectar less plants are excluded when calculating the percentages. This rule is valid only if the honey contains few honeydew elements (HDE/P < 1). Honeys produced mainly from honeydew contain many honeydew elements (in general HDE/P > 3). The percentage of pollens from nectarless plants is usually higher in honeydew honeys than in flower honeys.

3.721 *Special cases*

The pollen grains of some flowers are over-represented, i.e. the percentage of poller. in the sediment is greater than the percentage of the corresponding nectar in the honey With some other pollens the situation is reversed; they are under-represented.

3.721.1 *Over-represented pollens*
The most extreme case of over-represenration of pollen known is *Myosotis* spp. (forget-me-not). If a honey sediment is rich in *Myosotis* pollen therefore, make a second count excluding *Myosotis.* The pollen of *Castanea sativa* is also likely to be over-represented, and only honeys containing 90% or more of pollen from *Castanea* can be regarded as *Castanea* honey. In sediments with a high percentage of *Castanea* pollen make a second count excluding *Castanea. Cynoglossum* and *Mimosa pudica* pollens are also thought to be over-represented. In general, honeys from flowers with over-represented pollens have a higher absolute pollen content than honeys from sources with normally or under-represented pollens. In doubtful cases it is best to count the absolute number of microscopic particles (see 4).

3.721.2 *Under-represented pollens*
The most important pollens known to be under-represented are listed below. If the frequency of any of these pollens is as high as the percentage quoted, the honey is mainly from that source.

Citrus	10-20%
Lavandula spica X *L. latifolia* (lavandin)	10-20%
Rosmarinus	10-20%
Salvia (European)	10-20%
Robinia	20-30%
Tilia	20-30%
Medicago	20-30%

Epilobium and Cucurbitaceae pollens are also under-represented.

In general, honeys from flowers with under-represented pollens show a low absolute number of pollen grains. In doubtful cases a quantitative evaluation should be made (see 4).

3.721.3 Certain anomalies in representation must be taken into account: plants which produce extrafloral nectar; dioecious plants (whose female individuals do not produce pollen); plants which do not disperse their pollen, e.g. many Orchidaceae and Asclepiadaceae which produce pollinia comprising all the pollen grains from a theca.

3.721.4 The botanical origin of honeys which contain a high percentage of unknown pollen grains must be treated with reserve, because the degree of representation of these pollen grains will be unknown.

3.721.5 Some unifloral honeys have specific chemical or physical properties, which may be used to confirm the results of microscopical analysis. For instance the thixotropy and protein content of *Calluna* (heather) honey can be measured by simple methods; *Robinia* and tupelo honeys contain much fructose; honeydew honeys have a high electrical conductivity. Some honeydew honeys contain much melezitose. Kirkwood et al. (1950, 1961) and Cowan and Mitchell (1964) characterized honeydew honeys by a discriminant function based on the pH value and the percentages of ash and reducing sugars.

3.8 Reproducibility

Vergeron (1964) published a paper on the repeatability of pollen grain counts these findings were taken into account in compiling the rules for counting described in 3.52 and 3.62.

The reproducibility of counts from preparations of the same honey in different laboratories showed a good conformity. A systematic "ring-analysis" has not yet been carried out. However, the counting and identification of pollen grains depend greatly on the experience and performance of the operator, and a ring-analysis might well give more information about the competence of the various analysts than about the reproducibility of the method used.

4. QUANTITATIVE MICROSCOPICAL ANALYSIS

4.1 Determination of the amount of sediment in honey

4.11 *Scope*

This determination is not strictly speaking a microscopical procedure, but it is described here because its result shows whether or not certain methods of evaluation are admissible (see 2.2).

4.111 Determination of sediment provides information about the method of honey extraction pressing centrifuging, straining), and

4.112 about any contamination with large numbers of particles not usually present in honey (of dirt, pollen substitutes," yeast, etc.).

4.12 ***Field of application***

The amount of sediment can be determined for all honeys.

4.13 ***Principle***

A honey solution is centrifuged and the water-insoluble material obtained as a sediment, which is measured in calibrated centrifuge tubes.

4.14 ***Apparatus***

This comprises:

centrifuge tubes *c.* 50-ml capacity (see 3.3)
centrifuge (see 3.3)
water-operated or other vacuum pump
centrifuge tubes about 10-ml capacity with a calibrated tubular end;
capacity of the calibrated part 0.2 ml, graduation 0.01 ml *(Trommsdorfrohrchen).*

4.15 *Samples:* see 3.4

4.16 *Procedure*

Weigh 10 g of honey to 0.01 g, and dissolve in 20 ml of hot distilled water or clean tap water (not above 40°C) Centrifuge the solution for 10 min and draw off the supernatant liquid carefully leaving 1-2 cm above the sediment Disperse the sediment in distilled water and pour it quantitatively into a calibrated centrifuge tube of appropriate capacity; centrifuge again for 10 min. If the amount of

sediment is very high or low, use half or twice the amount of honey, respectively. During centrifugation, protect the tubular calibrated end of the centrifuge tube with vacuum rubber tube or a metal tube.

4.17 *Results and evaluation*

The amount of sediment is measured from the graduated end of the tube. Honeys extracted by centrifugation contain little sediment. In general, 10 g of honey yields 0.0015-0.0035 ml of insoluble matter. A high amount of sediment (>O.01 ml) indicates a pressed honey, unless most of the sediment is due to material not usually present in honey (dirt, pollen substitutes, yeasts, etc.). A low amount cf sediment may indicate honey naturally poor in pollen (e.g orange), excessive filtering, or falsification , (e.g. by feeding sugar to the bees)

4.2 Determination of the absolute number of plant elements in honey

4.21 ***Scope***

The absolute number of plant elements in a given quantify of honey is determined

4.22 ***Field of application***

It is applicable to all honeys.

4.23 *Maurizio's method*

4.231 ***Principle***
The sediment of a given quantity of honey is concentrated, and then dispersed in a known volume of water. A known volume of the suspension is spread over a circumscribed area on a slide. The microscopic elements are counted, and the number per unit weight is calculated.

4.232 *Apparatus*
This comprises:

Erlenmeyer flasks marked at 100 ml
centrifuge tubes 10-ml capacity, calibrated
centrifuge tubes 100-ml capacity, with conical ends
centrifuge (see 3.3)
Breed pipettes 0.0l-ml, calibrated
Slides
microscope (magnification about 300 X) with a mechanical stage and an eyepiece fitted with a graticule.

4.233 *Samples:* see 3.4

4.234 *Procedure*

4.234.1 *Preparation of slides*
Weigh two 50-g portions of honey in separate Erlenmeyer flasks. Dissolve the honey in a water-bath and fill the flask up to 100 ml. For honeys rich in sediment, two portions each 10-30 g are sufficient. Centrifuge the honey solution in 100-ml tubes. Decant the supernatant liquid carefully, disperse the sediment with a platinum rod, and transfer

it quantitatively to a 10-ml calibrated centrifuge tube by rinsing the large tube several times with distilled water Centrifuge the dispersion again for 5 min, decant or draw off the supernatant solution and disperse the sediment in an appropriate volume of water to give a convenient concentration of the plant constituents see Maurizio (1939) for statistical evaluations. In general, a dispersion volume of 0.5 ml gives a suitable concentration of the sediment from 50 g honey extracted centrifugally. For honey rich in sediment, a volume of 1 ml or more is recommended.

Stir the sediment with a platinum rod. Using a calibrated Breed pipette, transfer 0.01 ml of the dispersion to a slide, spread it, by blowing, over an area of 1 cm^2 which has previously been traced out underneath the slide. Spread out 2 drops of the same dispersion, side by side, on each slide. (Altogether 4 smear preparations are made. 2 from each honey portion) Dry the smears; the sugars remaining in the dispersion form a glaze which allows microscopic examination without use of a cover-glass.

4.234.2 *Counting plant constituents*

Count 100 fields of view in a graticule for each smear, at a magnification of 300 X. Start from the middle of one side and progress to the opposite side, displacing the field of view by the mechanical stage. This allows identical examination of the edges and of the centre of the preparation.

Pollen grains, fungal spores and algae are counted and noted separately in each field of view.

4.235 *Calculation and interpretation*

The absolute number of pollen gratias fungal spores and algae is based on the average number in the 400 fields of view, the area of a field of view, the dispersion volume, and the quantity of honey used The result is expressed as The number of plant elements in 1 g or 10 g of honey.

Experience shows that the absolute number of plant constituents in unifloral honeys poor in pollen grains (e.g. *Robinia or Citrus) is* generally below 20 000 in 10 g (group I). In the majority of Flower honeys and mixed flower-honeydew honeys the absolute number is between 20 000 and 100 000 (group II). In group III, with an absolute number of 100 000 to 500 000, are flower honeys rich in pollen *(Myosotis* and *Castanea)* and honeydew honeys. Group IV, with 500 000 to 1 000 000, comprises flower honeys extremely rich in pollen, and some pressed honeys. Group V, with more than *1 000 000,* includes only pressed honeys rich in pollen.

The counts are customarily expressed by a formula such as 32/38/0.7-II. This means that 10 g of honey contained 32 000 pollen grains, 38 000 fungal spores, and 700 algal cells; the 70 700 plant constituents place the honey in group II. It is a mixed honey, from both nectar and honeydew sources, whereas a floral honey might give 28/1/0-II. The result could be expressed as the number of plant constituents in 1 g of honey, but 10 g is normally used.

4.24 Z. ***Demianowicz's method***

4.241 *Principle:* see 4.231

4.242 *Apparatus:* see 4.232

4.243 *Samples:* see *3.4*

4.244 ***Procedure***

4.244.1 ***Preparation of slides***

4.244.11 ***Experimentally obtained single-species honey****
Place a drop of undiluted honey on a previously weighed cover-glass, and reweigh it. Then invert the cover-glass and place it on a microscope slide. Choose the amount of honey so that none is squeezed out beyond the edge of the cover-glass.

4.244.12 ***Unifloral honeys****
Use Maurizio's method (4.23); Demianowicz recommends drawing 1-cm squares on the slide with Indian ink; the ink keeps the dispersed sediment within the prescribed square.

4.244.2 Counting
Instead of counting 100 fields of view per smear, count plant constituents in strips across the preparation The number of strips depends on the absolute number of particles in the honey; if this is below 12 000, count 8 strips; if it is between 12 000 and 96 000, count 4 strips. With 96 000, 2 strips are sufficient. From the counts, calculate the number of plant constituents per square centimetre, and thus the absolute number per unit weight of honey.

4.245 ***Interpretation:*** see 4.235

4.25 ***Louveaux's method***

4.251 ***Principle***
Separate the sediment from a weighed quantity of honey. Disperse the sediment with water; pass through a filter of known surface area and whose pores are smaller than the diameter of the particles to be counted. After drying, clear the filter with immersion oil. Count the elements retained on the filter under the microscope. Calculate the number of particles per unit weight, from the number of fields of view and the area of each, the number of elements counted, the area of the filter, and the weight of honey used.

4.252 Reagents
Use immersion oil Millipore, refractive index 1.515.

4.253 ***Apparatus***
This comprises:

centrifuge tubes 50-ml capacity, and centrifuge (see 3.3)
water-operated vacuum pump or other device to draw off solutions
Pyrex micro-filtration apparatus of the Millipore Filter Corporation (XX 10 025 00) filter 25 mm diameter, white, smooth, pore width *c.* 1 μm
microscope (3.3)
slides at least 2.5 cm wide

' In this text a "unifloral honey" means normally obtained extracted honey, mostly from a single plant species (see 3.72); the term "single-species honey" is reserved for honey produced by bees allowed to forage on one flowering species only, under experimental conditions that prevent their access to any other source of food.

4.254 *Samples:* See 3.4
4.255 Procedure

4.25S.1 *Preparation* Weigh out 10 g of honey to the nearest mg. For honeys very poor in pollen, use 20 g; for honeys rich in pollen use less than 10 g. Dissolve the honey in 20 ml of distilled water (preferably hoc, but not above 40°C), and centrifuge for 10 min. Draw off the supernatant fluid leaving about 2cm above the sediment. Refill the tube with distilled water and centrifuge again. After drawing off the supernatant solution as described below, disperse the sediment in about 10 ml of distilled water, and put it into the cylindrical upper part of the filtration apparatus. Use a slight vacuum to draw the dispersion through the Millipore fi1ter; the addition of a drop of a detergent solution facilitates this operation. Refill the centrifuge tube several times with distilled water, and filter this too. In order to obtain a uniform sedimentation on the filter, turbulence must be avoided when new portions of liquid are put into the cylinder Finally wash the sides of the cylinder before the filter dries. Remove the filter from the apparatus and dry it by slight warming. Wash the cylinder thoroughly with a detergent solution. Put a few drops of immersion oil on a slide and cover with the filter, which becomes clear and translucent; put 1 or 2 additional drops on the upper surface, and enclose the preparation with a cover-glass.

4.255.2 *Counting and evaluation* Count the plant constituents in 100 fields of view at a magnification 800 X . Give the same attention to the periphery and the central part of the filter. The diameter of filter surface used can generally be identified without difficulty because the plant constituents give it a slightly yellow or brownish colour. The area of the surface can be measured easily after s solution of Indian ink has been strained through the filter. The number of microscopic elements fn the weight of honey used is given by the formula:

$$N = (F \times n) / (f \times a)$$

N = total number of elements in the weight of honey used
F = surface of the filter used, in mm^2
f = field of view in mm^2
n = number of elements counted
a = number of fields of view counted.

4.255.3 *Evaluation* see 4.235

4.26 *Reproducibility*

A repeatability study and a ring-analysis between different laboratories are still lacking

5. PREPARATION OF REFERENCE SLIDES

5.1 Purpose

A collection of reference slides is an indispensable supplement to the literature on melissopalynology.

5.2 Reagents

These are:
- diethyl ether analytical grade
- fuchsine (NB) powder
- fuchsine solution, alcoholic 0 1%
- Kaiser's glycerine gelatine
- fructose solution: 20 g fructose + 0.5 g crystallized phenol in 100 ml of distilled water, filtered through paper
- Alcohol
- acetolysis chemicals (see 3.2)
- glacial acetic acid
- saturated sodium chlorate solution
- concentrated hydrochloric acid
- KOH solution 5%
- Glycerine.

5.3 Apparatus

This comprises:
- warming plate (40°C)
- water-bath (up to 100°C)
- centrifuge (see 3.3)
- slides
- cover-glasses, circular or square, 18 mm in diameter or length
- platinum needle.

5.4 Samples

Use flowers of identified plants, fresh or dry. Zander recommends collecting the flowers as buds and letting them open in the laboratory. Out of doors, open flowers are often contaminated with pollen of other plants, by wind or by insect visitors.

5.5 Procedure

6.51 *Unstained preparations of degreased pollen*

Wash out the anthers, or whole flowers, or inflorescences, in a watch-glass filled with ether. Decant the ether, and rinse the pollen with fresh ether; decant this also. After drying, transfer the pollen to a slide and spread it out. A drop of fructose solution makes transfer easier, and accelerates the swelling of the pollen grains. Dry the preparation, preferably by slight warming (not above 40°C), and mount it with glycerine gelatine. If the pollen grains are slow to swell, keep the preparation warm until appropriate swelling has occurred. If the pollens have a tendency to burst, remove the fat from them with ether, directly on the slide. Mount the pollen quickly, with minimal warming.

5.52 *Unstained preparations without removal of fat*

Strip the pollen from the flowers or anthers with a microscope slide, or empty ripe anthers on to a slide with needles. Spread out the pollen and remove any remains of anthers and dirt particles. Then mount the preparation with glycerine gelatine.

5.53 *Stained degreased preparations* Prepare as in 5.51, but use a stained mounting material instead of unstained gelatine Stained gelatine is prepared by adding some drops of alcoholic fuchsine solution to melted glycerine gelatine. Pollens show different affinities to fuchsine, some being stained easily, others slowly. The preparation of a set of glycerine gelatine specimens with different concentrations of fuchsine is recommended; add 0.2-1.5 ml of alcohol solution to 10 ml of liquefied glycerine ge1atine.

5.54 *Acetolysis method* (Erdtman, 1960)
Put the pollen material into a heat-resistant centrifuge tube and cover with 5 ml of a mixture of acetic anhydride and sulphuric acid. Prepare the mixture by adding the acid, drop by drop, to nine times the volume of acetic anhydride; make the mixture afresh each day. Insert a glass rod into each tube, and transfer the tubes to a water-bath at 70°C, if possible in a fume cupboard. Heat the water-bath to boiling point, then immediately stop heating, and stir the liquid in the tubes and transfer these to the centrifuge After centrifuging, decant the reaction mixture into a reserve receptacle. Add 10 ml of water-alcohol mixture to the sediment and shake the tube thoroughly. It is usually not necessary to chlorinate thin-walled pollen grains In routine work, however, it is nearly always desirable to chlorinate grains and to mix, under the same cover-glass, grains that are acetolysed but not chlorinated with grains that are acetolysed and then chlorinated.

After acetolysis and washing transfer about a third of the suspension in the centrifuge tube to another tube. Centrifuge and decant again, then add to the sediment about 2 ml of glacial acetic acid, 1 or 2 drops of saturated sodium chlorate solution, and finally 2 or 3 drops of concentrated hydrochloric acid. Stir the liquid with a glass rod. Chlorine is produced immediately, and bleaching is usually obtained in a few seconds. After centrifuging the reaction mixture again, decant it and wash the sediment twice with distilled water. Then mix the suspensions of acetolysed pollen grains and of acetolysed and chlorinated grains. After centrifuging and decanting once again suspend the sediment in a few drops of a mixture of glycerine and water (1:1). Leave for at least 10 min, centrifuge decant, then invert the centrifuge tubes on filter paper. Fix a minute amount of glycerine jelly on a platinum needle, and carefully dip it into the pollen-bearing sediment. Then transfer to a slide the jelly and the pollen material adhering to it. Cover with a carefully cleaned, very thin circular cover-glass (diameter about 18 mm) or a square or rectangular cover-glass; but circular glasses are more easily sealed. Take great care in assessing the amount of glycerine jelly to use.

After applying the cover-glass the jelly should cover a more or less circular area with a diameter about 5 mm less than that of the cover-glass. J. Muller (Palynological Conference, Stockholm, 1950) suggests sealing with paraffin. Transfer a drop of melted paraffin to the margin of the cover-glass with a glass rod. If the slide has been gently heated, the paraffin will spread quickly under the cover-glass (see also 5.6) Then turn the slide upside down to allow small pollen grains and pollen fragments to settle close to the cover-glass.

Pollen grains embedded in glycerine, and thus mobile, can be sealed in the same way. Fresh material from Cannaceae, Juncaceae, Lauraceae, Maranthaceae, Musaceae and certain species of Zingiberaceae usually become more or less shriveled and wrinkled after acetolysis; chlorinate such material only, or warm it with 2-5% KOH or NaOH solution for a few minutes, instead of acetolysing it.

5.6 *Stability of preparations of non-acetolysed pollen grains*

Pollen grains in reference preparations alter in the course of time. If the fatty oil is not removed from them, they become pale, and the exine also loses colour. In all types of preparation the pollen grains increase in size, because of swelling. Old preparations are not useless, as they sometimes show particular characteristics better than fresh ones, but they should not be used for comparing diameters. The collection should be renewed from time to time with fresh preparations. The cover glasses may be sealed with Caedax, liquid Canada balsam or a suitable lacquer.

In hot and humid climates, preparations may be ruined because the gelatine melts and gets mouldy; the collection should therefore be stored in a refrigerator or cool-room. Sealing with parafin wax (see also 5.54) is advantageous in these conditions. The pollen is spread over a restricted area and covered with a small quantity of glycerine gelatine to prevent the mounting medium reaching the edges of the cover-glass Melt paraffin wax in a porcelain crucible, and transfer a few drops to the edge of the cover-glass, with a glass rod. If the slide was slightly warm beforehand the wax will spread into the gap between cover-glass and slide, and seal the preparation hermetically. The gelatine is prevented from flowing out even if it becomes liquid, and is protected against moulds.

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