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A new microsieving technique in pollen analysis

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ABSTRACT

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A new procedure has been added to the standard pollen preparation protocols to address the issue of microdebris and clay removal to facilitate easier and better microscopic observation and counting. This procedure consists primarily of a technique that uses a 5µm membrane filter under water pressure to filter out particles < 5µm and concentrate polliniferous material from core sediments and surface samples. The procedure includes a brief (optional) ultrasound pre-treatment followed by filtration using a membrane microsieve, the innovation highlighted herein. This technique is observed to be efficient in palynomorph retrieval from different kinds of sediment samples - the quantitative efficiency being superior to that of the conventional procedure and comparing favourably with ultrasonic microfiltration. The paper describes the new method, discusses its advantages and provides an illustrative, quantitative and qualitative comparison with the usual method (without microsieving) and recommends its widespread adoption in quantitative palynological studies.

Key-words—Pollen analysis, Microdebris, Microsieving, Membrane filter.

पराग विश्लेषण में एक नवीन सूक्ष्मछानन तकनीक

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सारांश

सूक्ष्ममलबा व मृत्तिका हटाने का पता लगाने की समस्या को आसान एवं बेहतर सूक्ष्मदर्शीय प्रेक्षण व गणना सुगम बनाने को मानक पराग निर्मिति पूर्व लेख में एक नवीन विधि समाहित की गई है। इस प्रक्रिया में यह तकनीक है जिसमें जल दबाव के अंतर्गत 5 µm कणों को छानने तथा क्रोड अवसादों व पृष्ठीय नमूनों से परागधर पदार्थ सांद्र करने के लिए 5 µm कला फिल्टर का प्रयोग होता है। प्रक्रिया में कला सूक्ष्मछन्नी का प्रयोग करते हुए अनुवर्ती संक्षिप्त (वैकल्पिक) अतिस्वन पूर्व-उपचार समाहित है, यहाँ पर नवीनता प्रमुख विशेषता है। यह तकनीकी अवसाद नमूनों के विविध प्रकार से परागाणुसंरुप सुधार में कुशल प्रेक्षित की गई है- मात्रात्मक कुशलता रुढ़ प्रक्रिया के उच्च है एवं पराश्रव्य सूक्ष्म छनाई सहित तुलना के अनुकूल है। शोध-पत्र नवीन विधि, इसकी श्रेष्ठता पर विचार करता है एवं सामान्य विधि (सूक्ष्म छानन रहित) से व्याख्यात्मक, मात्रात्मक व गुणात्मक तुलना प्रदान करता है तथा मात्रात्मक परागाणविक अध्ययनों में इसके अंगीकार की सिफारिश करता है।

मुख्य शब्द—पराग विश्लेषण, सूक्ष्ममलबा, सूक्ष्मछानन, झिल्ली फिल्टर।

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INTRODUCTION

POLLEN analysis of sediments and surface samples involves rigorous microscopic studies of the chemically treated samples, and inevitably, the final residue mounted on the slide contains a certain proportion of microdebris. Presence of microdebris in slides is very often a hindrance to a detailed observation of finer morphological characters of pollen. This is very often the case in different kinds of samples, organic or clastic, such as surface samples, samples from tree hollows, marine samples, archaeological deposits and samples from peat and other organic deposits such as river or lake sections. Even though organic and clay-rich samples usually contain more pollen, the quantity of the minute silicate particles, which usually do not get digested with HF treatment, poses a hindrance in the observation of pollen characters as well as their enumeration. The removal of clays, fine silts and microdebris is obviously a difficult and time-consuming process (Bates *et al*., 1978; Tomlinson, 1984; Heusser & Stock, 1984). Various processes have been used to reduce the amount of debris present in the sample (Funkhouser & Evitt, 1959; Tschudy, 1960; Gray, 1965; Hansen & Gudmundsson, 1979). Producing clean residues with high concentrations of palynomorphs from sediment/soil samples for microscopy is of paramount importance, even if labour intensive at the stage of chemical preparation (Heusser & Stock, 1984). One of the vital steps for achieving this is microsieving, in addition to or in conjunction with disaggregation and deflocculation (Cwynar *et al*., 1979; Lentfer & Boyd, 1999).

Present day studies on palaeovegetation reconstruction rely on quantitative methods of pollen analysis unlike earlier qualitative procedures that relied more on relative pollen abundance. To be able to use the pollen counts generated in quantitative and statistical applications, and finally, in order to validate models, the state-of-the-art requires 'clean datasets'. Achieving this implies precision and refinement in every step of pollen analysis starting from the field to the lab to the microscope because, among the events influencing a pollen analytical registration, the effects of sampling procedures and analysis technique are important as they influence, the relation between the pollen flora of the deposit and the registration of it by the analysis (Faegri & Iversen, 1989).

Fig. 1—Apparatus for microseiving using a membrane filter.

Sieving (screening/filtering) has long been used as a part of palynological sample processing, primarily to separate different size fractions of both organic and inorganic constituents of various samples (Cwynar *et al*., 1979; Caratini, 1980; Tomlinson, 1984; Faegri & Iversen, 1989; Jemmett & Owen, 1990; Moore *et al*., 1991; Ediger, 1986). The use of ultrasonic microsieving in the field of palynology has been propounded for a long time now (Marceau, 1969; Hideux, 1972; Caratini, 1980; Tomlinson, 1984). Caratini (1980) has suggested the usage of fine-sieving using an ultrasonic generator and a microfilter and fabricated a new device, which is very handy in sediment preparation for pollen analysis. The ultrasound technology is used for the acceleration of sieving processes alternatively or complementary to the classical low-frequency vibrators. This procedure involves the usage of ultrasonic waves, i.e. sound waves with a frequency above the upper limit of human hearing (60-80 KHz) in order to get evenly suspended particles of very minute size for a better sieving. So far, palynologists have prepared the samples by coupling both ultrasound treatment and sieving (Caratini, 1980; Tomlinson, 1984). In this paper, we present a new microsieving technique involving sieving with the aid of membrane filter. We have attempted to concentrate pollen from sediments and surface samples by treating first with ultrasonic waves, if necessary, for fine dispersal of residue, followed by microsieving using a cellulose nitrate filter to sieve out particles < 5 µm.

MATERIALS AND METHODS

The apparatus used in this study and a flow diagram of the main steps involved, highlighting the newly introduced step of microsieving (MS) with a membrane filter are illustrated in Figs 1 & 2.

The filter used in this method is a cellulose nitrate filter of 5 µm pore diameter. Membranfilter GMBH was used in our trials but we recommend any other filter with similar properties. The device used is a vacuum filtration device fitted with water pump (Fig. 1).

After the standard treatment procedure involving hydrofluoric acid treatment to remove silica and silicates (Faegri

& Iversen, 1989; Moore *et al*., 1991), the residue in water is poured into a test tube and allowed to undergo ultrasonic vibration for about 2-3 minutes using an ultrasonic generator emitting a minimum frequency range of 60-80 KHz. This ultrasonic treatment is optional and used on a case by case basis, depending upon parameters such as the concentration of the sample and nature of the sample.

Then the residue in the test tube is poured into the filtration device fitted with the cellulose nitrate filter, which in turn is fitted with a water suction apparatus (Fig. 1). By using a vacuum suction force created below the filter by applying water pressure on it, particles less than 5µm size are eliminated. The material on the filter is kept in constant movement by frequently squirting water on the filter using a wash bottle jet, which also serves the purpose of unclogging the blocked pores. This can be done for 5 minutes or until complete filtration is ensured.

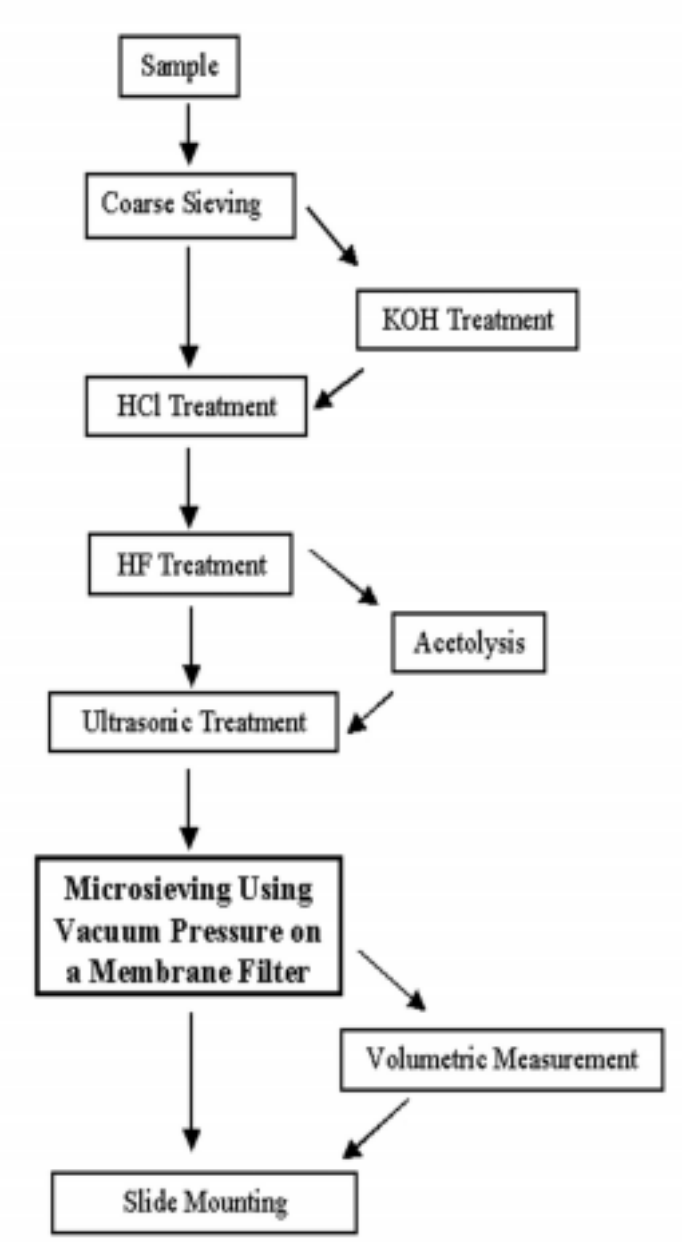

Fig. 2—Flow chart of the standard pollen analyses technique incorporating microsieving with membrane filter.

The material on the filter is ready for centrifugation and mounting. The membrane filter has to be disposed off after every preparation.

To summarize, the main principle used in this method is that a great majority of particles smaller than the size of the palynomorphs required to be isolated, will be eliminated by micro-filtration. The optional use of ultrasound is to get even dispersion of the particles in the sediment to facilitate the easy removal of clays during the preparation of the samples.

RESULTS AND DISCUSSION

In the absence of microfiltratrion there is a large likelihood of microdebris being present in large quantities along with the extracted palynomorphs, eventually hindering pollen analyses under the microscope. The main objective of the proposed method is to achieve a clean residue, with minimal loss of and maximum concentration of palynomorphs. This method has been tested on more than thirty samples including surface sediments and cores and is being actively used in our lab in ongoing analyses.

To illustrate the efficacy of the method, a quantitative comparison of trials using both treatments, namely, the conventional one without any microfiltration and the newly proposed procedure, using a membrane microfilter, is presented here on two samples, the first, sediment from a core and the second sediment from a surface sample (Fig. 3).

Different measures are used to compare the results from the different treatments: the first is number of pollen identified and counted per line (N_l), second, the total volume of residue obtained (V_{tot}) and third, the ratio between V_{tot} measured using the two treatments – namely, without MS (i.e. unfiltered residue UF) and using MS with a membrane filter (MF) (Fig. 3).

The first measure gives an idea of the ‘ease of pollen counting and identification’, which naturally increases with increasing number of grains per line. As counting more number of grains per line implies a better concentration of palynomorphs in the residue, in effect this is also a measure of the residue richness. This is always greater in the MF method of preparation (Fig. 3).

The second is an absolute quantitative estimate of palynomorph retrieval and concentration from the raw sediment sample. As expected, because the microdebris remain in the final residue, this measure is always greater in the UF method of preparation (Fig. 3). The third measure, the ratio of V_{tot} between UF and MF methods is around 1.6 (Fig. 3).

Sample No.	Pollen per line (N_l)		Total volume of residue V_{tot} (µl)		Ratio: UF V_{tot} / MF V_{tot}
	UF	*MF*	*UF*	*MF*	
S227/8 (Sediment-Core)	9	16	72	120	1.66
S231/8 (Sediment-Surface)	6	15	508	792	1.56

Fig. 3–Quantitative comparison of two samples under different treatments *UF–Without microsieving, MF–Microsieving with a membrane filter.*

Actual views of the microscopic field of the samples under the two different treatments are also presented (Pl. 1). The photographs represent the microscopic field under different magnifications (Pl. 1.1a & 1.2a: 16X objective, Pl. 1.1b & 1.2b: 40X objective and Pl. 1.1c & 1.2c: 100 X objective respectively). For comparison, we focused on a field with a grain of Poaceae in both cases. To illustrate the effect of microsieving both the microdebris and the pollen visibility have been highlighted in Plate 1.

After processing, the microfilter was treated separately to recover any possible palynomorphs that may have adhered to the membrane. None were recovered and it was concluded that there is no loss of palynomorphs through the use of the microfilter. Another validation of the same result was obtained by estimating the pollen per gram of sediment using the method of Cour (1974) modified by Anupama (1996) for aeropalynological samples and Sutra (1997) for sediments. A consistently higher value was obtained using the MF method for both samples.

Further, the pollen per line in both cases are very different with more pollen counted per line using the MF method (Fig. 3). Evidently, filtration has helped remove a large proportion of the microdebris and this in turn has helped isolate and present the palynomorphs in a clearer microscopic field – a great help in terms of the ease of counting as well as the time spent under the microscope, which, at the minimum is reduced by a factor of two to four.

The method presented here is only a small addition to the existing standard protocol for pollen preparation, but as it addresses the important issue of microdebris, this can contribute immensely to a pollen analytical study.

In a sense, this new method modifies the method proposed and used by Caratini (1980) where a plexi-glass 5µm microfilter mounted on a metallic frame was used. That method, though well known for nearly twenty years now, has not gained wide acceptance due to the cost and difficulty in procuring the plexi-glass filters of pore size 5µm. Additional reasons for modifying the existing method were the longer time required for sample processing due to the mandatory requirement of thoroughly cleaning the mesh after each sample preparation to avoid any contamination; there is also a gradual clogging of the microsieve's pores so that eventually, after around 50 to 60 samples, the microsieve is rendered ineffective. To minimize loss of pollen, it was, however, decided to persist with sieving at 5µm as recommended by Caratini (1980) rather than the more widely recommended and used 7µm mesh (Cwynar *et al*., 1979; Faegri & Iversen, 1989; Moore *et al*., 1991) because the proposed method deals with the issue of pore clogging in the former. In the tropical context of the sediments we analyzed, this gains further importance due to the occurrence of pollen types that may easily pass through a 7µm filtration, such as *Elaeocarpus*, *Poeciloneuron* and members of Moraceae, especially *Ficus* (Tissot *et al*., 1994; Bush & Colinvaux, 1988; Bush *et al*., 1992; Bonnefille *et al*., 1999; Anupama *et al*., 2000; Barboni & Bonnefille, 2001; Barboni *et al*., 2003; Weng *et al*., 2004).

Several caveats have been posed regarding the use of ultrasonic vibration in palynomorph preparations. For palynomorphs, such as globigerinids, poorly cemented arenaceous foraminifers and thin shelled ostracodes, 1 or 2 minutes ultrasonic treatment helps avoid breakage and abrasion (Stevens *et al*., 1960). Even those who seldom observed any damage due to ultrasonic treatment observe that even if normally, the fragile, thin walled and brittle specimen can withstand vigorous treatment, occasional ill effects such as broken up walls, may be observed (Funkhouser & Evitt, 1959). Though effective in ensuring a better retrieval of pollen grains, it has been claimed that ultrasonic vibration given for a longer duration of time can disrupt and destroy the pollen grains (Marceau, 1969; Dodson, 1983; Lentfer & Boyd, 1999; Hideux, 1972; Tomlinson, 1984; Ellin & McLean, 1994). It is however true that careful tuning and control of exposure time can help avoid possible pollen damage (Hodgkinson, 1991). The use of an ultrasonic generator emitting a minimum frequency range of 60-80 KHz is recommended as lesser frequency device may damage the palynomorphs (Marceau, 1969; Hideux, 1972; Caratini, 1980).

Despite the stated caveats, ultrasonic vibration, used judiciously always helped us to obtain a cleaner, palynomorph rich residue suspension rather than the contrary. Hence we do recommend its conditional use – first, it should never be below the frequency range specified above, second it should never exceed three minutes and finally it needs to be used only when it is essential as, for example, in clay rich samples for getting

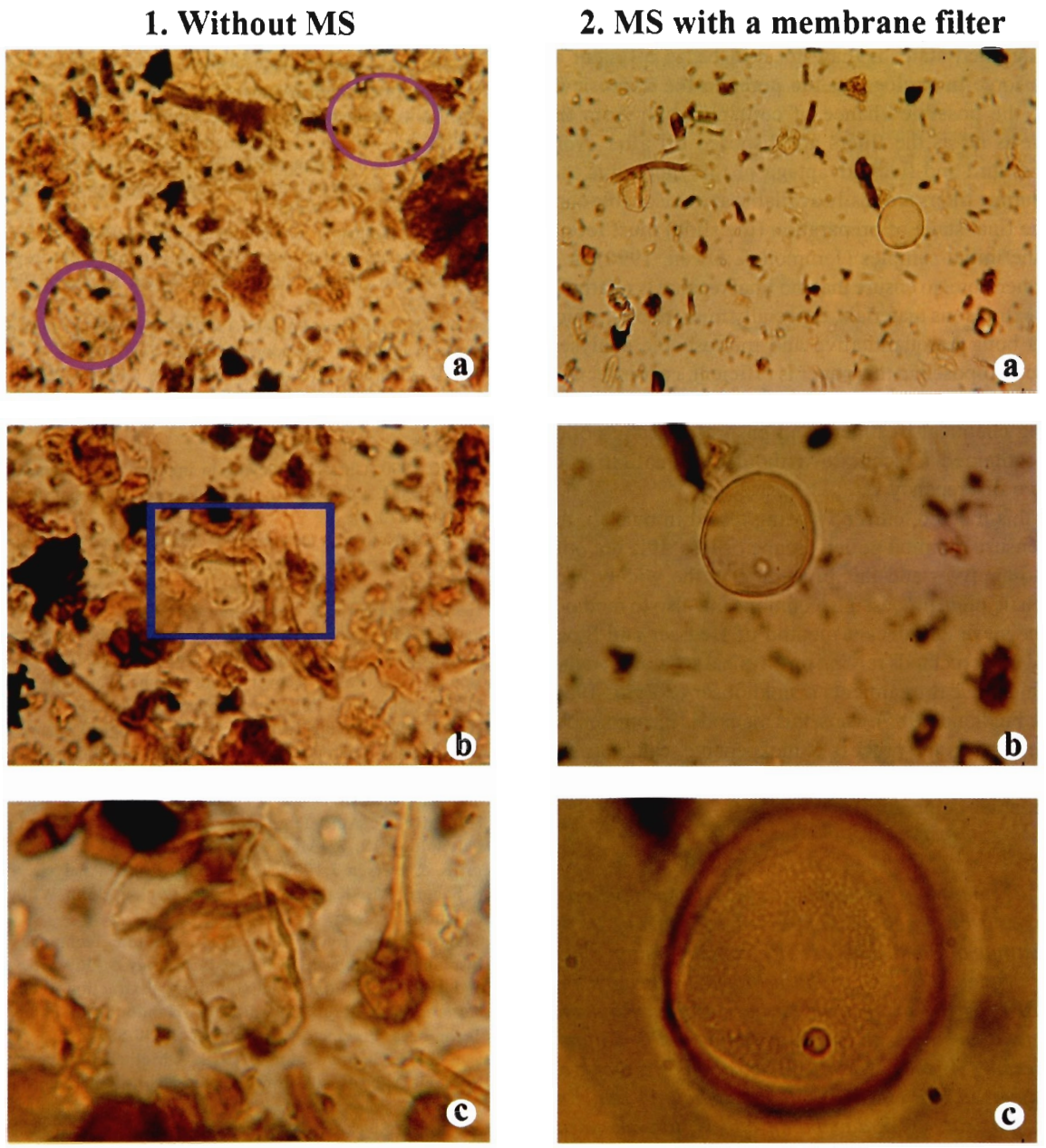

PLATE 1

(1, 2) : Views of the microscopic field of the samples under the two different treatments (a, b, c): under different magnifications (Pl. 1.1a & 1.2a: 16X objective, Pl. 1.1b & 1.2b: 40X objective and Pl. 1.1c & 1.2c: 100 X objective respectively). To compare the single grain visibility/clarity, a field with a grain of Poaceae is focused under both treatments.
The kind of microdebris removed by microsieving in Fig. 1.2a are encircled in Fig. 1.1a by a pink circle.
The poor visibility of the grain of Poaceae is highlighted by a blue rectangle in Fig. 1.1b.

an even suspension. For easily applying the second condition, we have separated the steps of ultrasonic vibration and microfiltration. An added advantage of this separation is that possibility of the mesh distortion during the ultrasonic treatment is also ruled out. Microfiltration is, however, indispensable for most samples and a 5μm sieve seems ideal as there are no species with pollen size below that and only a few species even in the range of 6-8 μm.

In our proposed method, the first advantage is the usage of water suction force, that, even when applied for a long time,

does not harm the polliniferous material; a caveat being the fact that though suction force keeps the suspension in a constant agitated state, it may not still be as efficient as ultrasonic force in unclogging the pores in the microsieve. Secondly, the possible chance of contamination from the microsieve is nil as the filters are disposed off after every usage. The third and final advantage of this method is that cellulose nitrate filters are easily available at a reasonable cost.

The final stage of preparation (the slide) must reflect those in the initial samples (Jemmett & Owen, 1990). It is essential, therefore, to ensure that the final residue is relatively free from extraneous materials, especially microdebris, which influences both the quantitative and microscopic analyses. The method proposed in this paper is efficient, cost-effective, pollen-protective and reduces the quantity of sediment undergoing chemical treatment and ensures that the pollen spectrum obtained is accurate without any qualitative or quantitative discrepancy.

In this method, currently being tested in our lab, the aim is to ensure minimal pollen escape or loss. It is not only possible to apply or add this procedure to the widely used conventional pollen processing technique but also to methods such as the sieve and decant method of Heusser and Stock (1984) and other methods relying on physical separation rather than harsh chemical treatments (Funkhouser & Evitt, 1959; Johnson & Fredlund, 1985). Thus, this method is recommended for a wider use, as the process is inexpensive, can be easily added on to the existing method and requires only simple, easily available equipment.

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