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Note

Sample introduction in gas chromatography: simple method for the solventless introduction of crude samples of biological origin

B. V. BURGER*, ZENDA MUNRO and DENICE SMIT
Laboratory for Ecological Chemistry, University of Stellenbosch, Stellenbosch 7600 (South Africa)
U. SCHMIDT
Zoologisches Institut der Universität Bonn, Poppelsdorfer Schloss, D-5300 Bonn 1 (F.R.G.)
and
CHIA-LI WU and FENG-CHANG TIEN
Department of Chemistry, Tamkang University, Tamsui 25137 (Taiwan)
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The preparation of samples of biological origin normally involves extraction of the organic volatiles with a suitable solvent. This step in the work-up procedure has the advantage that it can be employed as a concentration step by extracting a sufficiently large amount of material with a relatively large volume of solvent and concentrating the extract to a suitable volume before injecting it into the gas chromatograph. The efficiency with which organic compounds are extracted from biological matter depends on factors such as the physical state of the sample and the polarity of the organic compounds and of the solvent used for extraction. If known compounds have to be determined, incomplete extraction is not such a serious problem, as quantification can be carried out relative to one or more internal standards which have properties similar to those of the analytes and are added to the biological sample before extraction.

However, if complex mixtures of unknown organic compounds with divergent physical and chemical properties have to be extracted from samples which might, for instance, contain considerable amounts of water and might further be relatively viscous or difficult to mix with the organic solvent, simple extraction techniques offer little hope of obtaining an accurate quantitative picture of the composition of the volatile organic fraction. The determination of the volatile organic constituents of the preorbital gland secretion of the small antelope *Raphicerus melanotis*, which contains water, mucous, water-soluble solid material and a complex mixture of organic compounds ranging from formic acid to long-chain alcohols and formates [1], is an example of the formidable analytical problems posed by such samples. One possible approach to the analysis of such complex samples is the direct solventless introduction of the crude sample into the injector of the gas chromatograph, provided that the

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analytes are present in high enough concentrations to afford reliable quantitative data with amounts of material that can be handled by such direct solventless introduction techniques. Although it is not common practice, the comparison of the results obtained by conventional injection of an extract of the material and those provided by solventless injection of the crude material could provide invaluable information on the reliability of the extraction and work-up procedure and on whether volatiles are lost during the solvent removal step or are obscured by the solvent peak.

Solid sample introduction systems developed by the manufacturers of gas chromatographic equipment or by workers in specific fields of research, such as the system recently elaborated by Attygalle and Morgan [2], can be used for this purpose. However, as most laboratories are only occasionally required to analyse samples that might be amenable to solid sample introduction, these systems are unfortunately not in general use in most laboratories.

In our own research on semiochemical communication in the animal world, results obtained with extracts of the exocrine secretions of insects and mammals are routinely compared with those obtained when the crude untreated secretions, or even the glands producing these secretions, are introduced into the injector of a gas chromatograph. For the past 8 years we have been using an extremely simple solventless sample introducing technique with excellent results. However, this method, which was first mentioned in a paper on the characterization of the defensive larval secretion of the citrus swallowtail butterfly *Papilio demodocus* [3] does not appear to have gained any recognition beyond a reference to it in the paper by Attygalle and Morgan [2]. This has prompted us to describe the method in more detail than in the paper in which it was first mentioned and to illustrate its application to the analysis of a number of different sample types. For this purpose we have selected a few examples from our own research and from work carried out in collaboration with other laboratories.

EXPERIMENTAL

Gas chromatographic (GC) analyses were carried out with Carlo Erba (Milan, Italy) Model 4160 and 5300 gas chromatographs and a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 5890A gas chromatograph equipped with flame ionization detectors, using helium as carrier gas. GC–mass spectrometric (GC–MS) analysis was done with a Carlo Erba QMD 1000 system. With the exception of the column employed for the analysis of the pheromone glands of the smaller tea tortrix, all columns were coated using either published methods [4] or procedures developed in the Laboratory for Ecological Chemistry.

A hole was made through the centre of a septum with a syringe needle (No. 18, 1.2 mm) with a sharpened 90° point. By rotating the needle slowly and forcing it through the septum, it was possible to produce a tapered hole through the septum. However, any septum which had been pierced a few times during normal use in a gas chromatograph could be used equally successfully without additional widening of the hole. The septum was then installed in the gas chromatograph. By tightening the septum cap sufficiently the hole through the septum can be closed and samples can be injected with a syringe in the normal manner. An empty sample introduction probe or a thin flame-polished glass rod can also be used to close the hole in the septum, which tends to widen if the solventless sample introduction technique is used repeatedly.

Sample introduction probes were made from capillary tubing (0.7 mm I.D. × 1.2 mm O.D.) drawn from soft glass tubing on a capillary drawing machine (Hewlett-Packard 1045A, Hupe & Busch or Carlo Erba GCM60). Ordinary melting point capillaries with similar dimensions can, however, also be used for this purpose. To manufacture a sample introduction probe, a 100-mm length of capillary is sealed off about 20 mm from one end by holding the capillary in the side of the flame from a small bunsen burner until a bead of glass has been formed at this point (Fig. 1A). The capillary is then removed from the flame, rotated to keep it straight and drawn out just enough to decrease the diameter of the soft glass bead to approximately that of the capillary itself. Using a glass-cutting tool or preferably the sharp edge of a fragment of silicon wafer, the shorter section of the capillary is scored a few millimetres from this position and about 15 mm of the capillary is broken off and discarded. The tip of the remaining capillary is flame polished to ensure smooth and unobstructed movement through the septum and to avoid fragments of the septum from being transported into the injector by the sharp edges of the capillary. The other end of the capillary is sealed off or flame polished to avoid hand injuries when the probe is inserted into the injector. To facilitate rapid and quantitative evaporation of volatiles from a sample, the probe is constructed with the shortest possible sample cavity which can still accommodate the sample without losing material when the probe is inserted through the septum. With some experience a sample probe can be made in a few minutes and, instead of cleaning used ones in an ultrasonic bath or muffle furnace, we prefer to use a new probe for each sample.

The sample probe is loaded with a sample by placing an appropriate amount of the material to be analysed in the sample cavity. Care is taken not to obstruct the free movement of the carrier gas into and the transportation of desorbed volatiles out of the sample cavity by blocking it with, for example, a large amount of viscous material.

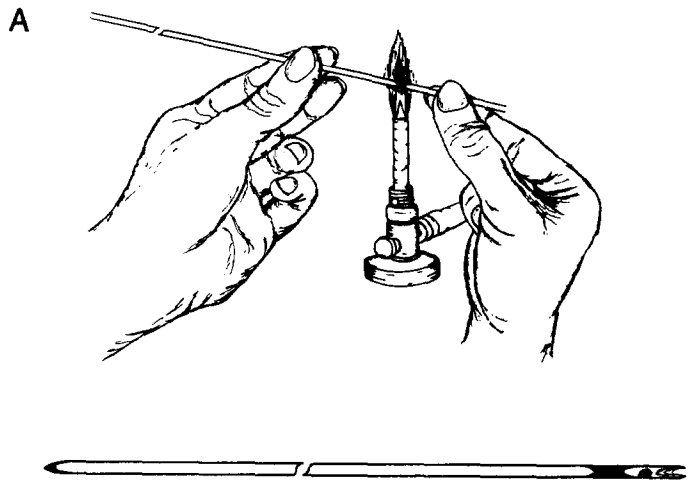

Fig. 1. Preparation and loading of a sample introduction probe. (A) Glass capillary tubing is sealed off about 20 mm from one end in the flame from a small bunsen burner; (B) sample probe trimmed to the required size and sample cavity depth, and loaded with a sample which is retained by a few glass fibres.

Such a sample is preferably spread out as a thin film on the inner surface of the sample cavity. In the case of solid material which might drop out of the sample cavity when the probe is brought into a vertical position, a few fibres of silanized glass-wool are inserted into the cavity to hold the sample in place as shown in Fig. 1B. This is best done under a stereo microscope with a thin glass rod. Fibres protruding from the cavity are removed with a pair of ophthalmic scissors or are cut off against a microscope slide with a razor blade or the sharp edge of a piece of silicon wafer.

To introduce the sample into the injector, the sample probe is pushed firmly down into and through the septum. The sample cavity is sealed gas-tight while it is passing through the septum, preventing any carrier gas or volatile components from escaping. Introduction of highly volatile samples can be done with the injector in the split mode. With solid or highly viscous samples, it is advisable to keep the split valve closed until all the volatiles have evaporated from the sample. It is also possible to use temperature-programmed sample evaporation if thermally labile compounds are expected to be present in the sample. Especially under conditions where volatiles are transported relatively slowly to the capillary column, cold or stationary phase focusing should be employed.

A section through an injector with a sample probe in place is shown in Fig. 2. In most gas chromatographs the needle-guiding holes through the septum cap and septum-supporting insert have to be enlarged to *ca.* 1.5 mm to accommodate the sample introduction probe, which is much thicker than a normal syringe needle.

RESULTS AND DISCUSSION

The application of this solventless introduction of samples into the injector of a gas chromatograph is illustrated in Figs. 3–6.

Larvae of the citrus swallowtail butterfly (*Papilio demodocus*) possess a defensive gland, the osmeterium, situated mid-dorsally behind the head. The gland is normally invisible, but can be extruded as a prong- or fork-like structure. A defensive secretion consisting almost entirely of water is produced by the osmeterium and was found to contain small amounts of terpenoid compounds in the pre-final instars. To collect the defensive secretion a larva was irritated by tapping it on the head with a sample probe. As the larva gradually extended its osmeterium, the two prongs were guided into the sample cavities of two probes. In this experiment sample cavities with a depth of about 5 mm were employed. Slow withdrawal of the osmeterium from the sample probes left a little opaque material in the sample cavities. Using this method, it was found that in some larvae one prong of the osmeterium produces high concentrations of a number of monoterpenes, while these compounds are almost entirely absent from the secretion collected from the other prong. Examples of the gas chromatograms obtained in such an experiment are given in Fig. 3.

In contrast to the defensive secretion of the citrus swallowtail, Lepidoptera mostly produce their sex pheromones in extremely small amounts. The first sex attractants to be identified were therefore isolated by extraction of many thousands of insects. Using modern analytical techniques it is possible, however, to identify the constituents of an insect pheromone by using the pheromone-producing glands of a single insect, preferably employing a direct solventless sample-introduction device [2]. This can be accomplished successfully and simply by using the technique described

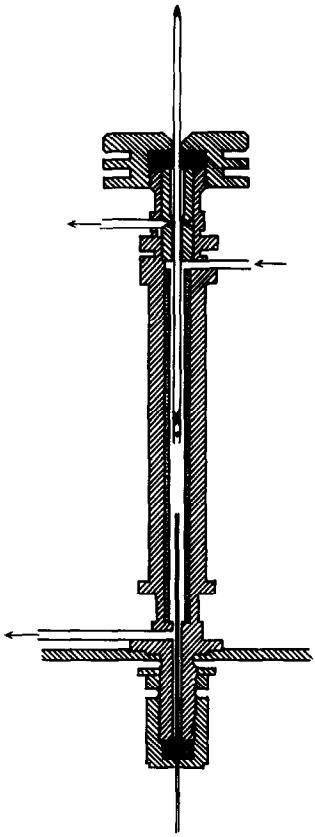

Fig. 2. GC injector with inserted sample probe.

in this paper. The clean profile of the constituents of the sex pheromone gland of the smaller tea tortrix (Taiwanese *Adoxophyes* sp.) shown in Fig. 4 was, for example, obtained by using two excized glands of the female moth.

An advantage of the technique described in this paper that is not offered by most of the other solid-sampling techniques is that the sample can be removed from the injector at any time after the volatiles have been desorbed. The formation of artefacts from the less volatile constituents in a sample at high injector temperatures and the transportation onto the column of heavy lipids which might adversely affect the performance of a column can thus be restricted by timely removal of the sample from the injector. It is essential, however, to carry out a few trial runs to determine the lowest temperature and the shortest time required for quantitative transportation of the volatiles of interest onto the column. This can be done, for example, by subjecting a sample to a second desorption in order to determine whether all the volatiles of interest had been removed from the sample during the first desorption. Gas chromatograms obtained in such an experiment are shown in Fig. 5.

Finally, the technique can also be used for sample introduction in GC–MS analysis. The total ion chromatogram shown in Fig. 6 was, for example, obtained with a trace amount of the waxy buccal secretion of the bat *Nyctalus noctula*.

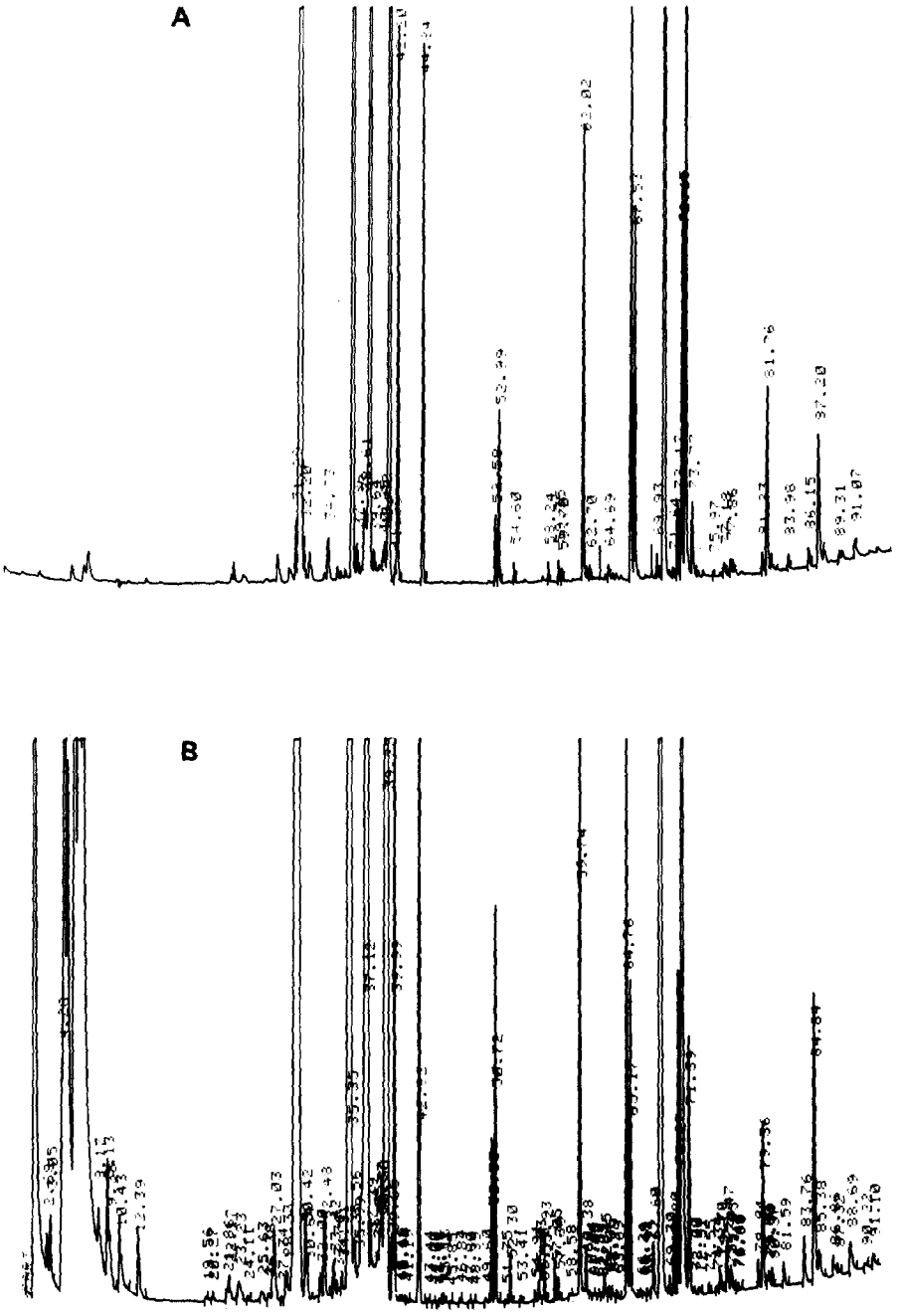

Fig. 3. GC comparison of the defensive secretions (A and B) collected separately from the two prongs of the osmeterium of a *Papilio demodocus* larva. The injector at 220°C was operated in the splitless mode for 2 min and the volatiles were trapped on the column at 30°C. Glass capillary column coated with Carbowax 20M (40 m × 0.3 mm I.D., film thickness 0.3 μm); temperature programmed from 40 to 220°C at 2°C/min.

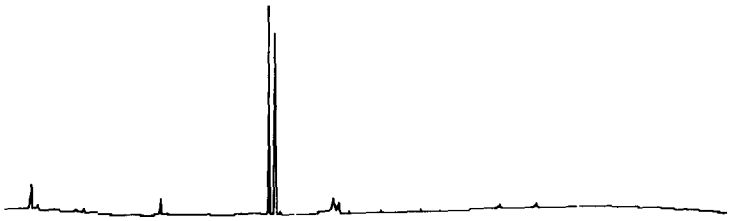

Fig. 4. Gas chromatogram of the organic volatiles desorbed from two excized sex pheromone glands of the smaller tea tortrix (Taiwanese *Adoxophyes* sp.). The injector at 220°C was operated in the splitless mode for 5 min and the volatiles were trapped on the column at 50°C. Fused-silica column (Supelco) coated with SP 2250 (60 m × 0.25 mm I.D., film thickness 0.2 μm); temperature programme, 50°C for 30 s, increased to 150°C at 4°C/min and from 150 to 260°C at 2°C/min, held isothermally at 230°C for 1 h.

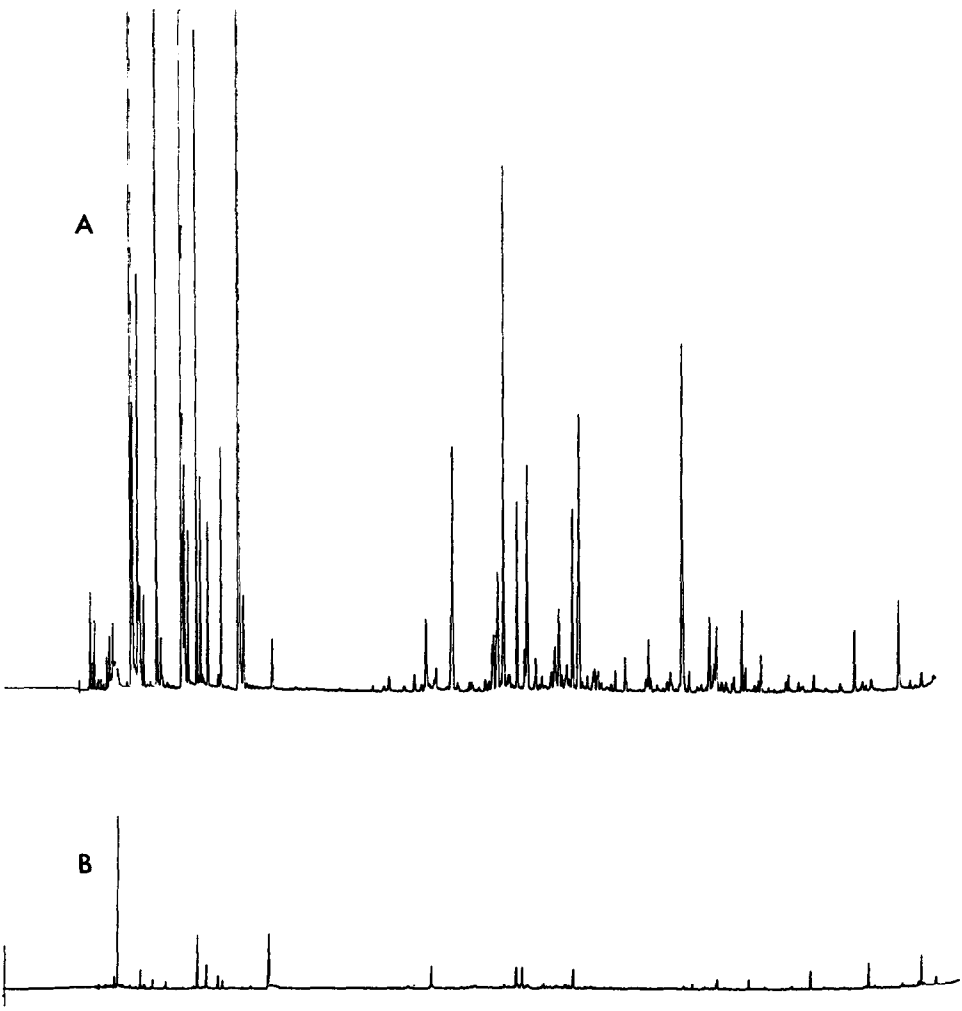

Fig. 5. Gas chromatograms obtained by consecutive desorptions of the volatiles from a 0.3-mg sliver of a *Eucalyptus linearis* leaf. The injector was programmed from 100 to 260°C at 30°C/min and held at 260°C for 15 min and the volatiles were trapped on the column with solid carbon dioxide. Fused-silica capillary column coated with Superox 4 (25 m × 0.25 mm I.D., film thickness 0.25 μm); temperature programmed from 40 to 80°C at 2°C/min and from 80 to 230°C at 4°C/min. (A) First desorption; (B) second desorption.

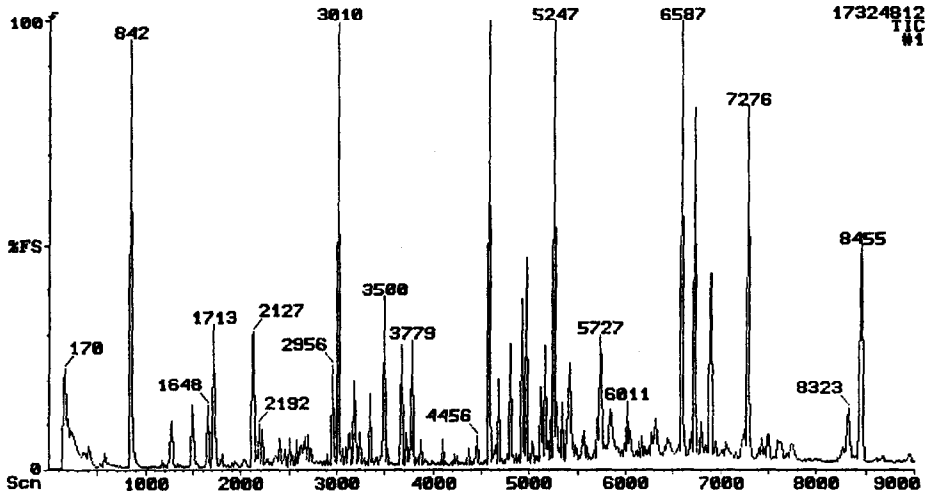

Fig. 6. GC–MS of the buccal gland secretion of a male bat, *Nyctalus noctula*. The injector at 220°C was operated in the splitless mode for 4 min and the volatiles were trapped on the column at 30°C. Glass capillary column coated with OV-1701-OH (40 m × 0.3 mm I.D., film thickness 0.5 μm); temperature programme, 30°C for 3 min, increased to 40°C at 25°C/min and from 40 to 250°C at 2°C/min.

When solventless injection techniques are used, the reproducibility of retention times depends on, *i.a.*, the degree of sophistication of the carrier gas pressure-regulating system, *i.e.*, whether the regulator will reset to exactly the same pressure after the pressure has been released to remove sample residues or glass fragments from the injector. If the injection technique described here is used with a relatively thick silicone-rubber septum and a properly tightened septum cap, it is possible to insert and slowly withdraw the sample introduction probe without losing carrier gas from the injector. In a study of the seasonal changes in the composition of the buccal gland secretion of *Nyctalus noctula* in which almost 100 samples of this secretion have been analysed over a period of more than 2 years, the retention time reproducibility obtained with this technique was found to be fairly good. It must be stressed, though, that cold or stationary phase trapping has to be employed in most instances in order to obtain sharp peaks and reproducible retention times.

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