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A MICRO METHOD FOR THE DETERMINATION OF VOLATILE METABOLITES IN BIOLOGICAL SAMPLES

A PRELIMINARY REPORT

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SUMMARY

A new method for analyzing volatile metabolites in biological fluids and tissues has been developed. The procedure involves extraction of the sample with diethyl ether, concentration on a short column of glass wool by elution of the diethyl ether, then thermal desorption of the trace constituents into a chromatographic system. Small samples of 100 μl are quite adequate. The method is applicable to serum, plasma, urine and other fluids. Small tissue samples can also be used with this technique.

INTRODUCTION

Low-molecular-weight volatile constituents of biological fluids have recently been subjected to intensive investigation by gas-phase analytical techniques[1–15]. Analyses are based on adsorption of head-space volatiles on a porous polymer, Tenax GC, thermal desorption, separation on high-efficiency open-tubular columns and identification by high-resolution mass spectrometry. Direct solvent-extraction techniques have also been used[1,10]. Profiles of biological metabolites have been developed for urine, serum, plasma and cerebrospinal fluid. Studies relating these profiles to specific diseases such as *diabetes mellitus* are also of current interest[4,11,13,14].

The use of headspace techniques presents some limitations to the analyses of biological fluids. These include size of sample, purity of sweeping gas and quantitation of analysis. Whereas 5 ml of biological fluid are normally no limiting factor as far as sample size is concerned, there are situations where smaller amounts are only available, *e.g.* (a) samples taken from animals at short intervals, (b) cerebrospinal fluid and (c) application of these procedures to tissues. It was therefore desirable to investigate the possibility of measuring the total volatiles present in 100 μl of biological fluid. The system developed involves extraction of the sample with diethyl ether, separation of the diethyl ether from the volatiles on an adsorbent and thermal desorption into a chromatographic column.

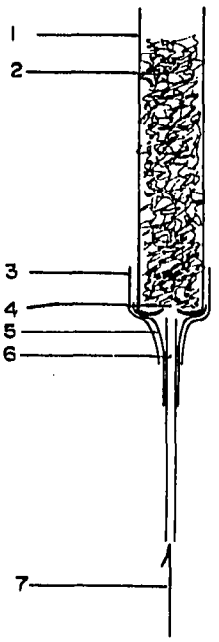
EXPERIMENTAL

Reagents

Diethyl ether, anhydrous, from Fisher Scientific Co. (Pittsburgh, Pa., U.S.A.), was purified by refluxing for 48 h over lithium aluminum hydride. Ammonium carbonate, analytical-reagent grade, was obtained from Mallinckrodt (St. Louis, Mo., U.S.A.) and glass wool, Pyrex brand, from Corning Glass Works (Corning, N.Y., U.S.A.).

Apparatus

The concentration column containing the adsorbent is shown in Fig. 1. The glass tube is 11 cm × 10 mm O.D. × 8 mm I.D. One gram of glass wool is packed into the tube to a depth of 9 cm. Helium enters the system at a flow-rate of 20 ml/min. The packed concentration tubes are conditioned in a stream of helium (20 ml/min) for a period of 1 h at 350° prior to use. The desorption system is illustrated in Fig. 2, and is similar to that previously described[3].



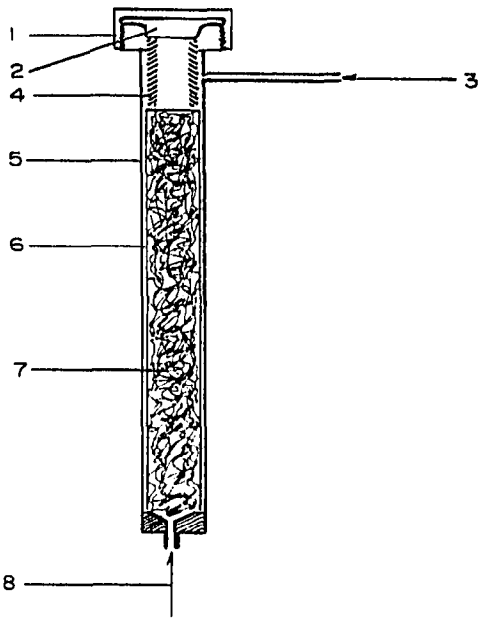


Fig. 1. Concentration tube. 1 = Glass tube; 2 = glass wool; 3 = heat-shrinkable PTFE; 4 = orifice of glass tube; 5 = supporting glass tube; 6 = PTFE tubing; 7 = helium inlet.

Fig. 2. Desorption system. 1 = Steel cap; 2 = PTFE plug; 3 = helium inlet; 4 = spring; 5 = steel supporting shell for concentration tube; 6 = glass tube (11 cm × 8 mm I.D.); 7 = glass wool; 8 = precolumn inlet.

Extraction of serum sample

To 100 μl of serum in a 3-ml glass tube (9 cm × 8 mm) with a PTFE-lined cap are added 1 ml of anhydrous diethyl ether and 0.05 g ammonium carbonate. This is mixed on a Vortex-type mixer for about 1 min until a gel has formed. The tube is then

centrifuged until the layers are well formed (20 min). The mixing and centrifugation are repeated. There are three layers in the tube. The top layer contains the diethyl ether extract, the middle layer some proteins, and the bottom layer the serum and salt. A 700-μl aliquot is taken from the top layer and injected by syringe into the concentration tube about one third from the bottom. This permits the solution to spread evenly over the glass wool. The diethyl ether is then stripped from the solution by passing helium through at 20 ml/min for 10 min. In the system shown in Fig. 1 the concentration tube is kept at room temperature. This tube is then removed and the moisture on the outside carefully wiped dry with adsorbent tissue (Scott microwipes). It is placed into the desorption system (Fig. 2) where a small trapping tube (precolumn) has been attached (75 cm $\times$ 0.5 mm I.D. coated with Emulphor ON870). The tube is heated to 250° and helium is passed through at 20 ml/min. Desorption of the volatiles takes place into the trap kept at liquid nitrogen temperature over a period of 10 min. This precolumn is then removed from the concentration system and placed into the chromatograph where it is attached to the front end of the capillary column.

Extraction of tissue sample

One gram of tissue (dog liver) is washed with 50 ml of 1% saline solution and twice with 50-ml portions of pure water ($KMnO_4$ distilled). Ten milliliters of this water are added and the mixture is homogenized for 1 min in a Polytron. A 100-μl aliquot of the homogenate is extracted in the same manner as the serum and subjected to the concentration procedure.

Chromatography

The column used in these experiments is made of 180 m $\times$ 0.5 mm I.D. nickel capillary tubing as previously described[16]. The chromatographic parameters are as follows: flow-rate, 9 ml/min nitrogen at 25°; temperature program, 10 min at 70°, then 2°/min to 150°; instrument, Varian 1200. Attenuation, 4.

RESULTS AND DISCUSSION

Diethyl ether was chosen as the extraction solvent because of its high volatility and its low retention on adsorbents such as glass wool. A dilute ether solution can be placed on the adsorbent and the solvent removed by passing helium through the system. Trace constituents of the solution will remain adsorbed on the glass wool. Thermal desorption into a chromatographic system yields the metabolic profiles.

Glass wool has good adsorptive properties for low-molecular-weight compounds such as those found on glass-fiber filters in air pollution studies[15]. It also has a very low affinity for polar materials such as ether and water. The thermal desorption temperature required for the volatiles in serum is 250°, which is relatively modest. When glass wool was used to obtain a blank for the analyses in the total system, a very small background was observed. All of these properties therefore make glass wool an excellent adsorbent for these analyses. Ammonium carbonate is used to facilitate the extraction of the serum components.

The chromatograms obtained from repeated runs of the same serum yielded almost identical results. It is also interesting to note that different sera give very similar

profiles (Fig. 3). This makes comparisons of the sera of normals with those of diseased patients much more useful.

This new procedure has many of the advantages of the Tenax head-space method and indeed has some of its own. (a) Samples may be stored after stripping of the diethyl ether from the glass wool. (b) Several samples of the same serum can be made by using aliquots. (c) Very little water is retained on the adsorbent thereby yielding no problems in the desorption step or for mass spectrometry. (d) The reagents and adsorbent are readily available in most laboratories.

In addition to the volatile metabolites the diethyl ether extract would be expected to contain compounds of high molecular weight including triglycerides, cho-

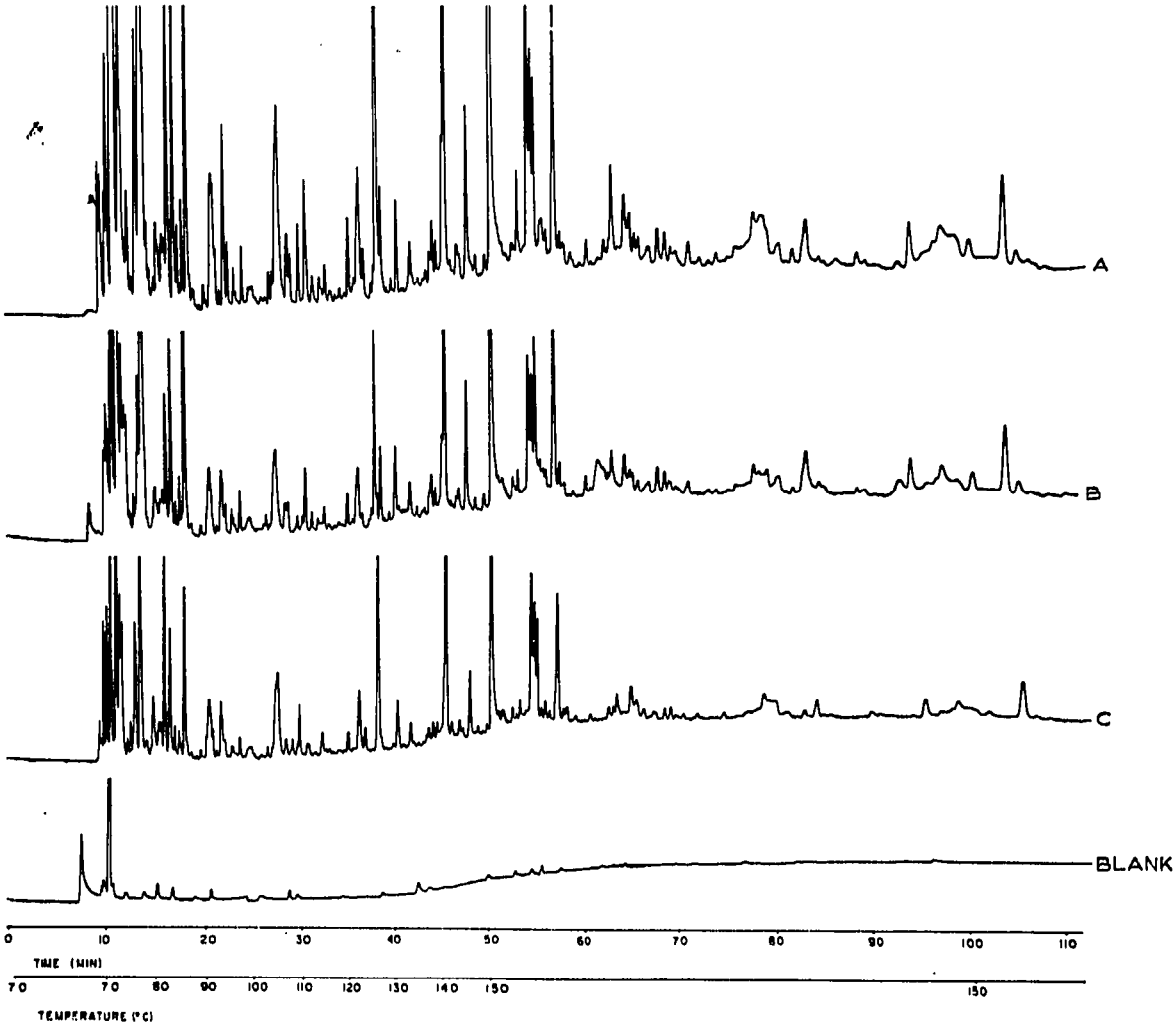


Fig. 3. Chromatogram of volatiles from three normal sera. The bottom chromatogram is the blank for the system. Column, 180-m × 0.5-mm-I.D. nickel tubing; stationary phase, Emulphor ON-870; operating conditions, as in text.

lesterol esters, free cholesterol, α-tocopherol, free fatty acids and traces of phospholipids. There is no evidence of carry-over of any decomposition products in the desorption step of the analysis described above.

The procedure is applicable to plasma, serum, urine, cerebrospinal fluid, saliva and semen. It can also be used for flavor studies such as tea, coffee, and alcoholic beverages. Although a 100-mg aliquot was taken of a 10-ml tissue extract, the method lends itself to the use of much smaller tissue samples for the determination of volatiles. This gives rise to its potential use in biopsies and in small animal studies. A typical chromatogram of dog liver tissue is shown in Fig. 4. The compounds in serum are presently being studied for identification by mass spectrometry.

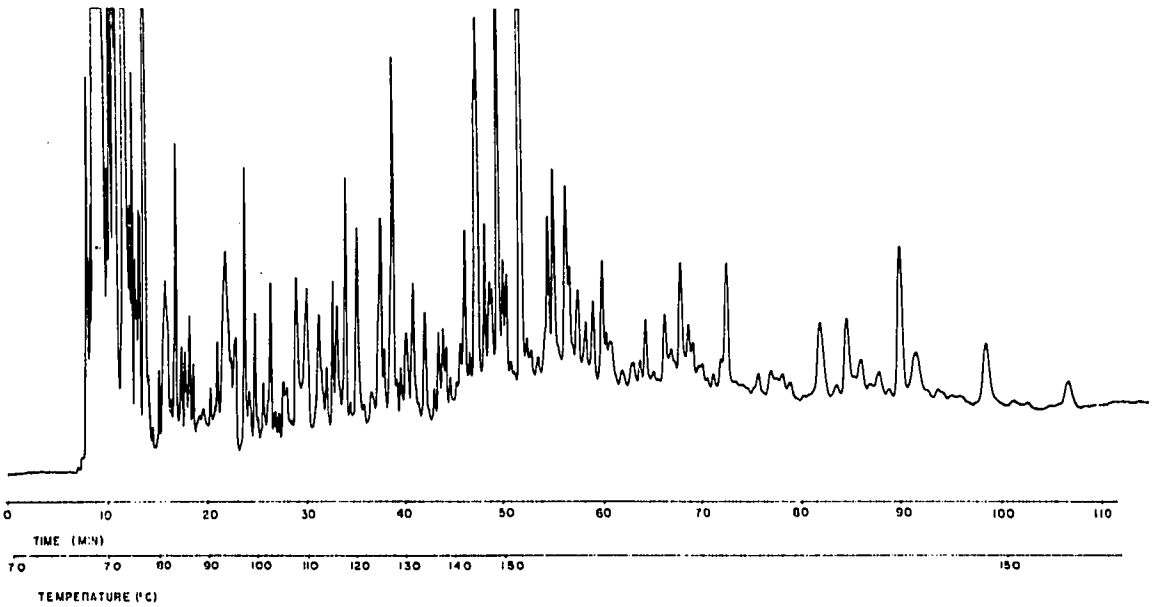


Fig. 4. Chromatogram of volatiles from dog liver tissue. Column, 180-m × 0.5-mm-I.D. nickel tubing; stationary phase, Emulphor ON-870; operating conditions, as in text.

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