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COLUMN ELUTION AND CONCENTRATION OF VOLATILE COMPOUNDS IN BIOLOGICAL FLUIDS

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SUMMARY

A new solvent elution method is described for the isolation and concentration of volatile metabolites in biological fluids. The procedure involves elution of the sample with a solvent through a micro-column of an adsorbent into glass-wool and evaporation of the excess solvent in a stream of helium at room temperature. The volatiles are recovered by subsequent heat desorption into a chromatographic system. Both elution and evaporation are made in a single step using a novel sampling device. Since the technique is based on liquid–solid adsorption chromatography, the adsorbent–solvent system which gives the maximum yield of volatile constituents was studied. The method is simple, highly efficient and reproducible, requiring only small volumes of biological fluids (less than 100 μl).

INTRODUCTION

Within the last few years, the profile studies of volatile metabolites in biological fluids have been the subject of considerable investigation. A primary goal of these studies has been to correlate characteristic chromatographic profiles to human normal and disease states. This area of research was pioneered by Zlatkis and Liebich[1]. Prior to gas chromatographic separation utilizing high-resolution columns, volatile components require isolation from their substrates, with subsequent concentration of these substances. Four techniques for isolation of volatile substances from dilute aqueous solutions are in common use: solvent extraction, steam distillation, vacuum distillation, and headspace analyses.

Solvent extraction[1,2], although relatively simple, introduces problems due to solvent impurities, selective extraction and loss of low-boiling compounds. On the other hand, headspace techniques frequently overemphasize the importance of the more volatile components. However, due to its good reproducibility, rapidity and simplicity, the headspace method has been a preferred technique. This technique has already been established and refined in detail in order to be more amenable for research and routine analyses in the laboratory[3,4].

The headspace method fails to provide a desirable sampling method in situations where only very small volumes of samples are available. For these circumstances,

a micro extraction procedure has been recently developed in our laboratory[5]. This sampling method involves single diethyl ether extraction of the sample saturated with ammonium carbonate, followed by concentration on a glass-wool trap by stripping off the excess diethyl ether. Since this simple method, like ordinary solvent extraction, requires vortex mixing, centrifuging and then transferring of the extract, the loss of some volatiles during these procedures is unavoidable. In order to isolate total volatiles from the micro sample without any loss and contamination, the sampling procedure should involve as few steps as possible.

As a continuation of the study on the total volatile metabolites in biological fluids, a more efficient, reliable, and rapid sampling method has been investigated. This paper describes the column solvent elution method for the sampling of volatiles present in serum in the order of 100 μl. The procedure utilizes the principle of extraction column chromatography developed on a macro scale[6].

EXPERIMENTAL

Reagents

Isopropyl chloride (Eastman-Kodak, Rochester, N.Y., U.S.A.) and methylene chloride, reagent grade (Mallinckrodt, St. Louis, Mo., U.S.A.) were distilled from phosphorus pentoxide, Baker Analyzed Reagent (J. T. Baker, Phillipsburg, N.J., U.S.A.), respectively.

Diethyl ether, anhydrous (Fisher Scientific, Pittsburgh, Pa., U.S.A.) was distilled from lithium aluminum hydride (Alfa Products, Danvers, Mass., U.S.A.).

Adsorbents

Porasil E, 80–100 mesh, and Anachrom 545A, 90–100 mesh, were obtained from Analabs (North Haven, Conn., U.S.A.); hydroxyapatite, spheroidal, from Gallard-Schlesinger (Long Island, N.Y., U.S.A.); Chromosorb P, regular, 80–100 mesh, from Varian-Aerograph (Walnut Creek, Calif., U.S.A.); Carbopack B-HT-100 from Supelco (Bellefonte, Pa., U.S.A.); and aluminium oxide, active, neutral (activity I) from EM Labs. (Elmsford, N.Y., U.S.A.).

Apparatus

The elution column was a glass tube (9.3 cm × 5 mm O.D. × 3 mm I.D.) containing an adequate amount of an adsorbent with a plug of glass-wool at both ends, shown in Fig. 1. Prior to packing, the adsorbents were conditioned at 380° by purging with nitrogen at 20 ml/min for 3 h. The glass-wool trap, serving as a concentrating column, was a glass tube (11 cm × 10 mm O.D. × 8 mm I.D.), tightly packed with glass-wool (Pyrex brand; Corning Glass Works, Corning, N.Y., U.S.A.). It was conditioned in the same manner as above. The elution apparatus consisted basically of a custom-made "transevaporator" and a conical-tip centrifuge tube with a glass stopper (Ⓢ 10/18), as shown in Fig. 1.

Sampling procedure

The volatile constituents in serum were isolated and concentrated in the following manner. The inlet of a "transevaporator" was connected to helium carrier and an elution column was attached to one end of the "transevaporator". A glass-wool

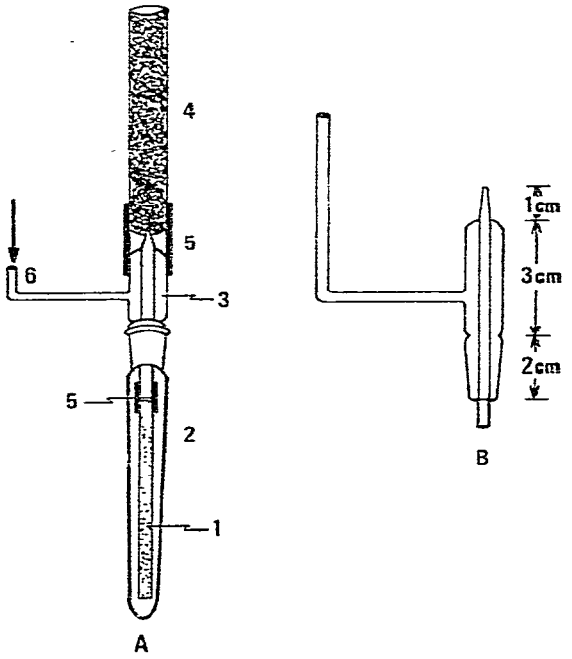

Fig. 1. (A), Elution sampling apparatus. 1 = Elution column; 2 = conical-tip centrifuge tube; 3 = "transevaporator"; 4 = glass-wool trap; 5 = heat-shrinkable PTFE sleeve; 6 = helium inlet. (B), Transevaporator.

trap was secured to the other end. A conical-tip centrifuge tube was fitted to the transevaporator. Gas-tight connections between glass and glass were made via shrinkable PTFE sleeves. The purified helium (cold trap) was allowed to flow through the elution column to the glass-wool trap and the needle valve was adjusted to give a 10 ml/min flow-rate at the trap. Once the flow had been controlled, the conical tube was disconnected from the transevaporator and a 100-μl serum sample was transferred into it with a syringe. The tube was connected back to the transevaporator and the serum was allowed to wet the elution column homogeneously for about 1 min with the aid of helium. After adsorption on the column, 1 ml of solvent, such as isopropyl chloride, diethyl ether or methylene chloride, was introduced to the tube, thereby starting the solvent-elution process.

High-molecular-weight substances, solids and water were retained by the column adsorbent. Only the volatile and soluble constituents were eluted from the column together with solvent and flowed to the glass-wool trap, from which excess solvent was stripped. Sample blanks were prepared by the above procedure, except for the addition of serum to the sampling tube. After the evaporation step, the trap was disconnected from the transevaporator, and inserted into a modified injector port[4], maintained at 280–300°, the outlet of which was connected to a pre-column cooled at liquid nitrogen temperature. The concentrated volatiles were thermally desorbed from the trap by purging with helium at 6 ml/min for 20 min and condensed in the form of a plug in the pre-column (stainless steel, 50 cm × 1 mm I.D., coated with the same phase as the separating column).

Gas chromatographic separation

A Hewlett-Packard Model 5830A gas chromatograph with flame ionization detector and a Type 18850A GC Terminal (Hewlett-Packard, Avondale, Pa., U.S.A.) was used in this study. A separating column (stainless steel, 100 m × 0.5 mm I.D.) was coated with 10% Witconal LA-23 (Witco, Houston, Texas, U.S.A.) in methylene chloride. After desorption, the pre-column was connected to a separating column and the gas chromatographic run was started at a flow-rate of 6 ml helium/min at 25°, according to the preset operating parameters: isothermally at 60° for 5 min, programming 2°/min to 160°, and held for 100 min.

RESULTS AND DISCUSSION

Solvents

Diethyl ether is the most widely used solvent for the extraction of the volatile components from biological fluids[1,2]. However, it has some disadvantages such as low stability and high contamination, and also purification by distillation requires the utmost care. Therefore, it was decided to search for another solvent which could serve the same purpose.

The best candidate should have: (1) high volatility, (2) high solvent power with low water solubility, and (3) high stability. With these requirements in mind, a thorough solvent survey was made. The two solvents isopropyl chloride and methylene chloride were the most promising from the standpoint of physical properties, as shown in Table I.

TABLE I

COMPARISON OF PHYSICAL PROPERTIES OF SOLVENTS

Physical properties	*Diethyl ether*	*Isopropyl chloride*	*Methylene chloride*
Boiling point (°C)	34.55	35.74	39.75
Density	0.71	0.86	1.33
Dielectric constant	4.33	9.82	8.93
Dipole moment	1.15	2.02	1.14
Water solubility (%)	6.04	1.30	0.34

A micro extraction technique[5] was employed for testing the solvent power. Isopropyl chloride was found to be comparable to diethyl ether, as illustrated by Fig. 2. Because of its higher density, methylene chloride was not applicable to the extraction method, but was satisfactory for the elution method. As expected, methylene chloride has a high solvent power. Its boiling point, however, is relatively high compared with those of diethyl ether and isopropyl chloride, thus requiring about 10 min more to strip off the excess methylene chloride. Isopropyl chloride was therefore the solvent of choice. It gave a considerably high yield of volatiles and the chromatograms did not reveal any artifacts.

Adsorbents

The elution column behaves as a selective filter for fractionating out the volatile components from the non-volatile matrix. Therefore, the proper adsorbent

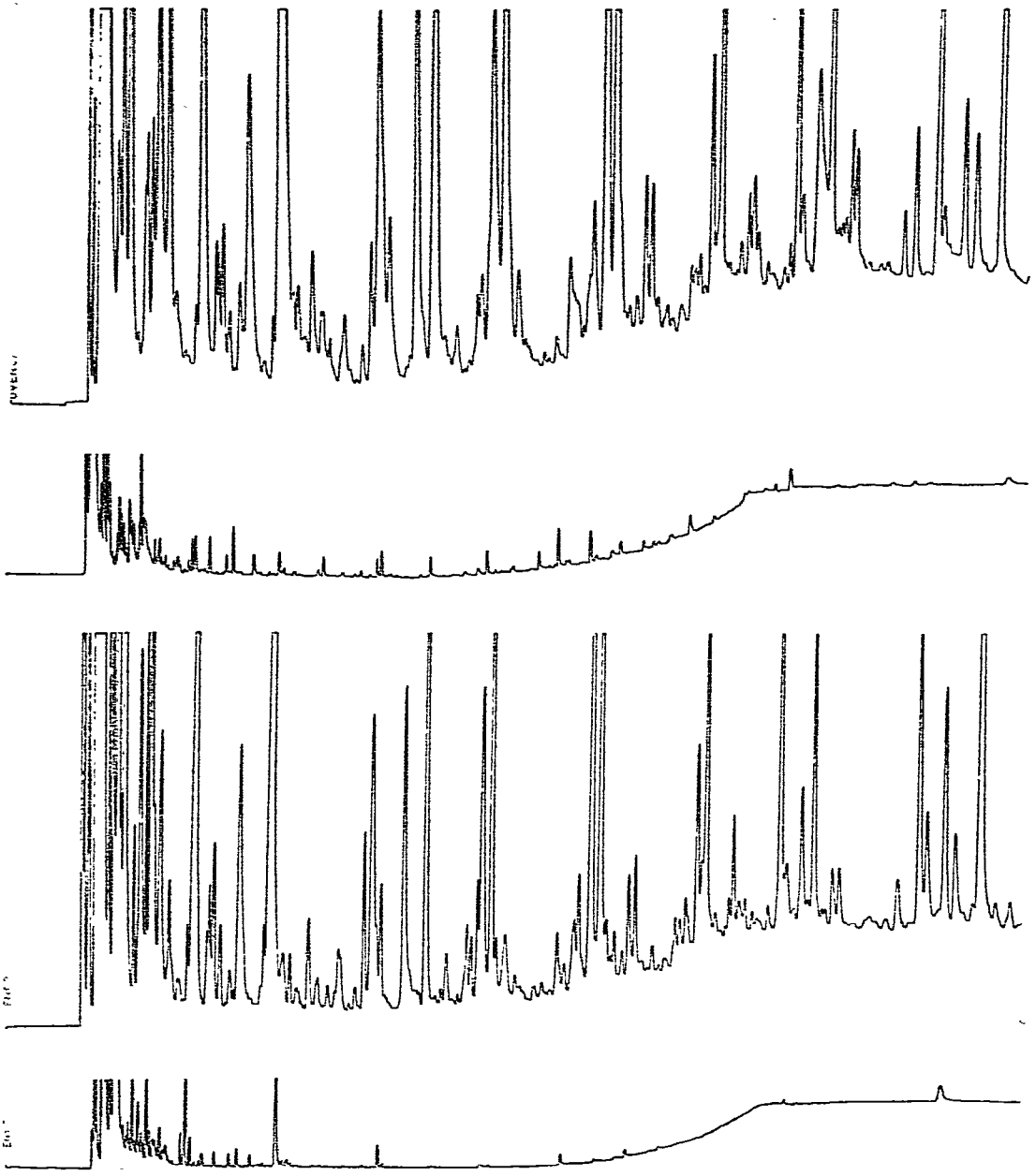

Fig. 2. Partial chromatogram of volatile compounds in 150 μl of serum extracted with 1 ml of solvent. Top: diethyl ether extract; bottom: isopropyl chloride extract. For sampling and operating conditions, see text.

of the elution column should be moderately hydrophilic, in order to hold the water component preferentially, and non-extractable with a given solvent. From these considerations, porous polymers are not applicable, and only the inorganic adsor-

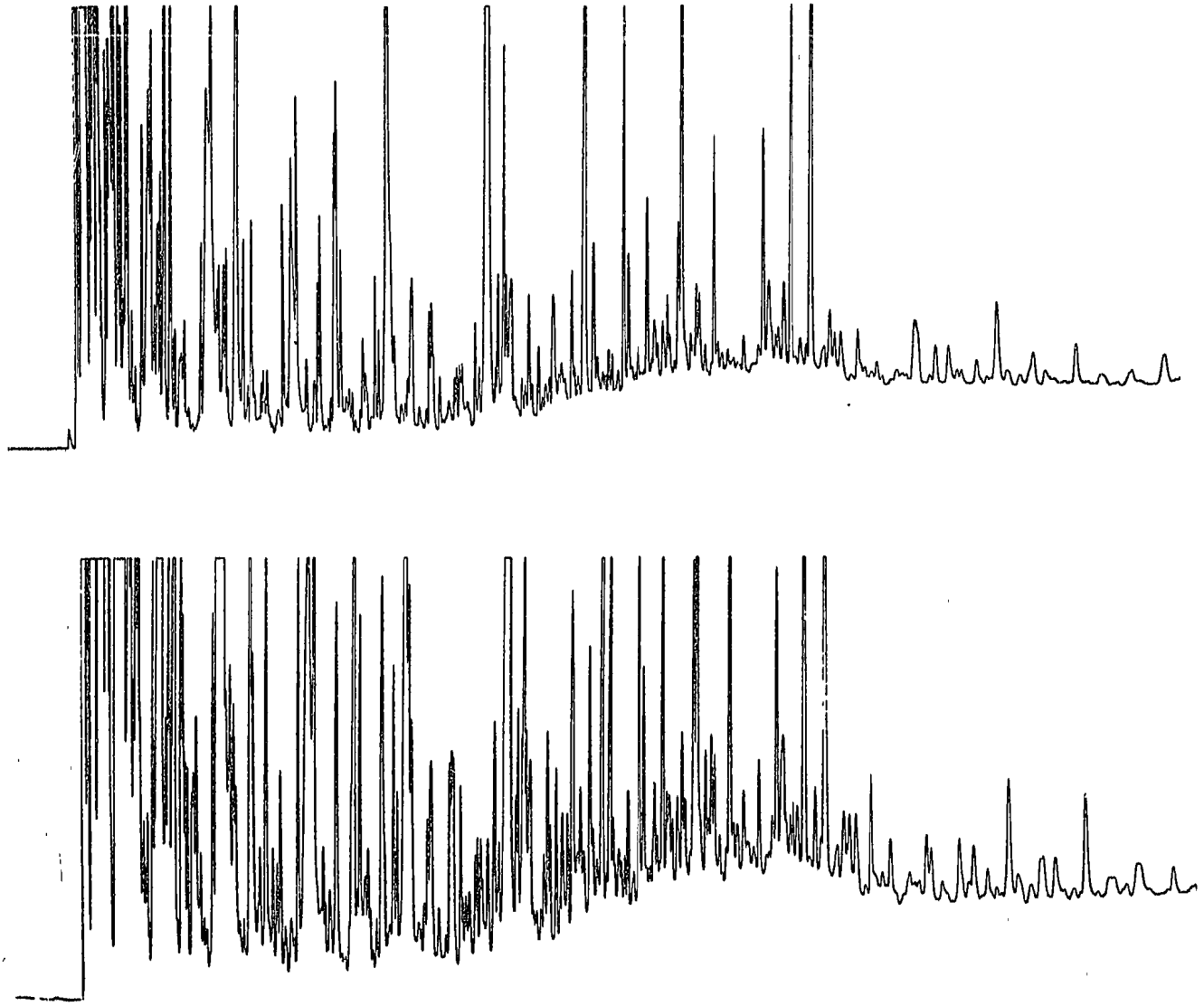

Fig. 3. Chromatogram of volatile compounds in 100 μl of serum eluted with 1 ml of isopropyl chloride through an elution column. Top:

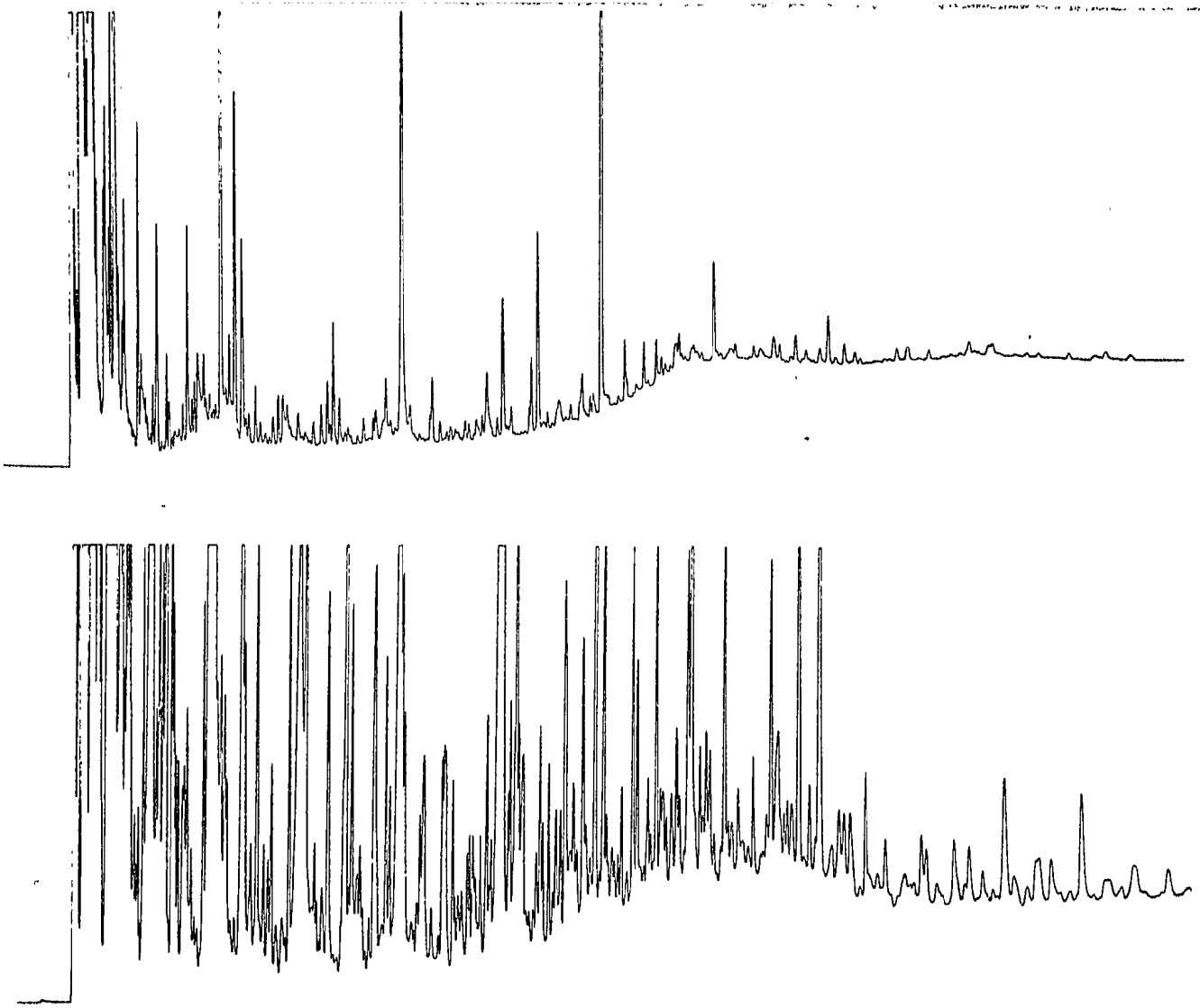

Fig. 4. Chromatogram of volatile compounds in 100 µl of serum obtained with elution method. Top: 1 ml of isopropyl chloride through alumina; bottom: 1 ml of isopropyl chloride through Porasil E. For sampling and operating conditions, see text.

bents are to be preferred. The adsorbent should be in the form of porous, mechanically stable and spherical particles, so that the particles can be packed into the micro column rapidly and uniformly, giving stable and incompressible columns with reproducible flow-rates.

With isopropyl chloride as the eluting agent, experimental selection of the proper adsorbent which would give the maximum yield has been made. All other conditions were kept constant except the amount of each adsorbent. Due to the varying adsorptivities, the optimum size of adsorbent for a given amount of sample differed for each of the adsorbents. Table II shows a list of properties of the adsorbents which have been examined. It was obvious that the sampling efficiency depends on the amount of a given adsorbent, as demonstrated by Fig. 3. The optimum result was obtained with a Porasil E column and the least desirable with that of alumina. Fig. 4 shows the profiles obtained from the two adsorbents. The other adsorbents gave about the same profiles as that with Porasil E in qualitation, but not in quantitation. Interestingly, in the case of diethyl ether as the eluting agent, both Porasil E and alumina gave good results.

TABLE II

PHYSICOCHEMICAL CHARACTERISTICS OF ADSORBENTS

Adsorbent	*Type of material*	*Polarity*	*Specific surface area (m^2/g)*	*Mesh*	*Relative adsorptivity**
Porasil E	Porous silica beads	polar	25	80–100	0.33
Chromosorb P	C-22 firebrick	polar	4	80–100	0.25
Alumina	Active aluminum oxide	polar	100–200	—	0.25
Hydroxyapatite	Porous, spheroidal hydroxyapatite of calcium phosphate	polar	—	—	0.2
Anachrom 545A	Flux-calcined diatomaceous earth	polar	1	90–100	0.17
Carbopack B	Graphitized carbon black	non-polar	100	—	<0.15

* Adsorptivity was calculated according to the following formula: amount of serum used/amount of adsorbent needed.

Porasil E is in the form of perfectly spherical beads of porous silica. According to Cadogan and Sawyer[7], upon heating from 200–500°, silica loses all hydrogen-bonded water and most of the coordinated water molecules from its surface, and surface free hydroxyls are converted to reactive hydrogen-bonded hydroxyls serving as the site of adsorption.

Since Porasil E was conditioned at 380° prior to use, it might become more polar. Water in serum was retained by Porasil E after elution, thus eliminating the water as a problem. The optimum amount of Porasil E for 100 μl of serum was in the order of 0.3 ml and the sampling time including both elution and evaporation was about 27 min.

Sampling procedure

The main purpose of this work was to develop a better sampling procedure in terms of efficiency, sampling time, and simplicity. Fig. 5 compares the efficiency of

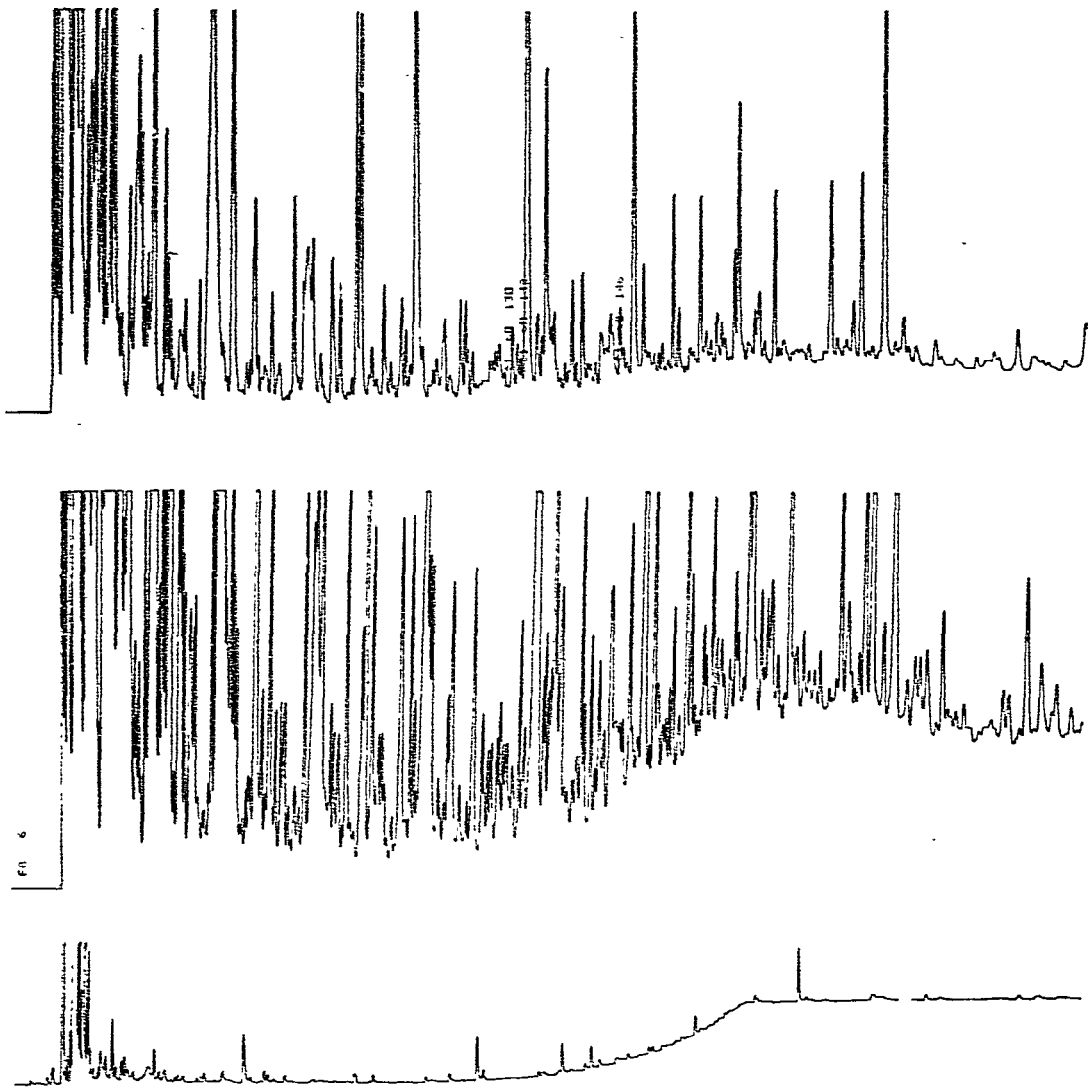

Fig. 5. Chromatogram of volatile compounds in 100 μl of serum. Top: extraction with 1 ml of isopropyl chloride; middle: elution with 1 ml of isopropyl chloride through a Porasil E column; bottom: blank. For sampling and operating conditions, see text.

extraction and elution (through Porasil E) methods for the same amount of the same serum sample. Qualitatively, the two profiles are comparable, but quantitatively, the elution profile is about three times better than the extraction profile. The sampling time needed for the elution method was less than 30 min, but more than 40 min for the extraction method. The elution apparatus provided both efficient elution and evaporation in a single step. In regular intervals, blanks were run to control the total sampling and gas chromatographic procedure for contaminants. This was particularly important for the sample of micro size. A 25-μl sample of serum was eluted through a col-

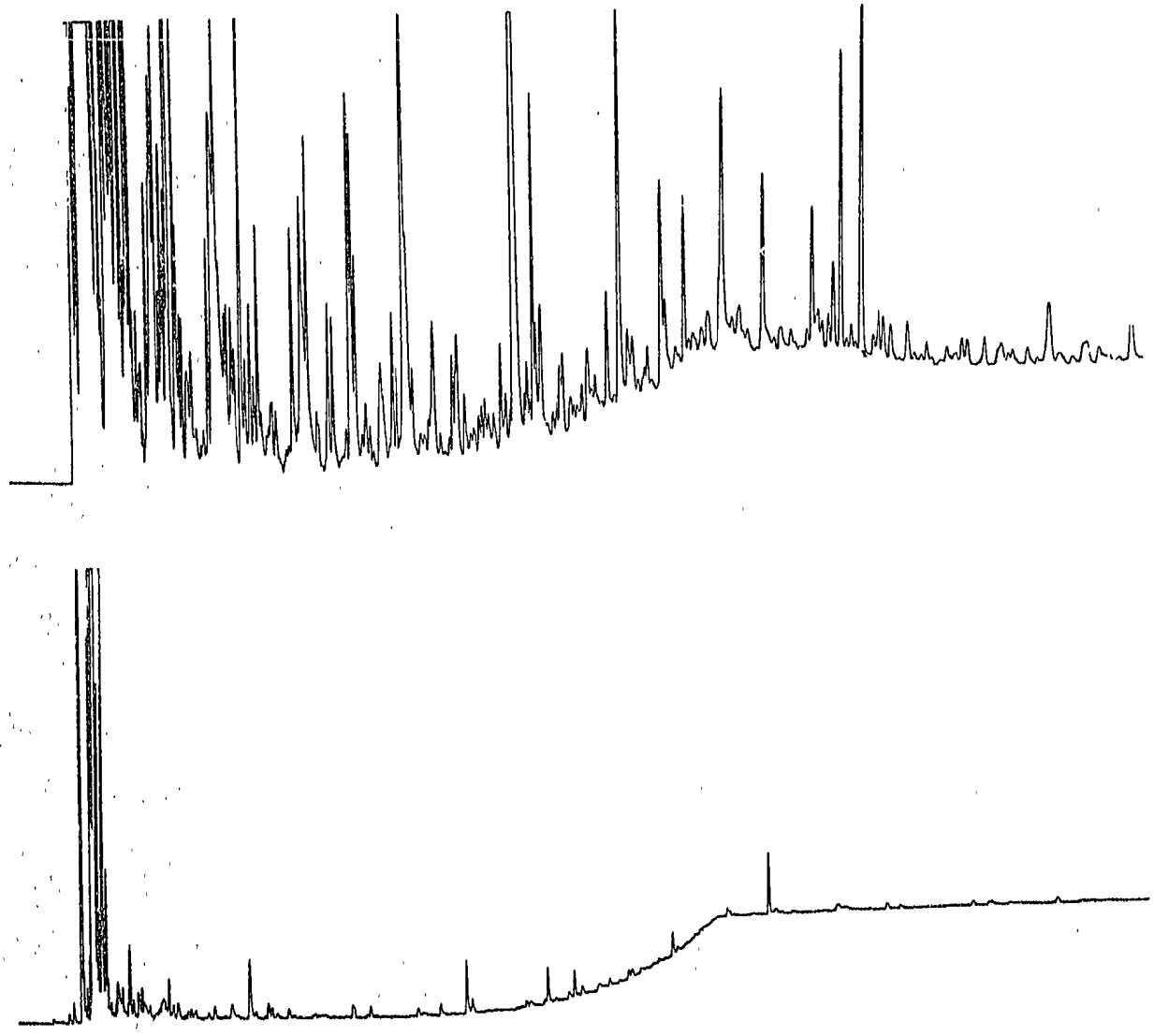

Fig. 6. Chromatogram of volatile compounds in 25 μl of serum eluted with 1 ml of isopropyl chloride through a column containing 0.1 ml of Porasil E. Bottom: blank. For sampling and operating conditions, see text.

umn (3 mm O.D., 2 mm I.D.) containing 0.1 ml of Porasil E with 1 ml of isopropyl chloride. Fig. 6 shows that such a sample was sufficient for a gas chromatographic profile.

The sampling procedure developed should prove useful for a wide range of materials other than blood specimens in determining trace amounts of volatiles in an aqueous medium such as urine, cerebrospinal fluid, saliva, flavors, and contaminated water. Identification of the compounds shown on the profiles is presently being made by mass spectrometry and will be reported subsequently.

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