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PROFILES OF VOLATILE METABOLITES IN BODY FLUIDS

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SUMMARY

A method for the analysis of volatile metabolites present in plasma, urine, breast milk and amniotic fluid collected from mother–infant pairs has been developed which requires only 100 μl of plasma, 3 ml of urine, 20 μl of breast milk and 500 μl of amniotic fluid. After extraction with diethyl ether, the volatile compounds were adsorbed on glass wool in a special concentration tube and subsequently desorbed and transferred to a 100-m nickel capillary column for analysis by gas chromatography and gas chromatography–mass spectrometry. The separations, carried out by temperature programming, were complete in 90 min.

INTRODUCTION

Methods for the analysis of the volatile compounds in human urine were developed by Zlatkis and co-workers[1–3] and by Matsumoto *et al.*[4]. Ketones, aldehydes, alcohols, furans, pyrroles and sulfides with molecular weights between 40 and 160 were separated and tentatively identified by capillary gas chromatography (GC) and mass spectrometry (MS). The volume of urine employed varied from 50 to 450 ml. The procedure has been employed in a study of the low-molecular-weight aliphatic alcohols excreted in the urines of hospitalized patients with diabetes mellitus[5].

A procedure for the analysis of volatile components in plasma based on liquid–liquid extraction followed by capillary GC was developed by Stoner *et al.*[5]; 15 ml of plasma was used. Subsequently, Zlatkis and Andrawes[6] developed a semi-micro-extraction procedure based on the use of ammonium carbonate–diethyl ether as a salt–solvent pair[7]. The volatiles extracted from 100 μl of plasma were concentrated by adsorption on glass wool, and then desorbed and transferred to the capillary column for GC analysis.

The results of Zlatkis and Andrawes[6] suggested that a method could be developed for the analysis of volatiles in biological fluids which involved considerably smaller volumes than had been employed in earlier studies. This paper describes a general method for the analysis of volatile compounds in plasma, urine, breast milk

and amniotic fluid. The size of the biological samples varied from 20 μl (breast milk) to 3 ml (urine).

EXPERIMENTAL

The biological samples analyzed in this study were obtained from mother–infant pairs. Maternal urine, plasma and amniotic fluid were obtained at the time of delivery; breast milk was obtained from the mother after delivery. Neonatal urine and plasma were collected during the first 48 h after birth. All of the samples were stored at −14° until analyzed.

The volatile compounds were extracted by the use of ammonium carbonate–diethyl ether as a salt–solvent pair. The sample (100 μl plasma, 3 ml urine, 500 μl amniotic fluid, or 20 μl breast milk) was transferred to a 12-ml centrifuge tube fitted with a PTFE-lined screw cap. After saturation of the aqueous phase with anhydrous ammonium carbonate, the volatiles were extracted with glass-distilled diethyl ether that had been refluxed over lithium aluminum hydride prior to distillation. The volume of diethyl ether used was 500 μl, 1 ml, 750 μl and 100 μl for plasma, urine, amniotic fluid and breast milk, respectively. The contents of the tube were mixed thoroughly on a Vortex mixer and the layers were separated by centrifugation. After chilling the tube in an ice-bath, the diethyl ether layer was transferred to a 2.5-ml glass stoppered centrifuge tube and stored at −14° until analyzed.

For analysis by GC, the diethyl ether extract was transferred with a 1.0-ml syringe equipped with a 5-inch 22-gauge needle to a small concentration tube packed with 0.5 g Pyrex glass wool[6]. The volatile compounds were desorbed and transferred from the concentration tube to a stainless-steel precolumn (12 in. × 1/16 in.), cooled in liquid nitrogen. The transfer was carried out by raising the temperature of the concentration tube to 240° while sweeping with helium (30 ml/min). After a 15-min trapping period, the liquid nitrogen reservoir was removed from the precolumn, and the temperature of the GC column was raised to 70° and held for 15 min. The separations were carried out by temperature programming from 70 to 150° at 2°/min and then isothermally at 150° for 50 min.

A Tracor 550 gas chromatograph equipped with a modified injection port and flame ionization detector was employed. The 100 m × 0.5 mm I.D. nickel column coated with Emulphor ON-870 was prepared according to the procedure of Bertsch *et al.*[8]. The flow-rate of helium, the carrier gas, was 30 ml/min. The flow-rates of the detector gases were: hydrogen, 37 ml/min; air, 100 ml/min; and nitrogen make-up gas, 10 ml/min.

The MS analyses were carried out with an LKB Model 9000 gas chromatograph–mass spectrometer; the separator was kept at 150° and the source temperature was 190°. For analysis by GC–MS, a heating block and concentration tube were placed in series with a 12 in. × 1/16 in. O.D. stainless-steel precolumn and the GC column. A PTFE micro-needle valve was placed between the precolumn and the GC column so that the precolumn and flash heater could be opened for insertion of the concentration tube while the GC column and the separator of the mass spectrometer were maintained under vacuum[9]. The separations were carried out by temperature programming from 70 to 150° as described above.

RESULTS

Plasma

Profiles of the volatile compounds present in maternal and neonatal plasma are shown in Figs. 1 and 2. The samples were obtained from a mother–infant pair

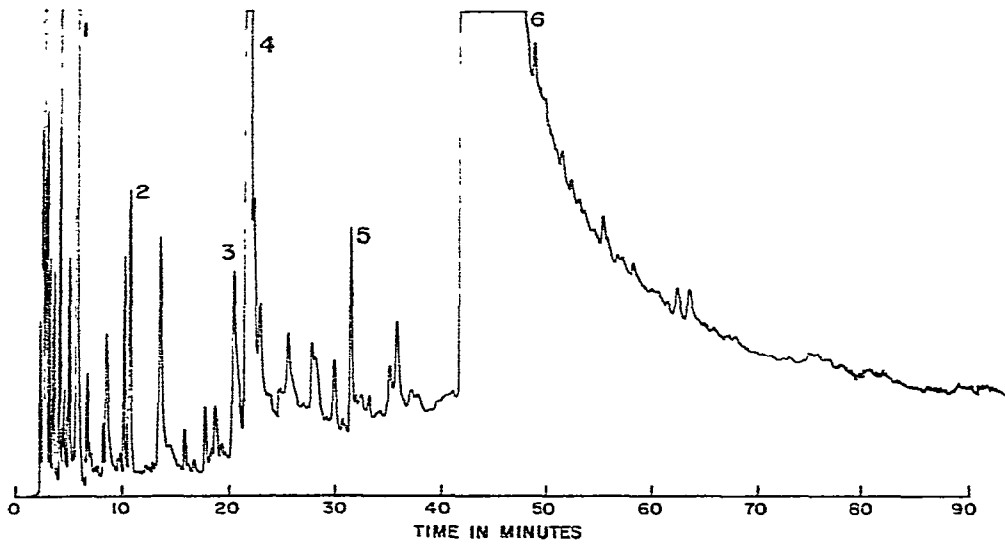


Fig. 1. GC analysis of the volatiles extracted from 100 μl of plasma obtained from a newborn infant. The compounds tentatively identified by GC–MS were: (1) 5-acetonyl-2-methylfuran; (2) ethylcyclopentanone; (3) ethylcyclohexanone; (4) benzaldehyde; (5) 1-cyclohexan-2-buten-4-ol; and, (6) benzyl alcohol. The GC conditions employed for all the analyses are described in the text.

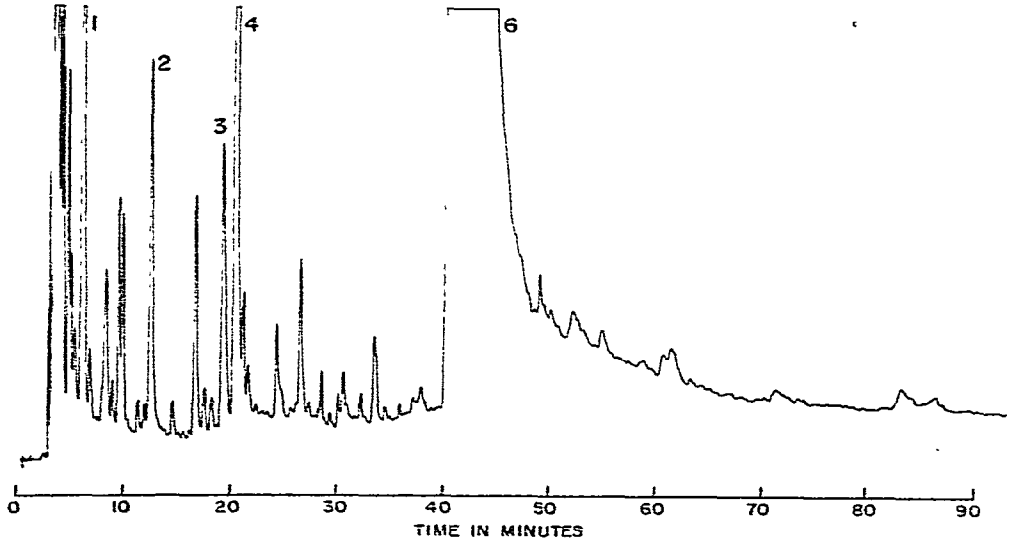


Fig. 2. GC analysis of the volatiles extracted from 100 μl of plasma obtained at the time of delivery from the mother of the newborn infant (Fig. 1). The compounds tentatively identified were the same as those listed in Fig. 1.

and the profiles were strikingly similar. The maternal plasma profile differed from the profiles of normal adults described by Zlatkis and Andrawes[6] in that a very large peak with a retention time (t_R) of 40–48 min was observed in the maternal plasma profile obtained at delivery. For comparison plasma samples were obtained from young adult females who were not pregnant and were not taking any medication. When the latter samples were analyzed for volatile components by the same procedure, it was found that the large peak with a t_R of 40–48 min was absent from the chromatograms. Fig. 3 shows a typical profile of the volatiles isolated from the plasma of a non-pregnant female, 27 years of age.

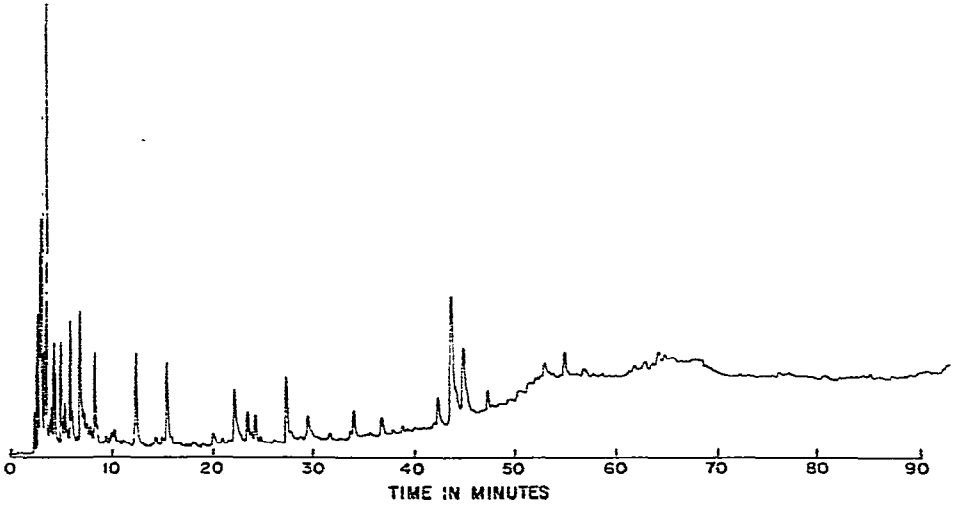


Fig. 3. GC analysis of the volatile components extracted from 3-ml aliquot of a urine sample obtained from a young adult non-pregnant female.

When the samples were analyzed by GC–MS, it was possible to tentatively identify several of the plasma volatiles (Fig. 1). The mass spectrum of the major component (t_R = 40–48 min) exhibited a molecular ion at *m/e* 108 and the spectrum was identical with the published spectrum[10] of benzyl alcohol (MW = 108).

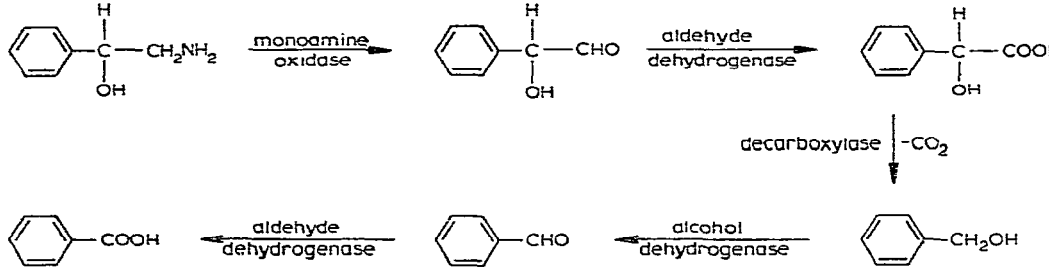
In order to confirm the identity of this component, a larger sample (300 μl) of maternal plasma was extracted with diethyl ether as described. After removal of the diethyl ether with a nitrogen stream, the residue was dissolved in 100 μl of pyridine and acetylated with 50 μl of acetic anhydride by heating at 60° for 15 min. When the derivatized sample was analyzed by GC–MS, the molecular ion of the major peak in the chromatogram had shifted by 42 a.m.u. from *m/e* 108 to *m/e* 150. The mass spectrum of the acetylated derivative in the biological sample was identical with the mass spectrum of benzyl acetate prepared by acetylation of benzyl alcohol.

A second characteristic peak present in the plasma profile had a t_R of 21–23 min. MS analysis of this peak indicated that the molecular ion occurred at *m/e* 106. The occurrence of a component with a molecular weight 2 a.m.u. less than benzyl alcohol suggested that this compound might be benzaldehyde. The t_R and mass

spectrum of this plasma component and the t_R and mass spectrum of a benzaldehyde standard were identical.

At the present time, the origin of the two volatiles, benzaldehyde and benzyl alcohol, is not known. Medication administered to the mother during labor and delivery, which included oxytocin, pethidine, promethazine and diazepam, did not appear to be the source. The two compounds were not artifacts introduced during the isolation procedure. Neither compound was present in the chromatogram when reagent blanks were analyzed; in the blanks, water was substituted for blood in a heparinized tube and the extractions were carried out as described.

If the benzyl alcohol and benzaldehyde found in maternal and neonatal plasma were of endogenous origin, they may have been formed from phenylethanolamine by a minor pathway of metabolism which has been described for phenylethylamine moieties[11]. In this pathway the side-chain is shortened and this conversion is most likely to occur with amines having a β-hydroxyl group. For example, 3,4-dihydroxybenzoic acid and 4-hydroxy-3-methoxybenzoic acid were identified as metabolites of noradrenaline in the rat. The corresponding alcohols were not found in rat urine[11]. However, the alcohols formed by decarboxylation of vanilmandelic acid and dihydroxymandelic acid were presumably intermediates in the metabolic conversion to the benzoic acid derivatives. It is possible that a related enzyme system which converts phenylethanolamine to benzyl alcohol, benzaldehyde and benzoic acid is operative in humans:



Urine

A typical profile of the volatile compounds present in maternal and neonatal urine is shown in Figs. 4 and 5. The compounds identified by MS analysis were acetonylfuran, acetophenone, 2-methyl-4-hexene, ethylcyclohexane, hexanol, acetic acid, and benzyl alcohol.

Two large off-scale peaks were usually present in the chromatograms of extracts of neonatal urine. The peak with a t_R of 23 min was identified by GC–MS as a mixture of 2-methyl-4-hexene and ethylcyclohexane. The identity of the peak with a t_R of 47 min is under investigation.

Breast milk

A chromatogram of the volatile components in a sample of human breast milk collected 30 days after delivery is shown in Fig. 6; only 20 μl of breast milk was used in the extraction. Characteristic peaks eluting at 5 min (hexanal), 19–20 min

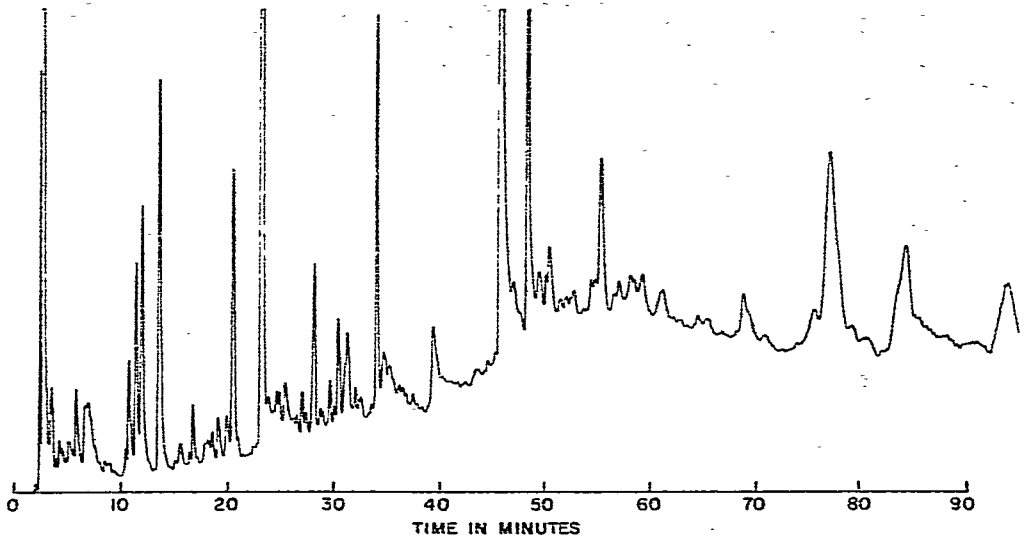


Fig. 4. GC analysis of the volatile compounds extracted from neonatal urine (3 ml).

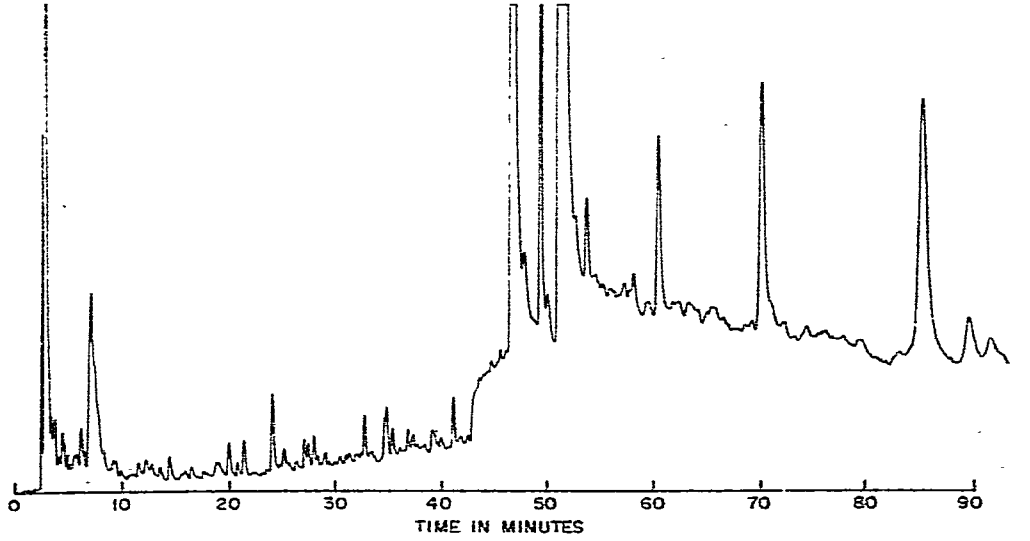


Fig. 5. GC analysis of the volatile compounds extracted from maternal urine (3 ml).

(ethylcyclohexenone andethylcyclohexanone), and a triplet of peaks at 38–42 min (pentenylcyclohexene, and aminopentanylfuran and its isomer) were tentatively identified by GC–MS. Profiles obtained from three different individuals were very similar; although there were quantitative differences, there were no significant qualitative differences in the profiles. The volatile compounds in breast milk that have been characterized by GC–MS are listed in Table I.

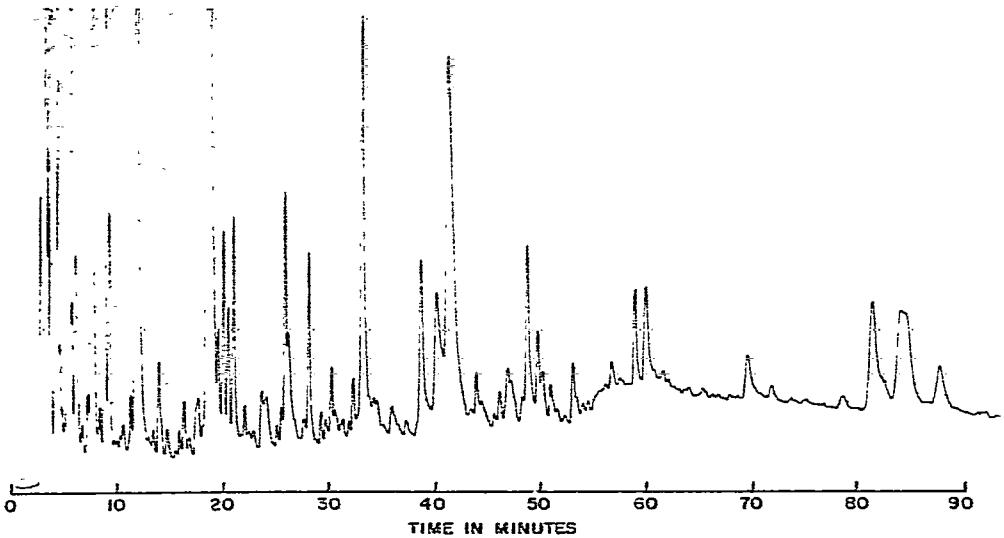


Fig. 6. GC analysis of the volatile compounds extracted from human breast milk (20 μl).

TABLE I

VOLATILE COMPONENTS OF HUMAN BREAST MILK

Tentative identification based on GC–MS analysis.

Compound	*Compound*
hexanal	methylhexanal
heptanal	2-methyl-4-hexene
6-methyl-2-heptanone	2-methylamylnitrile
2-pentylfuran	hexanol
ethylcyclopentanone	4-methyl-1-pentene
acetonylfuran	pentenylcyclohexene
ethylcyclohexanone	aminopentanylfuran
ethylcyclohexenone	

Amniotic fluid

A typical analysis of the volatiles present in amniotic fluid is shown in Fig. 7; the sample was collected 4 h prior to delivery. Two large peaks with retention times of 17 and 81–82 min were usually observed. Because of the small amount of fluid available and the low concentration of the volatiles in amniotic fluid, it was not possible to carry out MS analyses. It is of interest that the doublet eluted between 58–62 min was also present in breast milk.

From the examples cited, it is evident that characteristic profiles of the volatile components of body fluids can be obtained with quite small samples. The overall procedure including isolation and GC analysis requires 2–3 h. A number of components have been tentatively characterized by GC–MS. The excretion of relatively large amounts of benzyl alcohol is of particular interest if this compound is found to be of endogenous origin.

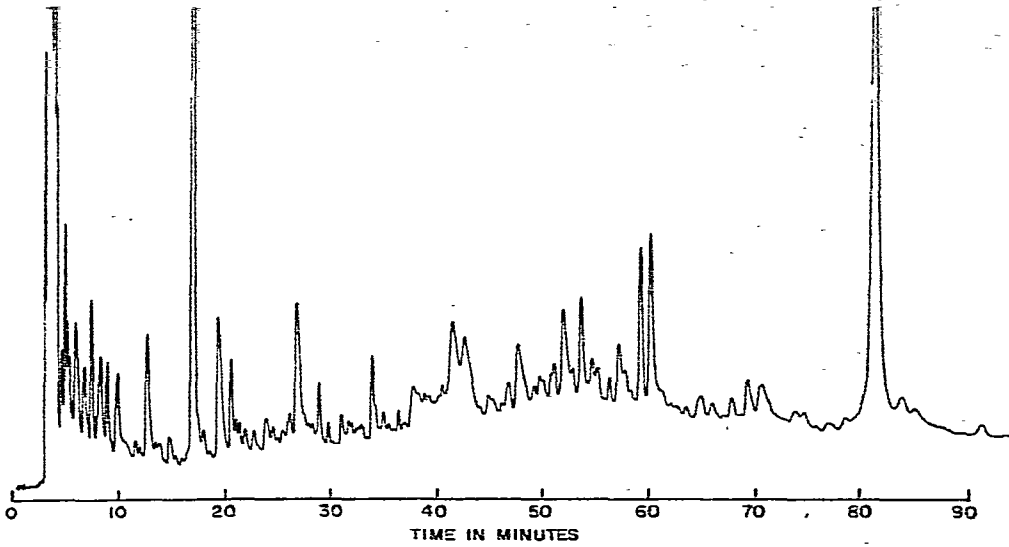


Fig. 7. GC analysis of the volatile compounds extracted from human amniotic fluid (500 μl).

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