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GAS CHROMATOGRAPHY OF VOLATILE FATTY ACIDS

METHOD INVOLVING SEPARATION FROM BIOLOGICAL MATERIAL BY VACUUM DISTILLATION

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SUMMARY

A method is described for the quantitation of C_2–C_5 volatile fatty acids present in biological tissues. It involved recovery of the acids from their biological matrix by vacuum micro-distillation at room temperature, followed by gas phase separation of aqueous solutions on orthophosphoric acid-modified Phasepak Q columns. The subsequent gas chromatographic procedure resolved iso from normal isomers and showed a linear response for each volatile acid over the range 10–400 ng. There was no evidence of ghosting, isomer peak broadening, or peak tailing. Relative molar response values were shown to be linear with carbon number for all the volatile fatty acids studied.

INTRODUCTION

Investigations, already reported elsewhere[1,2], of the volatile fatty acid content of carious enamel, dental plaque and other oral components, necessitated separation of the acids from these substrates for quantitative analysis by gas–liquid chromatography. Although direct injection of whole biological material into the gas chromatograph is possible in principle, this inevitably leads to rapid contamination and deterioration of the column, with resultant reduction in efficiency and ghosting[3]. Even after separation of the volatile acids from a substrate it is possible for ghosting and tailing to occur in the presence of water, if the column and column conditions are not correctly chosen. This is especially true at the low concentrations involved in the present work. Furthermore, good quantitative separation of the normal and iso-acids in aqueous solution is difficult, and as Kaplanová and Janák[4] have reported, published methods are often difficult to reproduce.

The present paper describes a system for isolating submicrogram quantities of volatile fatty acids (C_2–C_5) from their substrates, by vacuum distillation, before separation on a column of orthophosphoric acid-modified Phasepak Q. This modified polystyrene support material is similar to that recommended by Mahadevan and Stenroos[5] for the gas phase separation of volatile fatty acids in aqueous solution.

EXPERIMENTAL

Apparatus

The vacuum microstill is shown in Fig. 1. It consisted of an inverted U-tube with B10 sockets, one fitted with a 7-mm-bore distillation tube and the other with a small tapered receiver tube. The system was evacuated using a single-stage Edwards backing pump, operating through a liquid nitrogen vapour trap, and giving a vacuum of between 10^{-3} and 10^{-2} torr ($\approx$ 0.1 and 1 Pa). Joints and taps were sealed with silicone grease.

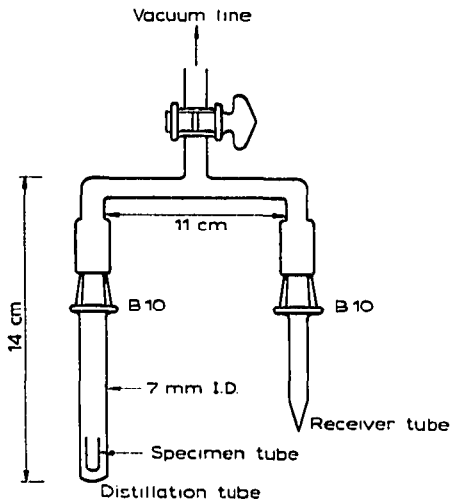

Fig. 1. Low-temperature vacuum microstill. Inverted borosilicate glass U-tube with detachable distillation and receiver tubes, lubricated with high-vacuum silicone grease.

The gas chromatograph was a Perkin Elmer Model F11, with dual flame ionisation detectors, operating under the following conditions: Column, dual 2-m glass, 3 mm I.D., containing 2% orthophosphoric acid-modified Phasepak Q; temperature programme, isothermal (column temperature 180°); injection, direct into column (temperature 180°); carrier gas, nitrogen, flow-rate 60 ml/min; hydrogen pressure, 18 lb. in.$^{-2}$ ($\approx 1.2 \times 10^5$ Pa); air pressure, 40 lb. in.$^{-2}$ ($\approx 2.8 \times 10^5$ Pa); attenuator range, 20–100 $\times$ 10^{-2} A for f.s.d.; chart recorder range, 0–2.5 mV f.s.d., speed 30 in./h; peak areas were measured by triangulation.

Materials

Phasepak Q (85–100 mesh), a cross-linked polystyrene support material, was obtained from Phase Separations (Queensferry, Great Britain). The acids used were purchased from BDH (Poole, Great Britain) without further purification.

Coating of support and column packing

A slurry of Phasepak Q (85–100 mesh) was made up with absolute methanol containing sufficient orthophosphoric acid to produce a 2% loading, evaporated to dryness in a rotary evaporator and finally dried at 100° for 3 h.

Packing was achieved by applying suction to the column and adding increments of the modified support phase. The columns were tapped to ensure uniform packing, avoiding excessive vibration which can lead to the production of fines. Porous PTFE plugs, with an approximate pore diameter of 46 μm, were used to retain the support phase within the columns. New columns were aged at 200° for 24 h, with a nitrogen carrier gas flow-rate of 20 ml/min.

Method

A standard stock solution was prepared, which contained 0.04% v/v of the following volatile fatty acids in 1 *M* hydrochloric acid: acetic, propionic, isobutyric, *n*-butyric, isovaleric, *n*-valeric, and *n*-hexanoic acids. This stock solution was successively diluted with 1 *M* hydrochloric acid solution containing 0.04% v/v of the internal standard *n*-hexanoic acid alone. In this way, known concentrations (10–400 ng/μl) of each acid up to C_5 were obtained, with the internal standard *n*-hexanoic acid maintained at a fixed concentration of 0.04% v/v (384 ng/μl) in all solutions. The 1 *M* hydrochloric acid was included in order to duplicate the situation when biological material (carious enamel for example) has been solubilized in this acid before analysis.

To avoid problems associated with the instability of dilute volatile fatty acid solutions, dilutions were freshly prepared each day.

For evaluation of the chromatography procedure alone, duplicate 1-μl aliquots of known standard volatile fatty acid solutions were directly injected into the column and, after correction for attenuation, the peak areas for each acid were related to the internal standard.

On-column injection into a soft column packing material, such as Phasepak Q, frequently results in loss of injection due to syringe blockage. To avoid this problem, the point of the syringe needle was sealed, and a slot cut 2 mm from the tip with a diamond disc. This modification of the needle permitted sideways injection into the column packing material and reduced injection losses to zero.

To evaluate the efficiency of the vacuum distillation procedure, it was necessary to ensure conditions reasonably similar to those occurring when an actual tissue sample is being analysed. For this, a 10-μl aliquot of each of the dilutions of the stock solution was used to dissolve milligram quantities of freshly ignited synthetic hydroxyapatite in a small specimen tube. This tube was then placed in the distillation tube of the vacuum microstill (Fig. 1), and externally cooled with liquid nitrogen to avoid the loss of the volatile components. The system was then evacuated until a pressure of less than 10^{-1} torr was reached, after which it was isolated by closing the tap.

On allowing the distillation tube to return to room temperature, spontaneous distillation into the tapered tip of the receiver tube occurred when this was cooled with liquid nitrogen. After a few minutes the apparatus was returned to atmospheric pressure, and then the receiver tube was warmed to room temperature. 1-μl Aliquots of the distillate were injected into the column as before, and the chromatograms obtained for comparison with those obtained without distillation. The procedure for dealing with biological specimens was exactly the same, except that weighed samples were dissolved in 10-μl aliquots of the 1 *M* hydrochloric acid (containing the standard *n*-hexanoic acid), and the distillate was obtained from these.

RESULTS AND DISCUSSION

Orthophosphoric acid-modified Phasepak Q effectively resolved the volatile fatty acids as shown in Fig. 2 within a 10-min period of separation. Excellent peak symmetry was observed with negligible peak tailing, or peak broadening of acid isomers[6]. These observations are in close agreement with a study by Mahadevan and Stenroos[5] using a similar column substrate. Hrivňák *et al.*[7] considered phosphoric acid to be the most suitable stationary phase additive for the base-line separation of sixteen C_2–C_6 fatty acids, using a polypropylene glycol capillary column.

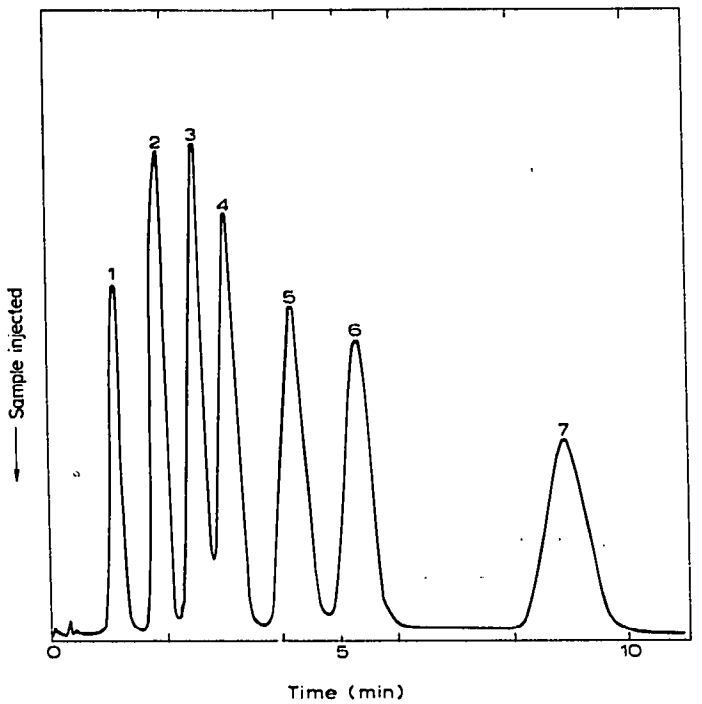

Fig. 2. Quantitative isothermal separation of volatile fatty acids in aqueous solution on a 2% w/w orthophosphoric acid-modified Phasepak Q column. 1 = Acetic acid; 2 = propionic acid; 3 = isobutyric acid; 4 = *n*-butyric acid; 5 = isovaleric acid; 6 = *n*-valeric acid; 7 = *n*-hexanoic acid (internal standard). Sample volume, 1 μl, containing *ca.* 200 ng of each volatile fatty acid.

Fig. 3 shows the proportionality of detector response to initial fatty acid concentration, in solutions which were put through the distillation procedure before injection. Coefficients of variation ranged between 4.0 and 9.8% for the C_2–C_5 acids. This compares with values of from 2.0 to 4.0% for direct aqueous injection without distillation.

Relative molar response factors calculated for the volatile fatty acids are shown in Table I, there being a linear relationship between response and effective carbon number for each acid (4th column). Thus the flame ionization detector responded in a linear manner to the carbon chain structure of the volatile fatty acid homologous series. These observations confirm the data given by Ackman and Sipos[8] and Boček

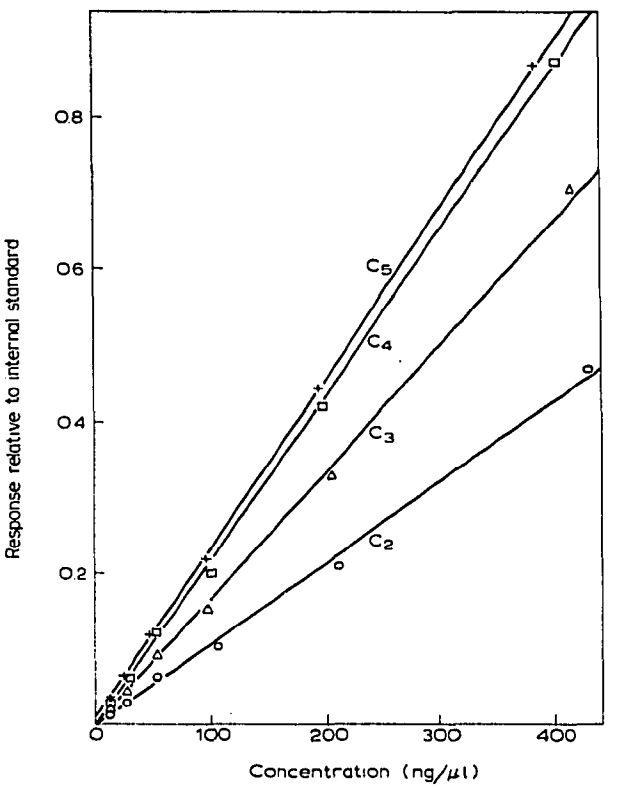

Fig. 3. Calibration curves for acetic (C_2), propionic (C_3), *n*-butyric (C_4), and *n*-valeric (C_5) acids; recovery by vacuum distillation from milligram quantities of hydroxyapatite. Volatile acid response values expressed in terms of peak area ratios relative to the internal standard, after correction for attenuation.

TABLE I

RELATIVE MOLAR RESPONSES (RMR) OF VOLATILE FATTY ACIDS RELATIVE TO THE STANDARD *n*-HEXANOIC ACID

RMRs and coefficients of variation were derived from calculated lines of regression for each acid. Calibration range: 10–400 ng of each acid per microlitre aqueous injection. Recoveries were calculated by comparison of the slopes of lines of regression, which included the distillation procedure, with those obtained by direct injection.

Volatile fatty acid	*RMR*	*Effective carbon number*	*Relative response (g-atom carbon)*	*Recovery by distillation (%)*	*Coefficient of variation (%)*
Acetic	0.212	1	0.21	103	8.4
Propionic	0.415	2	0.21	100	8.1
Isobutyric	0.650	3	0.22	103	9.8
n-Butyric	0.636	3	0.21	105	7.8
Isovaleric	0.810	4	0.20	106	6.4
n-Valeric	0.750	4	0.19	99	4.0

and Janák[9], which showed that the carbonyl group in fatty acids with less than six carbon atoms gives no effective contribution to molecular response. Relative responses for isobutyric and isovaleric acids were observed to be higher than the corresponding normal acids, coinciding with previously published data[8,10].

The quantitative extraction of volatile fatty acids from submicrogram quantities of biological material is difficult to achieve by classical methods of steam distillation. These techniques are known to have low efficiency as shown by the recovery of volatile acids from rumen liquor by Edwards *et al.*[11]. Extraction of sodium salts of volatile fatty acids with isopropanol, as used by Gardner and Thompson[12], for analysing blood plasma, provides an alternative recovery procedure. However, the vacuum distillation technique is more direct, and for the following reasons was expected to extract the volatile fatty acids efficiently from a biological matrix.

At the temperature of liquid nitrogen (—196°), acetic acid, the most volatile of the components analysed, has a vapour pressure of less than 10^{-10} torr, so that its evaporation rate from a sample maintained at this temperature during evacuation of the still is very low indeed. Furthermore, the quantity left in the vapour space of the apparatus when the receiver tube is cooled to —196° is less than 10^{-2} pg. Likewise, at this low vapour pressure, very little adsorption should occur on any of the internal surfaces of the apparatus that are kept at room temperature. As regards the distillation procedure itself, the boiling point of *n*-hexanoic acid, the least volatile

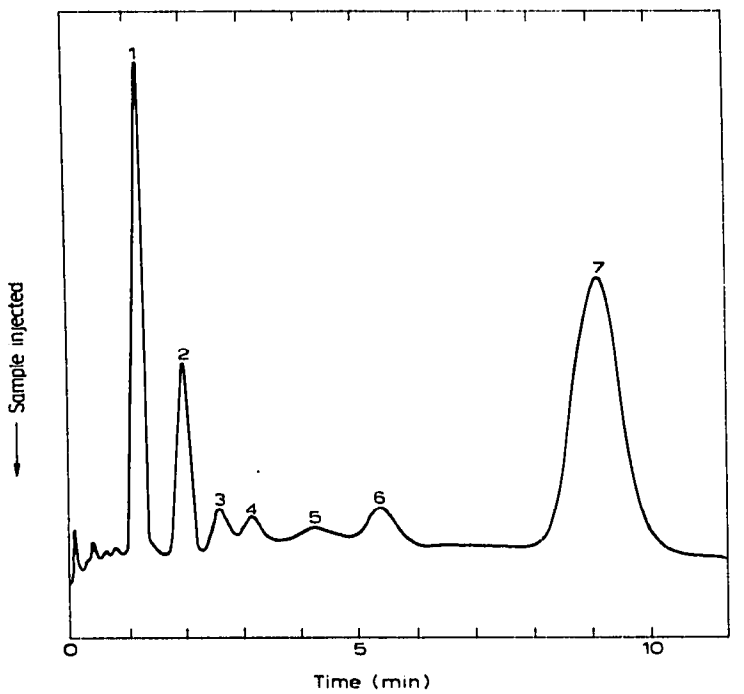

Fig. 4. Quantitative separation of volatile fatty acid components recovered from submilligram quantities of early carious (white spot) human enamel. 1 = Acetic acid; 2 = propionic acid; 3 = isobutyric acid; 4 = *n*-butyric acid; 5 = isovaleric acid; 6 = *n*-valeric acid; 7 = *n*-hexanoic acid (internal standard).

of the acids distilled, is approximately 35° at 10^{-2} torr, so that after a short period of evacuation with a good quality backing pump (pressure 10^{-2}–10^{-3} torr), warming to room temperature of the previously frozen sample should allow quite rapid distillation to occur, especially in view of the small quantities involved. In practice, as demonstrated in Table I, the method allowed satisfactory extraction of the volatile acids, from acetic to *n*-hexanoic, in a period of 2 or 3 min. The chromatogram in Fig. 4 demonstrates the volatile fatty acid constituents extracted from submilligram quantities of carious enamel by the vacuum distillation procedure. This technique is presently being used to evaluate the occurrence and role of volatile fatty acids in carious enamel and dentine, calculus, dental plaque, and saliva.

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