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QUANTITATIVE GAS CHROMATOGRAPHIC DETERMINATION OF LOW-MOLECULAR-WEIGHT STRAIGHT-CHAIN CARBOXYLIC ACIDS AS THEIR *p*-BROMOPHENACYL ESTERS AFTER EXTRACTIVE ALKYLATION IN ACIDIC MEDIUM

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SUMMARY

The measurement of acetate by gas chromatography at low concentrations in biological fluids is a difficult task. A significant improvement can be brought about by derivatization of acids as *p*-bromophenacyl esters.

By such a procedure, the determination of low-molecular-weight straight-chain carboxylic acids (acetic, propionic and butyric acids) was enhanced with respect to sensitivity and specificity. The volatile fatty acids were esterified by means of an extractive alkylation mechanism in acidic conditions from aqueous solutions. Tetrahexylammonium was used as counter-ion and the alkylating agent was *α,p*-dibromoacetophenone.

The phenacylation reaction was studied with respect to pH, concentration of counter-ion and kinetics. The yield of derivatization was *ca.* 70% after 2 h at 42°C.

A mass spectrometry study was performed in order to ascertain the structure of derivatives using electron impact and chemical ionization.

The gas–liquid chromatographic behaviour of the *p*-bromophenacyl esters of acetic, propionic and butyric acids was studied on an OV-225 packed column and an OV-1 capillary column. Flame ionization detection was shown to be linear between 0.2 and 4 nmol injected, with a quantitative limit of detection of 31 pmol (*i.e.* 1.8 ng of acetic acid).

INTRODUCTION

Many gas chromatographic (GC) methods for the measurement of acetate at low concentrations (10–100 μM) have been reported[1–5]. An extensive review of the techniques was compiled by Cochrane[6]. In most of these procedures, columns packed with liquid phases suitable for the chromatography of the free acid were used. Many pitfalls could be found in GC of volatile fatty acids (VFA). Among these, adsorption of the acid into the chromatographic systems (inlet and columns) resulted in tailing, irregular shape of peaks and ghosting[1–4]. Addition of formic acid to the carrier gas[1,7,11]

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and/or addition of phosphoric acid to the stationary phase[1–5,8] was proposed to improve this situation, but the lifetime of the columns then decreased. In addition, nonreproducible recoveries of VFA in aqueous biological fluids can occur during pre-chromatographic manipulation, such as extraction with organic solvents, concentration by evaporation or vacuum microdistillation[5,9,10,12].

Another problem occurs in the detection level of acetate. Owing to its structure (one methyl only), acetate has a very low response factor to the flame ionization detector (FID). Since the FID has a sensitivity of 20 millicoulombs per gram of carbon, the detection limit can be estimated at *ca.* 700 ng of injected acetate at the usual sensitivity of 10^{-10} A (attenuation $\times 100$) consistent with the noise level in biological analysis. In such conditions, acetate concentrations as low as 60 μM can be measured without pre-concentration. Such a value is close to the normal acetate concentration in human plasma. Accordingly, it is necessary to enhance the detectability of acetate by FID. This can be achieved by derivatization of the molecule.

Our objective was to select a suitable derivative for acetic acid that could be prepared quantitatively, chromatographed and detected in good analytical conditions.

MATERIALS AND METHODS

Reagents

α,p-Dibromoacetophenone (Merck, Darmstadt, G.F.R.) was dissolved in methylene chloride at 36 mM concentration. All organic acids (Merck) were dissolved in water, and freshly prepared solutions were used. Different counter-ions were tried: tetrahexylammonium hydrogen sulphate, tetrabutylammonium hydrogen sulphate or chloride or bromide, triethylbenzylammonium chloride, trimethylhexadecylammonium bromide, tributylhexadecylphosphonium bromide. They were all of analytical grade and were purchased from Merck or Interchim (Montluçon, France) or INC (Irvine, CA, U.S.A.) or Eastman (Rochester, NY, U.S.A.). They were dissolved at concentrations of 0.3–1 mM in phosphate buffer solution of variable pH.

Gas–liquid chromatography

The reaction conditions were evaluated with a Pye Unicam 104 Model 84 gas chromatograph equipped with a FID. The glass column, 2.50 m $\times$ 0.4 cm I.D., was packed with 2.1% OV-225 on Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.). Nitrogen was used as carrier gas at a flow-rate of 40 ml/min. The column oven and detector temperatures were, respectively, 185 and 250°C. Studies in the nanogram range were carried out with a Carlo-Erba 2150 gas chromatograph equipped with a FID and a splitless injector. The carrier gas was hydrogen at a flow-rate of 1 ml/min. The glass capillary column, 30 m $\times$ 0.3 mm I.D., was a soft persilylated glass as described by Grob[13] and coated with OV-1, film thickness 0.15 μm.

Mass spectrometry

Mass spectrometry (MS) was carried out on a Ribermag R-10-10B (Rueil Malmaison, France) apparatus. Sample introduction was via the GC inlet, OV-225 3% on Chromosorb W HP 100–120 mesh support at 180°C. Helium was used as carrier gas at 1.8 bar. The solvent peak was diverted from the GC–MS interface held at 250°C. The electron impact mass spectra were recorded at 70 eV; the ionization current was 210 μA. Chemical ionization mass spectra were recorded with ammonia as reagent gas.

Evaluation of reaction conditions

The derivatization was carried out as follows. For analytical purposes, 2 ml of diluted solution of acetic, propionic and butyric acids in the range 0.1125–1.125 m*M* were added to 8 ml of buffer solution containing the counter-ion. A 1 ml volume of methylene chloride containing the alkylating reagent (36 m*M*) and *n*-hexacosane (1.22 m*M*) was added. The reaction mixture was shaken in screw-cap PTFE vials for various times at 42°C. Then 2 μl of the organic phase were injected into the gas chromatograph. Reaction yields were calculated from peak heights ratios with *n*-hexacosane as internal standard.

For preparative purposes, 2 mmol of acetic acid in 150 ml of buffer solution (pH 5) were derivatized with 2.2 mol of α,*p*-dibromoacetophenone in 20 ml of methylene chloride. The organic phase was removed under vacuum. The residue, redissolved in benzene, was filtered through 20 g of dry silica gel, 35–70 mesh (Merck). The alkylated derivative was eluted with benzene and recrystallized from heptane.

RESULTS AND DISCUSSION

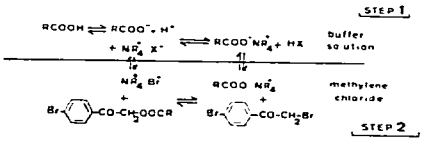
Critical examination of current techniques of synthesis of carboxylic acids esters prior to chromatographic analysis[14–17] indicates that a need exists for methods of derivatizing VFA, such as acetic acid, in very dilute aqueous biological samples. Indeed, many of these procedures were found to be tedious because they entailed successive steps of extraction, concentration and derivatization. Furthermore, when using solid–liquid phase-transfer catalysis[18,19] aqueous media were excluded, and a preliminary ion-exchange step was found necessary to resolve problems due to the interference of Na^+, Mg^{2+} and Ca^{2+} ions[20].

One interesting breakthrough was the application of extractive alkylation, which is well known in organic chemistry[21–25]. The present study applied this derivatization procedure while using a very reactive alkylating reagent, α,*p*-dibromoacetophenone[17].

The phenacyl esters so prepared exhibit good chromatographic properties. In GC, the separation and detectability are noticeably enhanced whereas in liquid chromatography such derivatives are nowadays largely used[26–28] because of their UV properties[19].

Derivatization reaction

Extractive alkylation, also called liquid–liquid phase-transfer catalysis[15], is a very convenient technique, coupling extraction and derivatization. (i) Extraction of the anion of the acid as an ion-pair. This ion-pair, with a bulky lipophilic radical R, is extracted into an organic phase where the alkylation takes place. As the anion present is highly reactive in poorly solvating solvents, the nucleophilic properties of the carboxylate anion are enhanced. (ii) Alkylation with a highly reactive reagent: α,*p*-dibromoacetophenone.



Study of the phenacylation reaction

The extractive alkylation of VFA was the subject of our investigation. Many parameters, such as pH and the nature and concentration of the counter-ion were studied.

pH influence. The influence of pH on the reactivity is shown in Fig. 1. The optimal pH for acetate and propionate is between 5 and 6 and for butyrate it is in the range 4–8.

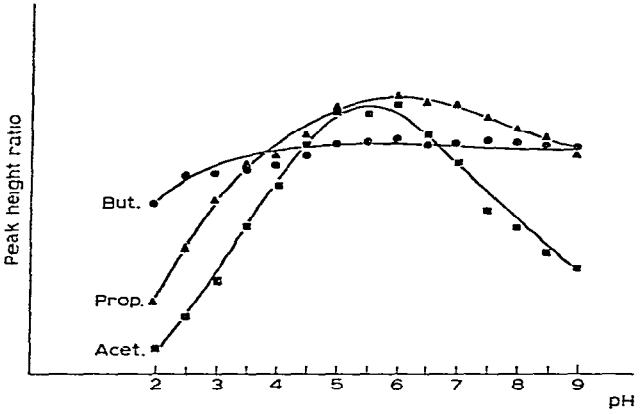


Fig. 1. Influence of pH on the reaction rate: 8 h at 42°C. Reactants: tetrahexylammonium hydrogen sulphate 0.3 m*M* in buffer solution 1 *M*, organic acids 1.5 μmol. Results are expressed in arbitrary units as peak height ratio of organic acids to internal standard (*n*-hexacosane). The peak height ratio was, respectively, 1.13, 1.11 and 1.04 for equal amounts of acetic, propionic and butyric acids with respect to internal standard.

The partition ratio of the carboxylate anion between the organic and aqueous phases is a function of the concentrations of the quaternary ammonium ion and the anion in the aqueous phase and of the extraction coefficient for the ion pair. Accordingly, it is to be expected that the dissociation coefficients of the organic acids will influence their reactivities. At a pH lower than the pK_a, VFA are not dissociated enough to be extracted as ion-pairs in the organic phase. In other respects, all fatty acids are totally dissociated at pH 9–10, which is the pH usually used for extractive alkylation[14,15]. Fig. 1 shows that, above the pK_a value of acetic acid, the reactivity decreases significantly. This abnormal behaviour can be explained by the well-known observation that the $CH_3COO^-Na^+$ salt is more stable than the $CH_3COO^-\overset{+}{N}R'_4$ salt. Consequently, at pH 9–10 the acetate ion cannot be extracted quantitatively as the $CH_3COO^-\overset{+}{N}R'_4$ ion pair.

When the length of the alkyl chain of the organic acid increases, so does the extraction coefficient of the ion-pair because of greater lipophilicity. Consequently, for the propionate and butyrate anions, the equilibrium of step 1 is displaced to the the right, *i.e.* the $RCOO^-\overset{+}{N}R'_4$ ion-pair.

Nature and concentration of counter-ion. The results are listed in Table I for acetic, propionic and butyric acids at pH 5 and 9.

The reactivity seems independent of the nature of the tetra-alkylammonium

TABLE I

INFLUENCE OF THE NATURE OF THE COUNTER-ION ON REACTION RATE

Reactants: counter-ion $0.3 \cdot 10^{-3}$ *M* in buffer solution, organic acid 1.5 μmol, internal standard: *n*-hexacosane. 8 h at 42°C. Results are expressed in arbitrary units relative to internal standard.

Counter-ion	*Buffer solution, pH 5*			*Buffer solution, pH 9*		
	Acetic acid	*Propionic acid*	*Butyric acid*	*Acetic acid*	*Propionic acid*	*Butyric acid*
Tetrabutylammonium hydrogen sulphate	0.03	0.06	0.10	0.05	0.12	0.31
Tetrabutylammonium bromide	0.04	0.05	0.16	0.04	0.06	0.18
Tetrabutylammonium chloride	0.05	0.11	0.24	0.05	0.08	0.23
Tetrahexylammonium hydrogen sulphate	1.45	1.40	1.23	0.45	1.23	1.21
Triethylbenzylammonium chloride	0	0	0	0.05	0.08	0.23
Trimethylhexadecyl-ammonium bromide	0.04	0.14	0.41	0.08	0.22	0.53
Tributylhexadecyl-phosphonium bromide	0.66	0.73	0.74	0.35	0.98	0.96

anion. On the other hand, as the extraction coefficient is a function of the lipophilic character of the counter-ion, the reaction rate is influenced by the length of the alkyl chain of the quaternary ammonium ion. Table I shows that the tetrabutylammonium salts are too hydrophilic. The best results were obtained with the tetrahexylammonium ion. This result is in good agreement with those published by Dehmlow[23], which showed that the counter-ion must contain not less than 15 carbon atoms. When the phosphonium salt was used, the reactivity did not increase. For the following experiments, tetrahexylammonium salts were used.

According to the scheme described above, the reactivity can be increased by a higher concentration of the quaternary ammonium ion. However, only the rate of derivatization of acetic acid increased with concentration of the tetrahexylammonium ion (Fig. 2). For propionic and butyric acids, the derivatization was practically independent of this parameter. This observation can be explained by the more lipophilic character of these acids.

Kinetics of reaction. Fig. 3 shows the results for the three acids at two concentrations of the quaternary ammonium ion. The yield of phenacyl acetate was constant at reaction times over 2 h for a 10^{-3} *M* concentration of tetrahexylammonium ion and over 18 h at a $0.3 \cdot 10^{-3}$ *M* concentration. It is clear that a fast reaction for hydrophilic acids will require a high concentration of the lipophilic counter-ion. The yield of the phenacyl ester ion remains constant at reaction times between 3 and 24 h, indicating that no hydrolysis takes place. The phenacyl esters are highly lipophilic and mainly present in the organic phase, which decreases the possibility of decomposition in buffer phase solution.

Choice of solvent. The distribution of the ammonium salt between the aqueous and organic phases depends to a large extent on the nature of the organic phase.

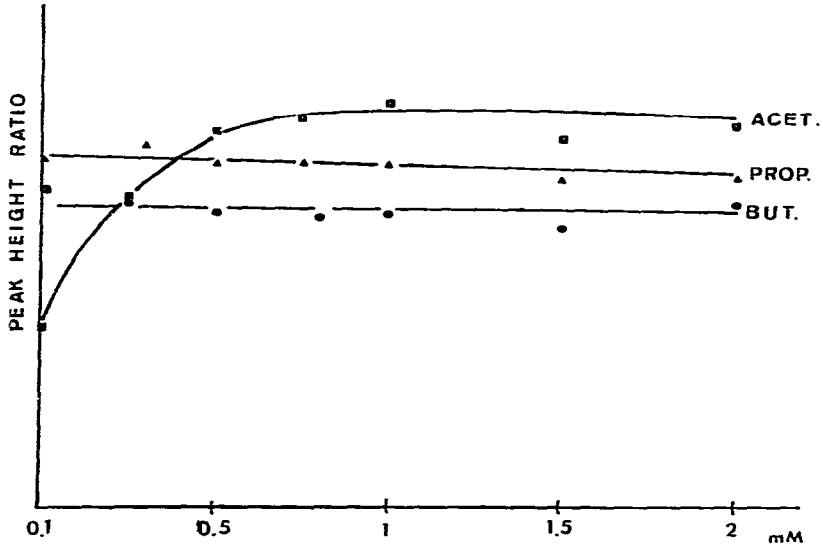


Fig. 2. Influence of tetrahexylammonium ion concentration on the reaction rate. Reactants: organic acids 1.5 μmol. For units see Fig. 1.

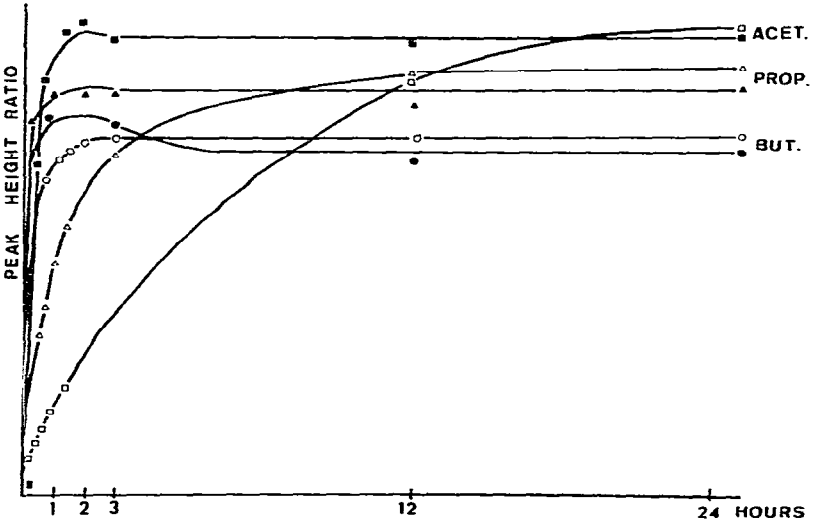


Fig. 3. Phenacyl esterification of acetic, propionic and butyric acids (1.5 μmol) at pH 5. □, Acetic acid; △, propionic acid; ○, butyric acid; all with tetrahexylammonium ion 10^{-3} *M*. ■, Acetic acid; ▲, propionic acid; ●, butyric acid; all with tetrahexylammonium ion $0.3 \cdot 10^{-3}$ *M*.

Methylene chloride, which is often recommended, showed by far the most favorable extraction coefficient. At 42°C, in optimal conditions, the yield of phenacyl acetate was *ca.* 70% (determined with a known amount of pure phenacyl acetate as reference). Benzene and dichloroethane were also tested as solvents, principally to increase the reaction temperature; however, interfering side reactions occurred.

Chromatographic analysis

Fig. 4 shows separation of acetic, propionic and butyric acids as phenacyl esters on an OV-225 packed column. All these peaks were quantitatively resolved when using this polar liquid phase. The retention indices (RI) of the *p*-bromophenacyl esters of acetic, propionic and butyric were, respectively, 2334, 2409 and 2491 at 185°C on OV-225, and the *α*,*p*-dibromoacetophenone reagent (RI = 2237) gave a pronounced peak with marked tailing. This tailing, if too great, made quantitative measurement of acetic acid difficult. Thus, the use of high-performance gas chromatography (HPGC) on glass capillary columns was attempted.

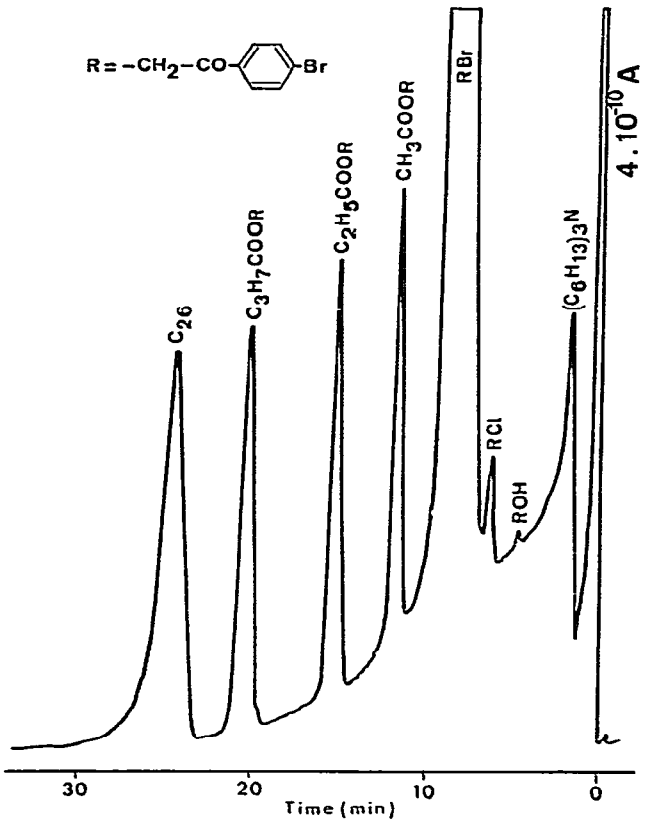


Fig. 4. Gas chromatogram of acetic, propionic and butyric acids as phenacyl esters on 2.1% OV-225 at 185°C.

Fig. 5 illustrates the effect of the efficiency of GC on the separation of the reagent, RBr, and the *p*-bromophenacyl esters of acids. This separation was carried out on a non-polar liquid phase OV-1. It should be noted that when such an analysis was carried on an OV-1 packed column, the acetic acid peak was obscured by tailing of the reagent peak. Retention indices of *α*,*p*-dibromoacetophenone and the *p*-bromophenacyl esters of acetic, propionic and butyric acids were, respectively, 1582, 1642, 1721 and 1778 on OV-1.

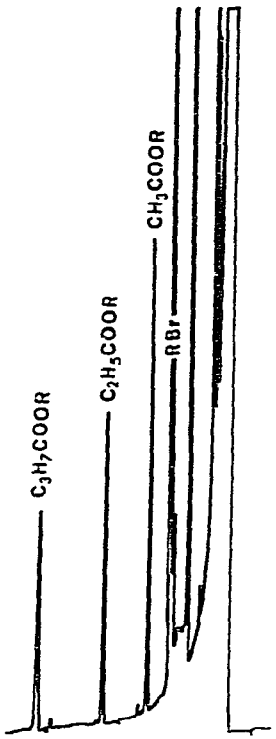


Fig. 5. Gas chromatogram of acetic, propionic and butyric acids as phenacyl esters on glass capillary column OV-1 30 m × 0.3 mm I.D., film thickness, 0.15 μm; oven temperature, 150°C; carrier gas, hydrogen at 0.5 bar. Split injection.

Mass spectrometry of p-bromophenacyl esters

In order to identify *p*-bromophenacyl ester derivatives, mass spectrometry was performed with a GC–MS coupled system using two ionization modes.

The electron impact (EI) ionization gave a base peak at *m/e* 183, 185, corresponding to the fragment ion $BrC_6H_4CO^+$. Such a fragment was present in the mass spectra of all the phenacyl esters studied (Fig. 6). It resulted from the elimination of the $RCOOCH_2$ residue from the molecular ion by a β-cleavage without hydrogen transfer. The presence of one bromine atom in the molecule provided a characteristic isotopic pattern. This doublet was present in all the fragments containing an bromine atom (^{79}Br and ^{81}Br) with a relative abundance of about one. The ions at *m/e* 155 and 76 corresponded, respectively, to the fragments $BrC_6H_4^+$ and $C_6H_4^+$. Finally, while using EI, the identity of the derivatives was confirmed only by the molecular ions at *m/e* 256–258, 270–272 and 282–284. These molecular ions were *ca.* 3% of the intensity of the base peak. However, fragments characteristic of derivatized acids were observed at low *m/e* 43, 57 and 71 for acetic, propionic and butyric acids, respectively. These ions corresponded to the fragments CH_3CO^+, $C_2H_5CO^+$ and $C_3H_7CO^+$.

Chemical ionization (CI) with ammonia as reagent gas gave quasi-molecular ions (QM^+) at $(M + 1)^+$: *m/e* = 257–259, 271–273 and 285–287 for the three VFA studied. A base peak (Fig. 7) was obtained at $(M + 18)^+$, corresponding to ions

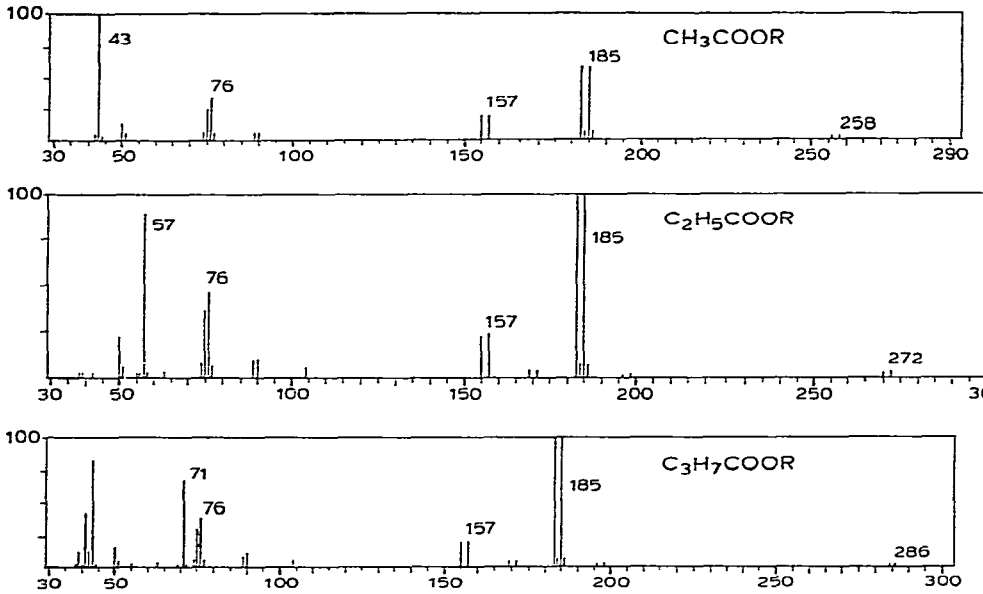


Fig. 6. EI mass spectra of phenacyl esters of acetic, propionic and butyric acids. R = Br–C_6H_4–CO–CH_2. For experimental conditions see text.

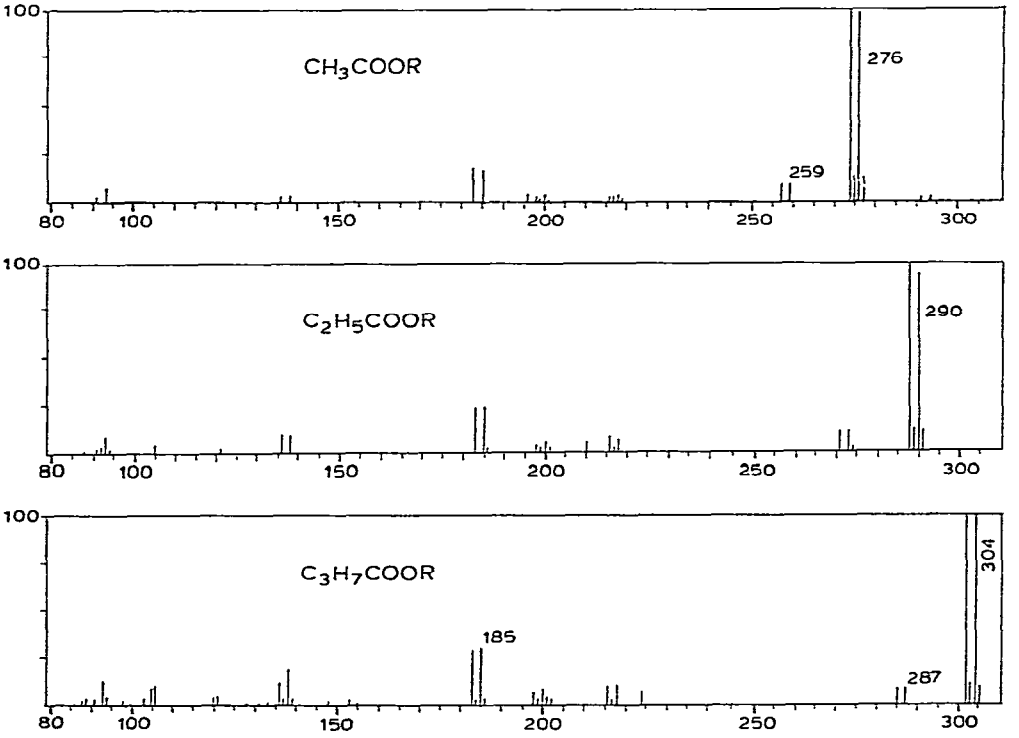


Fig. 7. CI mass spectra of phenacyl esters of acetic, propionic and butyric acids. Ammonia was used as reagent gas.

($Q - NH_4^+$). Furthermore, the fragment ions at $m/e = 183, 185$ were still present in all the mass spectra. This observation can be explained by the stabilization of the fragment ion, $p\text{-}BrC_6H_4CO^+$, obtained by a resonance mechanism.

Measurement of VFA

In order to establish the advantages of the derivatization of VFA for quantitative purposes, the linearity of response and the limit of detection were studied. A linear response from 200 pmol to 4 nmol injected of each derivatized acid was obtained, as shown in Fig. 8.

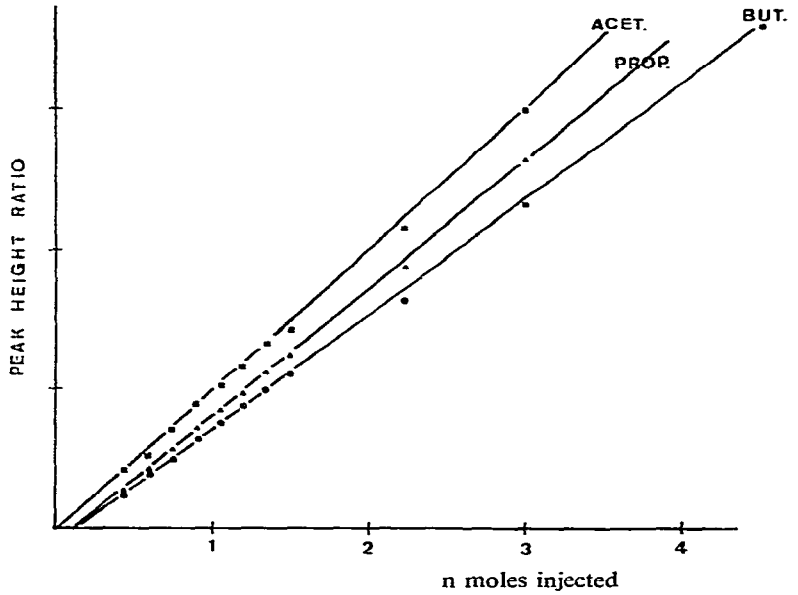


Fig. 8. Standard curves for acetic, propionic and butyric acids as *p*-bromophenacyl esters. Chromatographic conditions: 2.5 m × 0.4 cm I.D. column packed with 2.1% OV-225 on Gas-Chrom Q; oven temperature, 185°C; flame ionization detection; sensitivity, 2×10^{-10} A (full-scale deviation); internal standard, *n*-hexacosane.

By measuring the response of pure crystallized *p*-bromophenacyl ester of acetic acid, it was shown that the yield of derivatization was only *ca.* 70% in optimal conditions. However, this lack of total derivatization did not prevent quantitative analysis (Fig. 8). Nevertheless for quantitative analysis of biological samples, it will be necessary to check that the reaction yield is identical with that of the standards.

The limit of detection is an important criterion. According to Curie[29], a quantitative detection limit, Lq, can be defined as the level at which the precision of the measurement will be satisfactory for quantitative determination. This term Lq is calculated as $Lq = 10\,\sigma_0$, where σ_0 is the standard deviation of measurement at very low levels; for acetic acid it is 31 pmol or 1.8 ng injected in the gas chromatograph at 10^{-10} A sensitivity. Under these conditions the determination of the plasma acetate level in normal human subjects, 51 μM[5], could be performed satisfactory. This detection limit could be noticeably improved by using a capillary column.

CONCLUSIONS

Although several GC methods for the quantitative determination of acetate in biological fluids have been reported, almost all were concerned with pathological cases, such as hemodialysis or metabolic diseases. The main problem is due to the relatively low concentration of acetate in the plasma of healthy subjects. When such small amount have to be measured, the GC adsorption gives rise to tailing, irregular peaks and ghosting, making quantitative analysis difficult. Furthermore, owing to its structure, acetic acid gives a low signal in a FID (formic acid does not respond to a FID). Accordingly, in order to enhance detectability, volatile fatty acids have often been converted into their esters. However, owing to the non-quantitative recoveries of the esters, especially for the lower acids, these procedures are not suitable for quantitative analysis.

The authors attempted to improve the esterification procedure by using phenacyl esters via an extractive alkylation of acetic, propionic and butyric acids. Phenacyl esters can be prepared easily and quantitatively, allowing the detection of as little as 1.8 ng of C_2 acid. The method of preparation is inexpensive and rapid, coupling the extraction and derivatization steps. The reaction conditions do not require the exclusion of water. GC detectability and separation can be improved by using a glass capillary column. Furthermore, phenacyl esters are recognized as suitable tags for UV detection in HPLC analysis[19].

The formation of phenacyl esters of volatile fatty acids must thus be considered as a useful method for the quantitative analysis of acetate. Indeed, such a change in the polarity of the molecule cannot but enhance the specificity of the method.

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