PENTAFLUOROBENZOIC ANHYDRIDE AS A DERIVATIZING AGENT FOR ALCOHOLS AND HYDROXY FATTY ACID METHYL ESTERS DETECTED BY ELECTRON CAPTURE IN GAS CHROMATOGRAPHY

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SUMMARY

Pentafluorobenzoate derivatives of primary and secondary alcohols have been prepared using pentafluorobenzoic anhydride. The gas chromatographic properties of the homologous series of the methyl esters of 2-hydroxycarboxylic acids from C_{12} to C_{26} have been studied on the high-temperature stationary phase Poly-S 179. The suitability of determining trace amounts of these compounds using gas chromatography with an electron-capture detector and with electron-capture negative-ion chemical-ionization mass spectrometry is discussed.

INTRODUCTION

The development of this method for measuring hydroxy carboxylic acids was undertaken because of a need to quantify and identify hydroperoxides that may be formed in the retina[1]. The fatty acid side chains of these oxidation products of membrane phospholipids would be reduced to hydroxy fatty acids, transesterified to methyl esters and derivatized specifically at this hydroxyl to facilitate detection. Because of the small quantity of material available for analysis, it was imperative that the most sensitive detection method possible be used. Gas chromatography (GC) with electron-capture detection (ECD) provides good separation and detection of components in the picogram range[2]. For the development of this method a packed column was used. Those analysts interested in measuring only a few components will find the columns used here adequate. For those needing better separation, this method can be adapted to a capillary column. Useful work along these lines, preparing pentafluorobenzyl derivatives at the fatty acid ester linkage via transesterification of phospholipids with pentafluorobenzyl alcohol, has been carried out by Van Kuijk and co-workers[3,4].

After careful study, we decided that O-pentafluorobenzoylmethyl esters (as

shown in Figs. 2 and 3) would provide the properties desired: good stability[5,6], optimal detector response[7,8] and possession of the highest probability of separation[9]. Furthermore, it was necessary that the reaction proceeded to completion for all components in a short period of time under gentle conditions. For this reason, pentafluorobenzoic anhydride (PFBA) was chosen as the derivatizing reagent. This choice avoids the formation of HCl that occurs with the acid chloride. In addition, the derivatives formed have been shown to be very useful in negative-ion chemical ionization (NICI) mass spectrometry (MS)[10].

The homologous series of 2-hydroxycarboxylic acids (C_{12}–C_{26}) was studied so that a relative retention index could be reported for subsequently observed unknowns. Included as examples of the use of this index are 16-hydroxyhexadecanoic acid and ricinoleic acid.

EXPERIMENTAL

Chemicals

Pentafluorobenzoyl chloride, benzoyl chloride, 1,1,1-trichloro-3,3,3-trifluoro-acetone, 2-hydroxytetradecanoic acid and ethyl acetate were purchased from Fluka (Ronkonkoma, NY, U.S.A.). Potassium hydroxide, potassium bromide, 1-octanol, 3-octanol, decane, 1-decanol, 1,10-decanediol, 1-dodecanol, 1-pentadecanol, 1-octadecanol, 16-hydroxyhexadecanoic acid, cholesterol, cholesta-3,5-diene and trimethylcitrate were purchased from Aldrich (Milwaukee, WI, U.S.A.). 2-Hydroxy-dodecanoic acid and 2-hydroxyhexadecanoic acid were purchased from Lancaster Synthesis (Windham, NH, U.S.A.). Ricinoleic acid methyl ester (12-OH:18:1ω9), 2-hydroxystearic acid (2-OH:18:0), 2-hydroxybehenic acid (2-OH:22:0) and 2-hydroxycerotic acid (2-OH:26:0) were obtained from Serva (Westbury, NY, U.S.A.). Burdick & Jackson glass-distilled toluene was obtained from American Scientific Products (Boston, MA, U.S.A.). Silylation grade pyridine was obtained from Pierce (Rockford, IL, U.S.A.). N-Methyl-N-nitrosourea was obtained from ICN Pharmaceuticals. Diethyl ether was Fisher (Medford, MA, U.S.A.) HPLC grade. All chemicals were used without further purification.

Instrumentation

GC was performed on a Perkin-Elmer 3920 (purchased from Buck Scientific, Norwalk, CT, U.S.A.) with a flash vaporization injector interfaced to a Spectra-Physics (San Jose, CA, U.S.A.) SP 4100 integrator. When the baseline was extremely noisy, peak areas were determined by weighing photocopies of the peaks on a four-place Mettler balance. Emerson Electric (Hatfield, PA, U.S.A.) Model 8744A flow controllers were used for the carrier gas. The back pressure to the flow controllers was maintained at 78 p.s.i. The electron-capture detector was a Valco (Houston, TX, U.S.A.) Model 140 (180-μl volume) operating with a standing current of 0.6 nA. Periodically, it was cleaned by purging with hydrogen at a detector temperature of 370°C. The make-up gas was temperature equilibrated in the column oven prior to entering the detector. It was found that when the oven temperature was 230°C the optimal pressure for the make-up gas was 15 p.s.i. The injector temperature was maintained at its maximum temperature of 290°C and the column–detector interface at 330°C. The temperature of the column–detector interface was found not to influence

the response of the electron-capture detector. The oven temperature was calibrated with a Sensortek (Clifton, NJ, U.S.A.) Model BAT-12 thermocouple. It was found to be within 2°C of what it was set at. All oven temperatures are reported according to the setting and are ± 2°C.

A Perkin-Elmer (Norwalk, CT, U.S.A.) 1310 IR spectrophotometer was used to measure infrared spectra. Potassium bromide pellets of the samples were made in a hand-held press.

The nuclear magnetic resonance (NMR) spectra were recorded on a Varian (Palo Alto, CA, U.S.A.) XL-300 NMR spectrometer at ambient temperature under the conditions indicated in Table I. The reference and solvent for both spectra was $CFCl_3$. ^{19}F chemical shifts are reported relative to $CFCl_3$. ^{13}C chemical shifts are reported relative to tetramethylsilane. Chemical shifts are reported in parts per million (ppm) and coupling constants are reported in Hertz (Hz).

TABLE I

OPERATING CONDITIONS OF NMR

	^{19}F	^{13}C
Operating frequency (MHz)	282.2	75.4
Spectral width (Hz)	20 000	16 502
No. of repetitions	16	1024
Digital resolution (Hz)	3.36	0.55
Pulse repetition rate (s)	4.15	4.91

Mass spectra were recorded on a Finnigan (San Jose, CA, U.S.A.) 4000 quadrupole mass spectrometer interfaced to a Hewlett-Packard (North Hollywood, CA, U.S.A.) 5890 gas chromatograph. The gas chromatograph was equipped with a 25 m × 0.32 mm DB-1 capillary column having a film thickness of 0.25 μm (J&W Scientific, Rancho Cordova, CA, U.S.A.). The column was directly inserted into the ion source. The carrier gas was helium at a pressure of 13 p.s.i. The GC oven was initially set at 70°C and ramped linearly to 280°C at 30°/min. Sample concentrations were 3.5 ng/μl and injection volumes were one microliter. On-column injection technique and mass spectrometer operating parameters are described elsewhere[11] with the exception of scan time (for the m/z range 100–730) which was 1.0 s per scan for these measurements. The probe measurement was done on a VG Analytical SEQ-70 mass spectrometer.

Procedures

The 12 ft. × 2 mm column used to study GC properties was purchased from Supelco (Bellefonte, PA, U.S.A.). It was packed with 4.5 g of 3% Poly-S 179 (a polyphenyl ether sulfone) on 100–120 Gas Chrom Q II purchased from Alltech (Deerfield, IL, U.S.A.). Optimization of the derivatization reaction conditions was done with a 6 ft. × 2 mm Supelco column packed with 5% PPE-21 on 100–120 Chromosorb W AW (Supelco). Columns were installed with high-temperature Supeltex M-4 ferrules. The septum was an Alltech Ultrasep R and was changed every

50–75 injections (at room temperature). The carrier gas was grade 4.5 nitrogen and was passed through a molecular sieve/silica gel trap (Buck Scientific) and an R&D (N. Highlands, CA, U.S.A.) Model OT3 oxygen trap before entering the column. All bottled gases were purchased from Wesco (Billerica, MA, U.S.A.).

A Pasteur pipette connected to a Gilson Pipetteman was used to transfer organic liquids. Disposable micropipettes or Hamilton syringes were used to transfer quantities less than 50 μl. A 10-μl Hamilton syringe was used to inject samples into the gas chromatograph. A Bausch & Lomb 7 × magnifier was used to read injection volumes. The volume (*ca.* 0.2 μl) remaining in the syringe after an injection was taken into consideration. All solutions were stored in either 20 ml or 6 ml glass scintillation vials. PTFE liners (Thomas Scientific, Swedesboro, NJ, U.S.A.) were inserted in all scintillation vial caps. Vials and liners were used without treatment and only once. A Multi-Blok heater from American Scientific Products was used to control the temperature of derivatization reactions. An Electrothermal melting point apparatus was used. Thermometers were calibrated with water. An Alltech 100-ml soap bubble rotameter in conjuction with a seven jewel Kaltron stopwatch accurate to 1/5 s was used to calibrate the carrier gas flow meters. Flow-rates were corrected for the vapor pressure of water and found to be a parabolic function of the meter reading. The same stopwatch was used for all kinetic measurements.

Synthesis of pentafluorobenzoic anhydride

Pentafluorobenzoic anhydride was synthesized following the general method of anhydride synthesis of Abdel-Baky and Giese[12]. This method is easy to use, high yields are obtained and the reactions all occur in one flask at room temperature. The product was recrystallized in anhydrous diethyl ether at −20.0°C, then dried under nitrogen and vacuum desiccated overnight. This process was repeated until a constant melting point was obtained (66–68°C)[12]. The product was further characterized with ^{19}F NMR, ^{13}C NMR and IR spectroscopy as will be discussed in the Results section.

Benzoic anhydride was also synthesized using the same method. Its melting point[12] and infrared spectrum[13] were in agreement with previous studies.

Methyl esterification with diazomethane

All free acids were first methyl-esterified with diazomethane[14] at 0°C prior to derivatization. That is to ensure that the carboxylic group does not interfere with the PFBA derivatization and to provide volatility on the GC column. This reaction was carried out in the fume hood. An explosion may result from the use of chipped glassware or ground-glass joints. We recommend to use protective gloves. Decant from one flask to another; do not use Pasteur pipettes to transfer solutions of diazomethane. For α,β-unsaturated fatty acids it has been reported that diazomethane can add to this double bond to yield pyrazolines[15].

In a 50-ml Erlenmeyer flask (first flask) approximately 25 ml of diethyl ether was added to 10.0 ml of cool 50% potassium hydroxide. The resulting two-phase system was allowed to chill in an ice bucket. The free acid to be methyl esterified was dissolved in a small amount of diethyl ether in a second 50-ml flask and also allowed to chill. After the solutions equilibrated to 0°C, approximately 5 mg of N-methyl-N-nitrosourea was added to the first (two-phase) flask. Diazomethane was generated in the aqueous phase and it bubbled into the ether layer. Gentle swirling may be required.

After a sufficient amount of diazomethane dissolved in the ether, as indicated by a yellow color, the ether layer was poured onto potassium hydroxide pellets in a third flask to dry. Then, the dried diazomethane solution was added to the second flask containing the free acid. This solution was maintained at 0°C for 20 min. If a yellow color still persisted the reaction was assumed to be complete. Then, the entire reaction mixture was dried down under nitrogen and the fatty acid methyl ester was redissolved in toluene or decane–toluene (1:1), except that ethyl acetate was used to dissolve methyl 16-hydroxyhexadecanoate.

PFBA-derivatization reaction
        After the carboxyl groups were methyl esterified with diazomethane, the 2-hydroxy methyl esters were derivatized with PFBA to form 2-O-pentafluoro-benzoyl-methyl esters. Methyl 2-hydroxyhexadecanoate was used to optimize the reaction. The objective of optimization was to obtain the highest yield in the shortest period of time using the mildest conditions possible. The optimal conditions were as follows.
        A stock solution of 0.1 M PFBA in toluene was found to be stable at room temperature. This concentration was 25-fold in excess of the hydroxy methyl ester solutions. Hydroxy methyl esters were dissolved in toluene, decane–toluene (1:1) or ethyl acetate and stored at room temperature. Just prior to running the reaction, 20 μl of pyridine were added to 0.48 ml of 0.1 M PFBA in a 6.0-ml glass scintillation vial. The PFBA–pyridine solution and the hydroxy methyl ester solution were equilibrated to 50°C in a heating block. After temperature equilibration, 0.5 ml of the hydroxy methyl ester solution was added to the PFBA solution. Using these conditions, the reaction was found to be 98% complete after 1 h (see Fig. 1).

RESULTS

Characterization of pentafluorobenzoic anhydride
        We wanted to be certain that we had synthesized the correct compound. There are very few references to this compound in the literature. Only the melting point has been reported[12]. For identification purposes we measured the ^{19}F NMR, the ^{13}C NMR, and IR spectrum of our product. First-order analysis was used for the NMR spectra.
        The ^{19}F chemical shifts (ppm) were: *ortho*: 136.7, *meta*: 161.8, *para*: 145.3. Assignments were based on the assignments for pentafluorobenzoic acid[16] and pentafluorobenzoyl chloride[17]. The ^{13}C chemical shifts (ppm) and nearest neighbor coupling constants (Hz) were: C-1: 106.9, *ortho*: 147.5, J_{CF}: 263.3; *meta*: 139.1, J_{CF}: 252.7; *para*: 145.9, J_{CF}: 255.7; C=O: 153.2. These assignments were again based on assignments for pentafluorobenzoic acid[18] and pentafluorobenzoyl chloride[19].
        The IR spectrum compared favorably with the vibrational spectrum of pentafluorobenzoyl chloride[20]. In anhydrides, the carbonyl stretch is split[21]. The splitting was 64 cm^{-1} and the bands occurred at 1741 cm^{-1} and 1805 cm^{-1}. The reported[21] values for benzoic anhydride (in *n*-hexane) are: 1740 cm^{-1} and 1801 cm^{-1}.
        All measurements made on the product were consistent with its being pentafluorobenzoic anhydride. It was found to be stable (constant melting point) in a desiccator at room temperature.
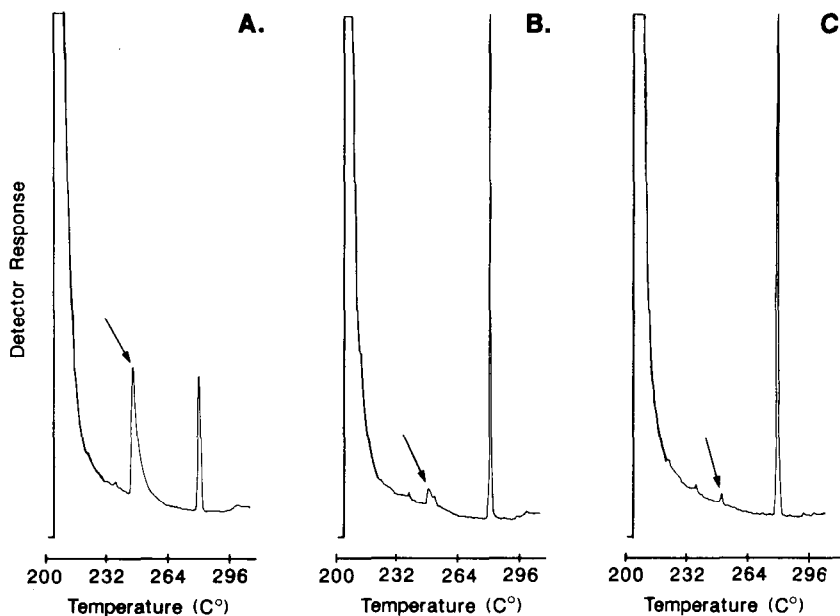
Fig. 1. Gas chromatograms of methyl 2-hydroxyhexadecanoate (arrow) and PFBA-derivatized methyl 2-hydroxyhexadecanoate during a derivatization reaction. The column was PPE-21. Chart speed is 0.5 cm/min. See Results and Experimental sections for further chromatographic conditions. Arrows indicate underivatized methyl 2-hydroxyhexadecanoate. (A) 1 min; (B) 36 min; (C) 62 min.

Optimization of the PFBA-derivatization

When optimizing the reaction parameters, we monitored the quantity of methyl 2-hydroxyhexadecanoate and derivatized methyl 2-hydroxyhexadecanoate using flame ionization detection (FID). The temperature program had an initial temperature of 200°C, a rate of 8°/min and a final temperature of 300°C (PPE-21 column). The reaction was 98% complete after 1 h using the optimal reaction conditions described in Experimental (Fig. 1). Based on unknown peaks in the chromatograms, which may be due to impurities, side reactions were limited to *ca.* 1.0% of derivatized methyl 2-hydroxyhexadecanoate. Upon standing for a few days the gas chromatogram of the reaction solution indicates that the reaction proceeded to 100% completion. From those observations, the FID response of derivatized methyl 2-hydroxyhexadecanoate is seen to be 1.36 times that of the underivatized compound. That relative response is consistent with the number of carbons found in each compound. Derivatized compounds in solution were found to be stable at room temperature for several months.

A plot of the disappearance of methyl 2-hydroxyhexadecanoate fits a first order curve with good correlation. The first order rate constant was found to vary linearly with PFBA concentration. The rate did not change appreciably with temperature. The pyridine concentration was found to be critical. Particularly at higher temperatures, the distillation of pyridine onto the sides of the vial and cap may slow the reaction and possibly the reaction may not proceed to completion, depending on how much

pyridine is lost from the solution. Consistent with previous results[10], when only pyridine was used as the solvent, the reaction solution turned yellow and then dark brown. Although it was not thoroughly investigated, derivatization with benzoic anhydride appears to proceed cleanly in pyridine.

The derivatization of 1-octanol, 3-octanol, 1-decanol, 1,10-decanediol, 1-dodecanol, 1-pentadecanol and 1-octadecanol all proceeded easily to completion, as in the case of the methyl 2-hydroxyhexadecanoate. The only tertiary alcohol attempted was trimethylcitrate. Even with a 50-fold excess of PFBA the reaction did not proceed to completion (overnight).

In this study, the reaction solution was injected directly into the gas chromatograph. Since these results indicate that this derivatizing agent may be used for measuring a large variety of primary and secondary alcohols, the mode of sample cleanup would vary according to the source of the alcohol. One way of removing excess reagents and contaminants is with an aqueous wash and subsequent use of a disposable silica column[10].

Mass spectra

To be certain of the structure of our PFBA-derivatized compounds, we measured the positive-ion chemical ionization (PICI) mass spectrum of derivatized methyl 2-hydroxyhexadecanoate (Fig. 2). Table II shows that the fragmentation pattern is consistent with the proposed structure. (See ref. 22 for a review of the CI-MS of lipids.) The similar relative abundances of $[M + H]^+$ and $[M - H]^+$ and the small relative abundances of $[M - CH_3OH]^+$ and $[M - H - CH_3OH]^+$, can be attributed to the relatively long alkyl chain [23]. Only the assignment of the ion at m/z 169 is questionable. It is probable that this ion is associated with the pentafluorobenzoyl moiety. Albeit remote, another possibility is $CH_3(CH_2)_{11}$. An ion at m/z 169 is often associated with perfluoroalkanes[24]. Possibly, this peak is due to a residual contaminant in the instrument[25].

The NICI mass spectra for derivatized methyl 2-hydroxyhexadecanoate and derivatized methyl 2-hydroxycerotate (C_{26}) at an ion-source temperature of 80°C are shown in Fig. 3. Contrary to 3-O-pentafluorobenzoyl-methyl myristate (3-OH:14:0) ref. 10, our compounds show significant fragmentation even at 80°C. Possibly, the greater fragmentation observed here is due to the closer proximity of the two ester groups to each other in our compounds, since the methane-PICI mass spectrum of methyl phthalate shows greater fragmentation than the methane-PICI mass spectrum
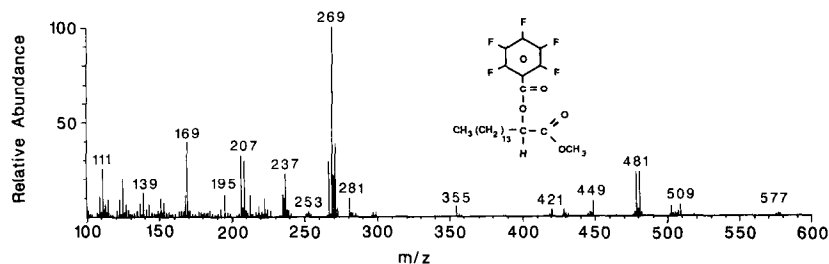

Fig. 2. Positive-ion chemical ionization mass spectrum of derivatized methyl 2-hydroxyhexadecanoate. Ion source temperature is 240°C.

TABLE II

PICI-MS FRAGMENTATION PATTERN OF DERIVATIZED METHYL 2-HYDROXYHEXA-
DECANOATE

M is the molecular ion m/z 480. Ion-source temperature was 240°C.

m/z	% Relative abundance	Positive ion assignment
509	5.8	$M + C_2H_5$
481	23.2	$M + H$
479	23.0	$M - H$
449	6.8	$[M + H] - (CH_3OH)$
421	2.5	$M - (CO_2CH_3)$
355	4.9	Column bleed $[Si_5O_5(CH_3)_9]$
281	9.3	Column bleed $[Si_4O_4(CH_3)_7]$
269	100.0	$CH_3(CH_2)_{13}CHCO_2CH_3$
253	2.5	$CH_3(CH_2)_{13}CHCOCH_3$
237	22.7	$CH_3(CH_2)_{13}CCO$
207	31.3	Column bleed $[Si_3O_3(CH_3)_5]$
195	11.2	C_6F_5CO
169	39.3	$C_6F_5H + H$ (?)
139	12.2	C_6F_2HCO
111	25.5	C_6F_2H

of methyl isophthalate[26]. Ion (m/z) assignments for fragments are: 130: C_6F_3H, 148: C_6F_4, 167: C_6F_5, 211: $C_6F_5CO_2$. Ion m/z 480 corresponds to the molecular ion for derivatized methyl 2-hydroxyhexadecanoate (Fig. 3A) and m/z 620 corresponds to the molecular ion for derivatized methyl 2-hydroxycerotate (Fig. 3B). The ion at m/z 193 has previously[8] been tentatively assigned as a tetrafluorohydroxybenzoyl system. Ion
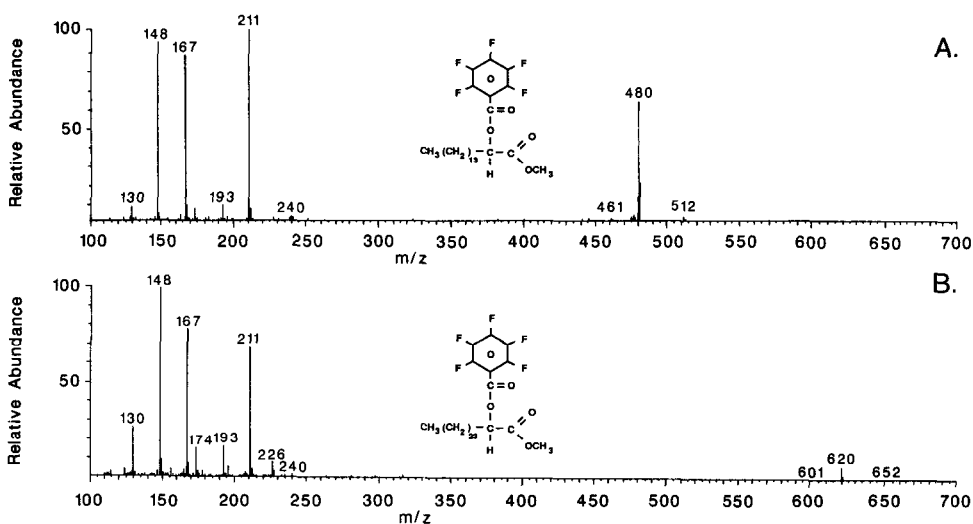

Fig. 3. Negative-ion chemical ionization mass spectrum of (A) derivatized methyl 2-hydroxyhexadecanoate; (B) derivatized methyl 2-hydroxycerotate. Ion source temperature is 80°C.

m/z 174 is consistent with this assignment (loss of fluorine). Another possibility is replacement of fluorine with hydrogen (m/z 211 − F + H). The ions at m/z 174 (m/z 211 − 2F + H) and m/z 130 (C_6F_3H) are consistent with this possibility.

Both compounds (C_{26} and C_{16}, see Fig. 3) show a small amount of $[M + 32]^-$. Possibly, this is due to the addition of CH_3OH. In a preliminary measurement using the direct-introduction probe technique (data not shown), the spectrum of *ca.* 1.0 μg of derivatized methyl 2-hydroxycerotate had $[M + 32]^-$ for its base peak (M^- had a relative abundance of 36%). The ions attributed to electron-capture processes approach a limiting value with large sample sizes[27]. If large sample concentrations are encountered, adduct ion formation may dominate the spectra[27] (leading to erroneous analysis of the spectrum). The presence of $[M + 32]^-$ in the spectra of Fig. 3 implies that even 3.5 ng of sample is slightly overloading the instrument. Taking into consideration that pentafluorobenzoyl derivatives can be observed in the femtogram region[8], this is not surprising.

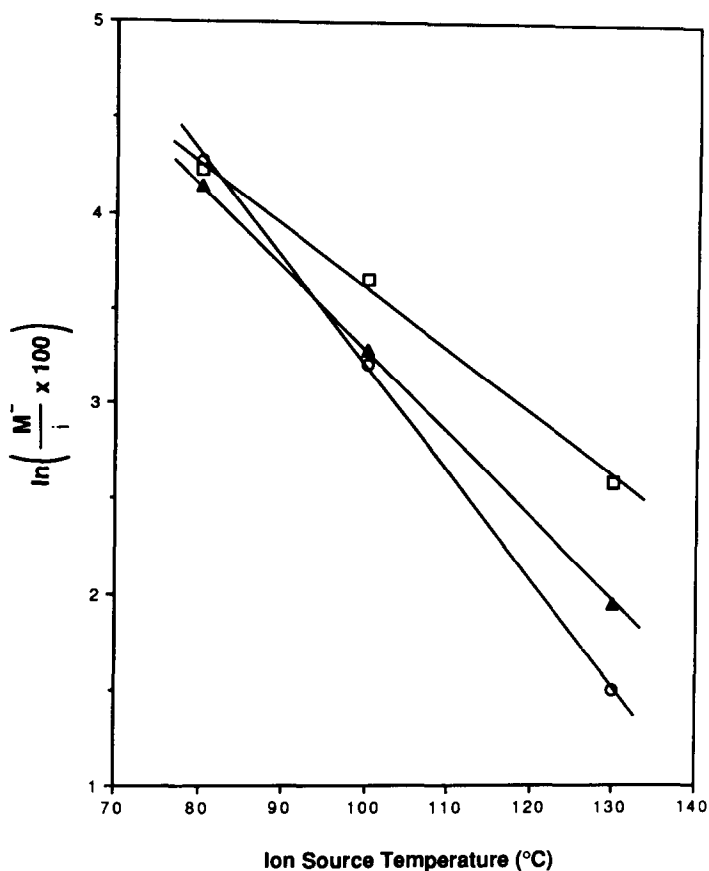Fig. 4 shows the ion-source temperature dependence of the NICI-MS frag-

Fig. 4. This plot shows the temperature dependence of the natural logarithm of the ratio (× 100) of the relative abundances of the molecular anion (m/z 480) to the pentafluorobenzoyl anion m/z 211 (▲), to the pentafluorophenyl anion m/z 167 (○), and to the tetrafluorophenyl anion m/z 148 (□) for the NICI mass spectrum of derivatized methyl 2-hydroxyhexadecanoate.

mentation for derivatized methyl 2-hydroxyhexadecanoate. When the ion-source temperature was higher (240°C), the molecular ion (m/z 480) was not observable; only m/z 148 and m/z 167 were present in the spectrum. These data suggest a dissociative electron-capture mechanism[28,29]. This is consistent with our results using ECD for derivatized methyl 2-hydroxystearate (see below). From Fig. 3B one can see that the molecular ion (m/z 620) for derivatized methyl 2-hydroxycerotate (C_{26}) is barely observable even when the ion-source temperature is 80°C. This indicates, in agreement with previous measurements on comparable compounds[28–30], that for proper identification of these derivatives, within the confinements of the instrument, one must find the optimum ion-source temperature.

The total positive-ion chromatogram and the total negative-ion chromatogram of derivatized methyl 2-hydroxyhexadecanoate indicate that the reaction goes to 100% completion and there are not any observable side reactions. This agrees with our measurements using the PPE-21 column (Fig. 1) and the Poly-S 179 column (data not shown).

The sensitivity of the electron-capture detector

The response of the electron-capture detector is a function of the compound being detected and the temperature of the detector. Assuming that $A + e^- \rightleftharpoons A^-$ is an equilibrium process, it can be shown that[31]:

$$K_{eq} = CT^{-3/2} \exp(E_a/kT)$$

where A is the electron acceptor, K_{eq} is the equilibrium constant, C is a constant, T is the absolute temperature, E_a is the electron affinity, k is the Boltzman constant.

When low detection limits are required, it is important to find the optimum detector temperature for the compounds of interest[32]. The data for detector response with respect to detector temperature is usually plotted in the form $\ln(CK_{eq}T^{3/2})$ *vs.* $1/T$. CK_{eq} is the intensity of the response. This plot indicates the optimal temperature and the mechanism of electron capture[33]. Discontinuities in these plots indicate that the mechanism can change with temperature. Fig. 5 indicates that derivatized 1-dodecanol has a non-dissociative mechanism that favors use of low temperatures. The pentafluorobenzoate derivative of *n*-hexanol[34] exhibits the same mechanism. Contrary to that, but consistent with the NICI-MS data for derivatized methyl 2-hydroxyhexadecanoate (Fig. 4), derivatized methyl 2-hydroxystearate (Fig. 5) has a dissociative mechanism up to approximately 250°C. For this derivatized hydroxy fatty acid 250°C is the optimal temperature. The decline in response observed above 250°C is probably due to thermal electron detachment[30].

The linearity of ECD response with amount may be very limited. For example, butyl 3-hydroxydodecanoate derivatized with heptafluorobutyric anhydride has a linear response only between 5.0 pg and 50.0 pg[35]. Fig. 6 indicates that with our apparatus, the linear region for derivatized methyl 2-hydroxystearate occurs between 0.07 pmol (34 pg) and 7.0 pmol (3.4 ng). The upper limit of linearity is consistent with our NICI-MS data. Using an on-column injector and a capillary column, Mohamed *et al.*[8] were able to generate a linear calibration curve from 1 fg to 1 ng for N^4-pentafluorobenzoyl-1,3-dimethylcytosine.
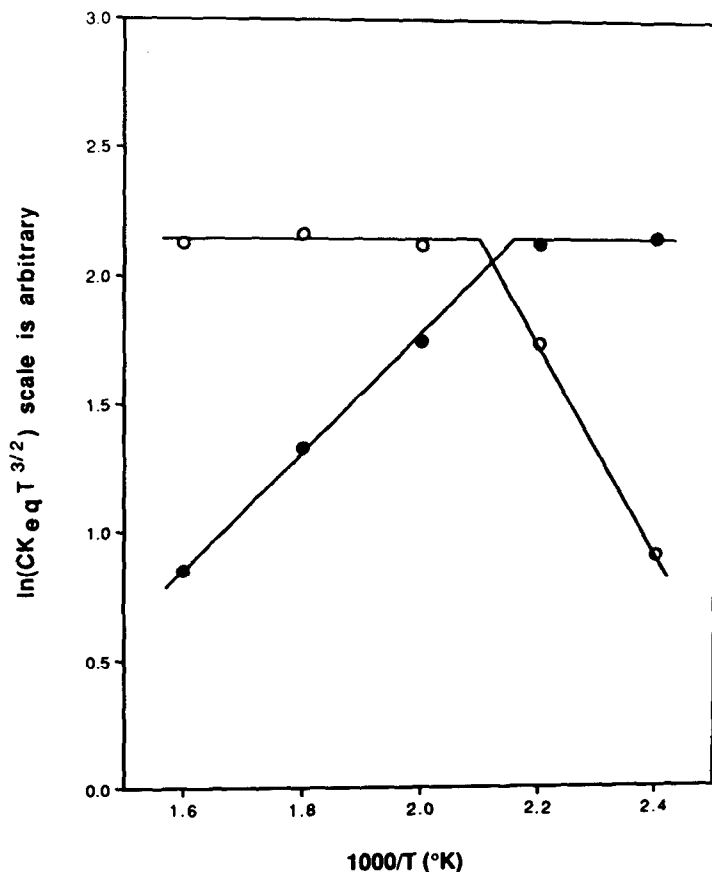
Fig. 5. Plot showing temperature dependence of ECD response. $\ln(CK_{eq}T^{3/2})$ vs. $1/T$ for derivatized dodecyl alcohol ($\bullet$) and derivatized methyl 2-hydroxystearate ($\bigcirc$). Scale has been normalized to same maximum response for both compounds.

Calculation of specific retention volumes

The goal here was to measure and report the retention volumes of the homologous series of derivatized 2-hydroxy fatty acid methyl esters so that they could be used as a standard for the identification of unknowns in subsequent measurements. It is customary to simply report retention times or retention temperatures (when using temperature programming); however, these were not measured directly because the carrier gas flow-rate was found to be a function of column temperature as will be shown. Therefore, measured retention times were converted to specific retention volumes taking into consideration that the carrier gas flow-rate was not constant.

Because of the large dead volume between the column and the electron-capture detector, the high temperatures employed in these measurements, the length of the column (12 ft.), and the use of 100–120 mesh support, maximum flow of the carrier gas was required to obtain reasonable peak shape. Under these conditions, the carrier gas flow-rate (as it entered the instrument) plotted against $1/T^{0.7}$ yielded a straight line
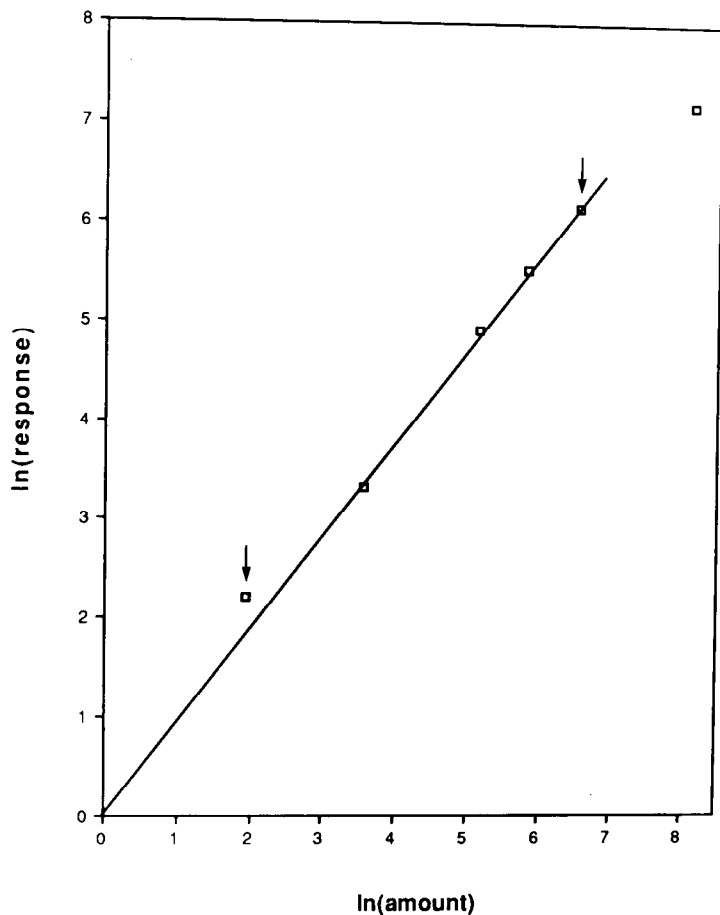
ln(amount)

Fig. 6. Standard curve of ECD response *vs.* amount of derivatized methyl 2-hydroxystearate. Arrows indicate 0.07 and 7.0 pmol.

(Fig. 7). Since the reciprocal of the viscosity of a gas as a function of temperature approximately fits the same curve[36], this indicated that the flow controllers were inoperative and the measurements were being made at constant pressure rather than constant flow.

For use in constructing temperature programs[37], isothermal retention time *vs.* temperature was measured. It is generally accepted[38] that:

$$V_r - V_{ds} = A \exp(\Delta H/RT)$$

where V_r is the isothermal retention volume, V_{ds} is the dead space volume, A is assumed to be independent of temperature and it has been shown[39] that $R\ln A$ is equal to the entropy of solution of the component in the stationary liquid phase, ΔH is the heat of evaporation of the component from the stationary liquid phase, R is the gas constant, and T is the absolute temperature. When operating under constant pressure, because of the required corrections, it is much more convenient to use retention volumes rather
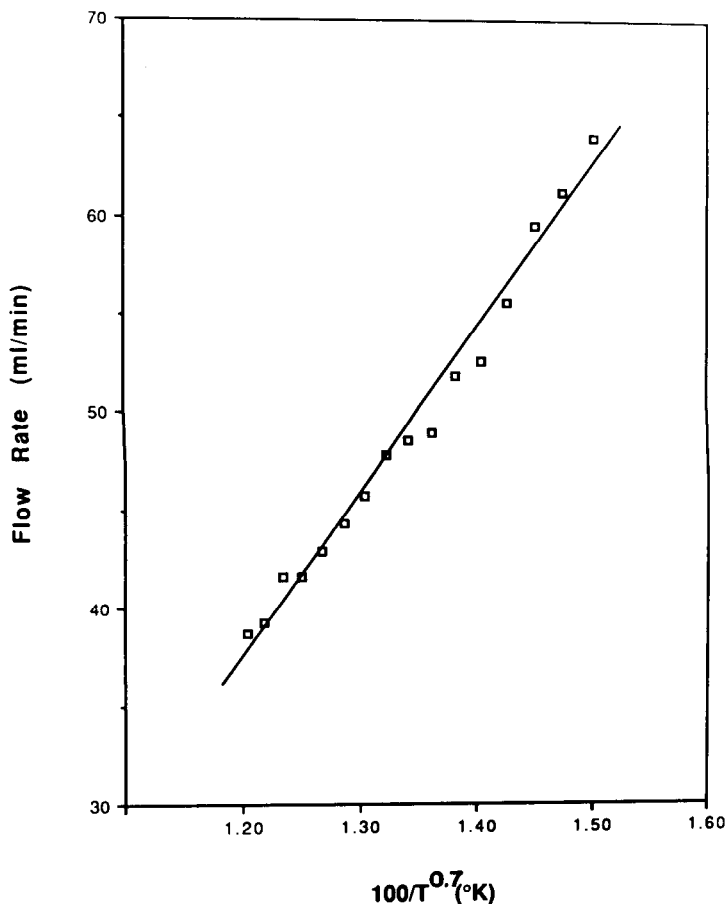
Fig. 7. Plot showing temperature dependence of flow-rate at column inlet. Flow-rate at column inlet *vs.* $1/T^{0.7}$ is plotted.

than retention times. Taking into consideration that the flow-rate was measured at the column inlet, the measured retention volume (V_{meas}) was corrected for the pressure differential in the column according to the method of James and Martin[40].

$$V_R = j V_{meas}$$

$$j = 1.5\{[(P_0^2/P_i^2) - 1]/[(P_0^3/P_i^3) - 1]\}$$

where V_R is the corrected retention volume, P_i is the inlet pressure (78.0 p.s.i.), and P_0 is the outlet pressure (14.7 p.s.i.). Then, according to the method of Littlewood *et al.*[41] V_R was converted to the specific retention volume, V_g.

All of the above corrections yield:

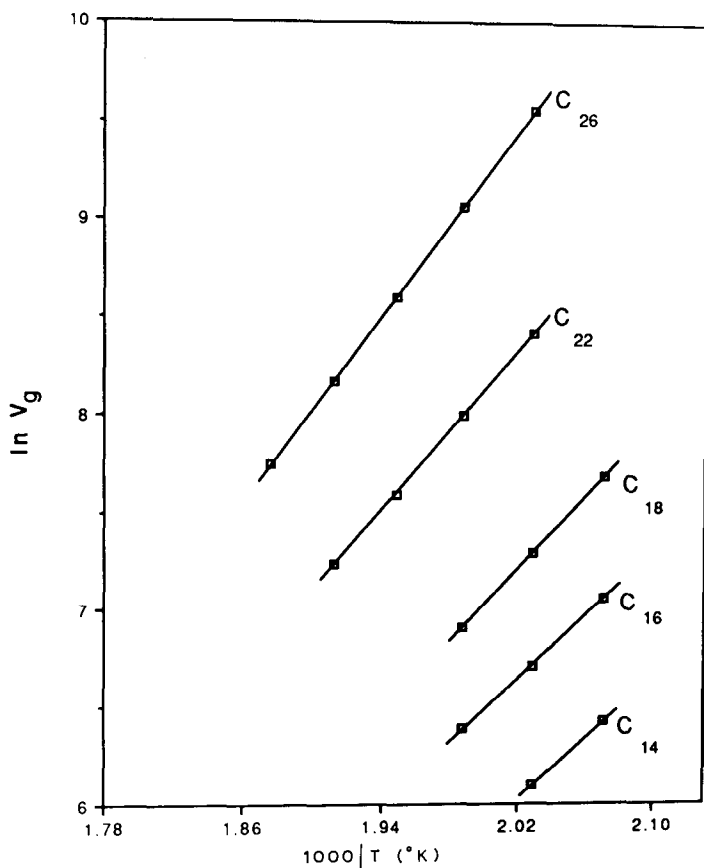$$F(T) = (79\,326.0/T^{0.7}) - 592.2$$

$$V_g = (t_R - t_{ds}) F(T)$$

Fig. 8. Temperature dependence of the specific retention volume (V_g). ln V_g of non-derivatized 2-hydroxy fatty acids vs. $1/T$ is plotted.

where $F(T)$ is the corrected flow-rate at 0°C per gram of stationary phase (ml/min-g), t_R is the retention time and t_{ds} is the t_R for dead space. According to Littlewood et al.[41]:

$$V_g = A \exp(\Delta H/RT)$$

where ΔH is the heat of evaporation of the component from the stationary phase at 0°C. From plots of ln V_g vs. $1/T$ for the homologous series of 2-hydroxy fatty acid methyl esters (Fig. 8 non-derivatized compounds and Fig. 9 derivatized compounds), Table III was generated. The difference in $\Delta H/R$ between non-derivatized compounds and derivatized compounds appears to be a constant independent of carbon number and has a mean of 2310. This implies that the derivatized products form a homologous series which is analogous to the homologous series of the commercially-obtained non-derivatized 2-hydroxy fatty acids.
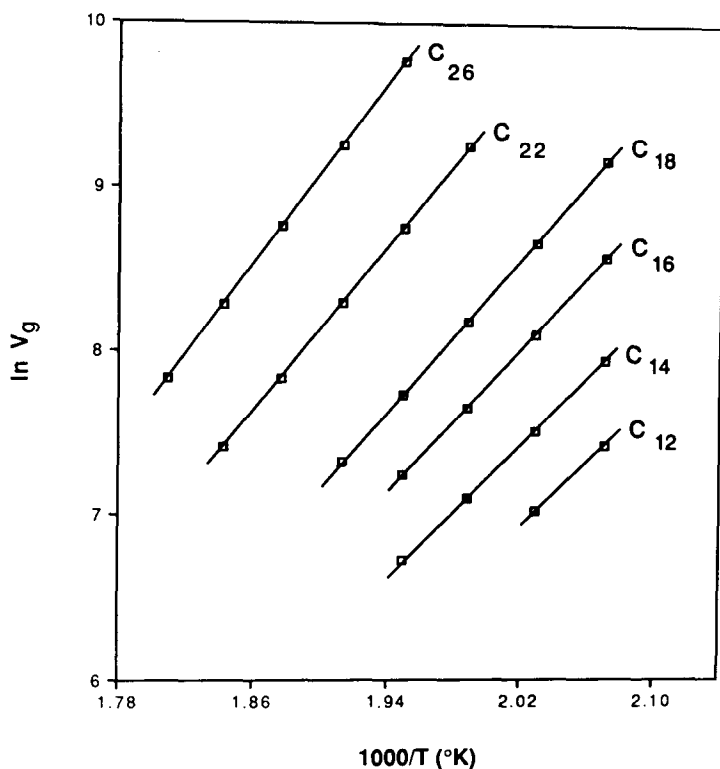
Fig. 9. Temperature dependence of the specific retention volume (V_g). $\ln(V_g)$ of derivatized 2-hydroxy fatty acids vs. $1/T$ is plotted.

TABLE III

THERMODYNAMIC PROPERTIES OF THE HOMOLOGOUS SERIES

The column packing was Poly-S 179. The carrier gas was nitrogen.

Carbon number	Non-derivatized		Derivatized		Difference[a]
	$\Delta H/R$	$\ln A$	$\Delta H/R$	$\ln A$	
12	—	—	9760	−12.78	—
14	7860	−9.84	10 160	−13.09	2300
16	8140	−9.80	10 930	−14.06	2790
18	9360	−11.69	11 570	−14.81	2210
22	10 160	−12.20	12 490	−15.58	2330
26	11 800	−14.40	13 710	−16.96	1910
				Mean:	2310
			Standard deviation:		283

[a] This is the difference between $\Delta H/R$ for derivatized compounds and $\Delta H/R$ for non-derivatized compounds.
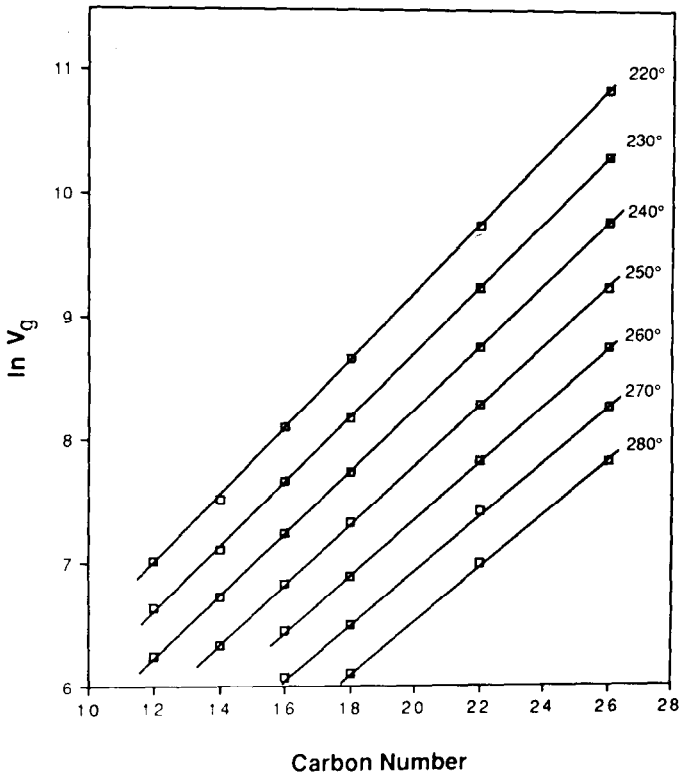
Fig. 10. Plot showing relation between ln V_g and the number of carbons for derivatized hydroxy fatty acid methyl esters.

TABLE IV

COMPARISON OF OBSERVED AND PREDICTED RETENTION TEMPERATURES FOR DERIVATIZED COMPOUNDS FOR THE TEMPERATURE PROGRAM $T_i = 210°C$, rate $= 2°/min$

A 12 ft. × 2 mm column was packed with 3% Poly-S 179. Carrier gas was nitrogen. Inlet pressure was 78 p.s.i.

Carbon number	T_R (°C)	
	Observed	Predicted
12	216.8	216.9
14	221.5	220.6
16	227.5	227.1
18	235.1	234.6
22	253.5	253.4
26	272.7	273.9

Temperature programming

To test the usefulness of the data in Table III, they were used to predict retention temperatures for the following temperature program: $T_i = 210°C$, rate $= 2°$ per min, $T_f = 310°C$. Since V_{ds} was found not to vary with temperature (over the range studied), this term was neglected. From Harris and Habgood[38], to calculate the retention temperature, one must evaluate the following integral:

$$r/F_{T_i} = (1/A) \int_{T_i}^{T_R} F'(T)\exp(-\Delta H/RT)dT$$

where r is the heating rate (deg/min), F_{T_i} is the corrected flow-rate per gram of stationary phase at the initial temperature, $F'(T)$ is the corrected flow-rate at the column outlet, T_R is the retention temperature and $\Delta H/R$ is assumed to be independent of temperature.

Since $PV = nRT$, where n is the number of moles,

$$F'(T) = \frac{F(T)}{T}$$

Consequently (for this system):

$$4.38 \cdot 10^{-3} = (1/A) \int_{T_i}^{T_R} [F(T)/T]\exp(-\Delta H/RT)dT$$

Since this integral can not be integrated in closed form[42], it must be evaluated numerically. This calculation was done on a personal computer (Tandy 1000 HD with an 8088 microprocessor and an 8087 math coprocessor) using Turbo Pascal Numerical Methods Toolbox (Version 4.0)[43] and the method of Akporhonor *et al.*[44]. Table IV indicates excellent agreement between observed and predicted values.

A retention index for ECD-sensitive derivatized hydroxy fatty acids

Fig. 10 shows that for the homologous series of derivatized 2-hydroxy fatty acid methyl esters there is a linear variation of $\ln V_g$ with number of carbons. Therefore, it is possible to define a retention index[45] of an unknown, I_x, for the derivatized compounds such that:

$$I_x = 100 \left[z + \left(\frac{\ln V_{g_x} - \ln V_{g_z}}{\ln V_{g_{(z+1)}} - \ln V_{g_z}} \right) \right]$$

where z is the number of carbon atoms. Using this index at a column temperature of 220°C with interpolated values, yields $I = 1887$ for derivatized methyl ricinoleate (12-OH:18:1ω9) and $I = 1989$ for methyl 16-hydroxyhexadecanoate (16-OH:16:0). The anomolously high retention index of this derivatized 16-hydroxy compound is consistent with previous work on acetoxy fatty acid methyl esters[46]. In subsequent

work, when mass spectral data are unavailable, unknown peaks will be reported according to this retention index, although it may be subject to modification if a capillary column is used.

Derivatization of cholesterol

Since our intent is to use this method to study lipid peroxidation in the retina, we were concerned that the large quantity of cholesterol[47] present might overwhelm the electron-capture detector. When the kinetic course of the derivatization of cholesterol was monitored using GC–FID, the cholesterol peak disappeared as a function of reaction time. The product peak appeared at a much shorter retention time than cholesterol, indicating that it probably was not cholesteryl pentafluorobenzoate. It has been shown that cholesteryl heptafluorobutyrate thermally decomposes to cholesta-3,5-diene[48]. The retention time of our product peak matched that of commercially obtained cholesta-3,5-diene. Since cholesta-3,5-diene has a very weak ECD response, it is anticipated that cholesterol will not interfere with future measurements.

DISCUSSION

Although the pentafluorobenzyl esters of carboxylic acids[49] and pentafluoro-benzoate esters of phenols[11,50] have been measured in the past, use of penta-fluorobenzoic anhydride to derivatize secondary alcohols has not been reported. Our results indicate that this is an excellent derivatizing reagent for primary and secondary alcohols.

From the chromatographic data it is clear that the retention temperature of hydroxy fatty acid methyl esters is significantly increased with the addition of the pentafluorobenzoate group (Figs. 8 and 9). With the use of Poly-S 179 packing the high temperatures required for elution were not a significant problem. This packing has been shown to be useful in the separation of a variety of compounds at temperatures as high as 395°C[51,52]. Although, we do not recommend using temperatures that high for these derivatives since they may undergo pyrolytic elimination of the pentafluoro-benzoyl group[53,54]. The polarity of Poly-S 179 has been shown to be comparable to Carbowax 20M[55].

The efficiency of the column for derivatized methyl 2-hydroxycerotate (C_{26}) and derivatized 2-hydroxybehenate (C_{22}) is 630 and 530 theoretical plates, respectively. This yields a resolution[56] of 6.3 between those two components, indicating that an additional five peaks may be resolved between these two homologues[57]. This resolution may be improved by reducing the dead volume between the column outlet and the detector. Comparable resolution occurs between derivatized methyl 2-hydroxystearate (C_{18}) and derivatized methyl 2-hydroxybehenate. A capillary column coated with Poly-S 176 (similar to Poly-S 179) has been shown to provide good separations of fatty acid methyl esters[58] and would provide much better resolution of the derivatized compounds. Recently, a Poly-S 179 capillary column was prepared and used to separate polycyclic aromatic hydrocarbons and triglycerides at temperatures as high as 380°C using GC–FID[59]. The favorable results reported indicate that this column may be very useful in studying the oxidation products of lipids in the retina using GC–ECD.

Previous methods for detecting trace levels of hydroxy fatty acids derivatize the

hydroxyl group with a trimethylsilyl group and esterify the carboxyl group with pentafluorobenzyl bromide[49] or transesterify the carboxyl group with pentafluoro-benzyl alcohol[3]. A disadvantage of trimethylsilyl derivatives is that they are vulnerable to hydrolysis and subsequent side reactions[3]. The result of using pentafluorobenzyl bromide or pentafluorobenzyl alcohol to esterify or transesterify the carboxyl group is that all fatty acids in the mixture to be analyzed have a large electron-capture response. Single-ion monitoring (SIM) MS alleviates that problem to a large extent[3], but coelution can still occur in complex biological mixtures. When PFBA is used to esterify the hydroxyl group, then the specificity of the method is increased substantially because only the fatty acids of interest [*i.e.* those that have been peroxidized (and subsequently reduced to alcohols)] are derivatized.

SIM-NICI-MS with the pentafluorobenzyl group on the carboxyl yields a sensitivity of *ca.* 10 pg[3]. This is extremely good considering that the observed ions are carboxylate anions sans the pentafluorobenzyl group. Without SIM, sensitivity only in the nanogram region has been reported for pentafluorobenzyl esters when using NICI-MS[49]. Our results indicate that when pentafluorobenzoate is on the hydroxyl group (O-pentafluorobenzoyl-methyl esters), a large amount of the sample introduced into the mass spectrometer does not undergo any fragmentation. Consequently, the observed anion still maintains its (extremely good) electron-capture sensitivity. When the electron-capturing group is on the hydroxyl group (where it remains), then a 20-fold increase in sensitivity (*ca.* 0.3 pg) is observed in the SIM-NICI-MS of 3-O-pentafluorobenzoyl methyl myristate[10]. Also, pentafluorobenzoyl derivatives of cytosine have a sensitivity of one femtogram using NICI-MS[8]. For the specific analysis of lipid hydroperoxides our method should be a useful complement to the method of Van Kuijk and co-workers[3,4].

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REFERENCES

1 G. J. Handelman and E. A. Dratz, *Adv. Free Radical Biol. Med.*, 2 (1986) 1.
2 C. F. Poole and S. K. Poole, *J. Chromatogr. Sci.*, 25 (1987) 434.
3 F. J. G. M. van Kuijk, D. W. Thomas, R. J. Stephens and E. A. Dratz, *J. Free Radical Biol. Med.*, 1 (1985) 387.
4 F. J. G. M. van Kuijk and E. A. Dratz, *Free Radical Biol. Med.*, 3 (1987) 349.
5 A. J. Francis, E. D. Morgan and C. F. Poole, *J. Chromatogr.*, 161 (1978) 111.
6 A. Sonesson, L. Larsson and J. Jimenez, *J. Chromatogr.*, 417 (1987) 366.
7 A. Zlatkis and C. F. Poole, *Anal. Chem.*, 52 (1980) 1002 A.
8 G. B. Mohamed, A. Nazareth, M. J. Hayes, R. W. Giese and P. Vouros, *J. Chromatogr.*, 314 (1984) 211.
9 L. Larsson, A. Sonnesson and J. Jimenez, *Eur. J. Clin. Microbiol.*, 6 (1987) 729.

10 A. Sonesson, L. Larsson, G. Westerdahl and G. Odham, *J. Chromatogr.*, 417 (1987) 11.
11 T. M. Trainor and P. Vouros, *Anal. Chem.*, 59 (1987) 601.
12 S. Abdel-Baky and R. W. Giese, *J. Org. Chem.*, 51 (1986) 3390.
13 C. J. Pouchert (Editor), *The Aldrich Library of FT-IR Spectra*, Aldrich, Milwaukee, WI, 1985, Vol. 2, p. 331.
14 A. I. Vogel, *Practical Organic Chemistry*, Wiley, New York, 3rd ed., 1962, p. 969.
15 G. Odham, L. Larsson and P. Mardh (Editors), *Gas Chromatography/Mass Spectrometry —Applications in Microbiology*, Plenum Press, New York, 1984, p. 58.
16 M. G. Hogben and W. A. G. Graham, *J. Am. Chem. Soc.*, 91 (1969) 283.
17 G. A. Olah and Y. K. Mo, *J. Org. Chem.*, 38 (1973) 2682.
18 J. M. Briggs and E. W. Randall, *J. Chem. Soc. Perkin II*, (1973) 1789.
19 A. I. Rezvukhin, G. G. Furin, G. A. Kalabin, S. A. Krupoder, V. G. Storozhenko, V. M. Polonov, D. F. Kushnarev and G. G. Yakobson, *Siberian Chem. J.*, 5 (1982) 94 (in Russian).
20 K. Singh, S. R. Singh and I. S. Singh, *Curr. Sci.*, 41 (1972) 471.
21 L. J. Bellamy, B. R. Connelly, A. R. Philpotts and R. L. Williams, *Z. Elektrochem.*, 64 (1960) 563.
22 Y. Y. Lin and L. L. Smith, *Mass Spectrom. Rev.*, 3 (1984) 319.
23 C. W. Tsang and A. G. Harrison, *J. Chem. Soc. Perkin Trans. II*, (1975) 1718.
24 F. W. McLafferty, *Interpretation of Mass Spectra*, W. A. Benjamin, Reading, MA, 2nd ed., 1973, p. 267.
25 H. Budzikiewicz, *Mass Spectrom. Rev.*, 5 (1986) 345.
26 A. G. Harrison and R. K. M. R. Kallury, *Org. Mass Spectrom.*, 15 (1980) 277.
27 E. A. Stemmler and R. A. Hites, *Anal. Chem.*, 57 (1985) 684.
28 B. J. Miwa, W. A. Garland and P. Blumenthal, *Anal. Chem.*, 53 (1981) 793.
29 J. Greaves, J. G. Bekesi and J. Roboz, *Biomed. Mass Spectrom.*, 9 (1982) 406.
30 P. Kebarle and S. Chowdhury, *Chem. Rev.*, 87 (1987) 513.
31 W. E. Wentworth and E. Chen, *J. Phys. Chem.*, 71 (1967) 1929.
32 E. C. M. Chen and W. E. Wentworth, *J. Chromatogr.*, 68 (1972) 302.
33 J. C. Steelhammer and W. E. Wentworth, *J. Chem. Phys.*, 51 (1969) 1802.
34 A. Zlatkis and B. C. Pettitt, *Chromatographia*, 2 (1969) 484.
35 Inder Jit Sud and D. S. Feingold, *J. Invest. Dermatol.*, 73 (1979) 521.
36 L. S. Ettre, *Chromatographia*, 18 (1984) 243.
37 A. S. Said, A. M. Jarallah and R. S. Al-Ameeri, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 345.
38 W. E. Harris and H. W. Habgood, *Programmed Temperature Gas Chromatography*, Wiley, New York, 1966, p. 146.
39 A. B. Littlewood, *Anal. Chem.*, 36 (1964) 1441.
40 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
41 A. B. Littlewood, C. S. G. Phillips and D. T. Price, *J. Chem. Soc.*, (1955) 1480.
42 R. Rowan, Jr., *Anal. Chem.*, 34 (1962) 1042.
43 *Turbo Pascal Numerical Methods Toolbox, Version 4.0*, Borland International, Scotts Valley, CA.
44 E. E. Akporhonor, S. Le Vent and D. R. Taylor, *J. Chromatogr.*, 405 (1987) 67.
45 E. Kováts, *Adv. Chromatogr.*, 1 (1965) 229.
46 J. S. O'Brien and G. Rouser, *Anal. Biochem.*, 7 (1964) 288.
47 S. J. Fliesler and R. E. Anderson, *Prog. Lipid Res.*, 22 (1983) 79.
48 C. F. Poole and E. D. Morgan, *J. Chromatogr.*, 90 (1974) 380.
49 R. J. Strife and R. C. Murphy, *J. Chromatogr.*, 305 (1984) 3.
50 N. K. McCallum and R. J. Armstrong, *J. Chromatogr.*, 78 (1973) 303.
51 R. D. Schwartz, R. G. Mathews, S. Ramachandran, R. S. Henley and J. E. Doyle, *J. Chromatogr.*, 112 (1975) 111.
52 R. Aneja, A. Bhati, R. J. Hamilton, F. B. Padley and D. A. Steven, *J. Chromatogr.*, 173 (1979) 392.
53 C. H. DePuy and R. W. King, *Chem. Rev.*, 60 (1960) 431.
54 F. L. Greenwood, *J. Org. Chem.*, 24 (1959) 1735.
55 F. Sellier, G. Tersac and G. Guiochon, *J. Chromatogr.*, 219 (1981) 213 (French).
56 W. Supina, *The Packed Column in Gas Chromatography*, Supelco, 1974, pp. 12–15.
57 R. A. Hurrell and S. G. Perry, *Nature (London)*, 196 (1962) 571.
58 S.-N. Lin and E. C. Horning, *J. Chromatogr.*, 112 (1975) 465.
59 R. Burrows, D. G. Gillespie, M. Cooke and O. Eddib, *J. Chromatogr.*, 404 (1987) 248.