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Note

Pentafluorobenzyl esters as derivatives for the semi-preparative high-performance liquid chromatography of fatty acids

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Our work on the metabolism of leaf fatty acids requires a high-performance liquid chromatographic (HPLC) method for the isolation of individual fatty acids. Such a system must overcome the widespread problem in reversed-phase systems of critical pairs, that is, fatty acids such as palmitoleic and arachidonic acid that elute as single peaks[1]. In addition it was shown[1] that on a preparative scale palmitic and oleic acids would not be sufficiently separated to allow clean isolations of each component. Initially we investigated two types of columns containing Ag^+, in order to separate according to the number of double bonds, but we were not encouraged by the results and therefore turned our attention to normal-phase HPLC on silica. Schwarzenbach[2] has shown that oleic and linoleic acids can be readily separated on silica that has been pre-treated with citric acid–sodium citrate (pH 3.0). It appears that this separation can be made, once the ionization of the carboxyl group is controlled, according to the adsorptive properties associated with the double bonds. We felt that an alternative to controlling the ionization would be to synthesize esters which would then afford the possibility of a separation on silica according to the number of double bonds. As we wished to use ultraviolet detection (254 nm) we investigated pentafluorobenzyl esters and these proved to be suitable. This paper describes their separation on silica according to the number of double bonds and also according to chain length by C_{18} reversed-phase HPLC.

The chromatographic separation of cyclopropanoid fatty acids from their monounsaturated isomers in biological extracts is a difficult problem[3]. We report here that pentafluorobenzyl esters of *cis*-9,10-methylenehexadecanoic and heptadec-10-enoic acids, and similarly their C_{19} homologues, can be completely separated using the described HPLC technique.

EXPERIMENTAL

A Waters Assoc. M45 or M6000A pump, a U6K injector and an M440 detector with filters for 254 nm detection were used. Samples for normal-phase separations were dissolved in benzene and those for reversed-phase separations in isopropanol.

Pentafluorobenzyl (PFB) esters were synthesized from 2,3,4,5,6-pentafluoro-

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benzyl bromide (Fluka, Buchs, Switzerland) by a procedure based on that of Ehrsson[4]. Up to 1 mg of fatty acid sample was dissolved in 1 ml of dichloromethane and 1 ml of a solution containing 0.1 mmol of tetrabutylammonium hydrogen sulphate (Fluka) and 0.2 mmol of sodium hydroxide was added. Pentafluorobenzyl bromide (20 μl) was then added, the mixture shaken vigorously at room temperature for 30 min and the dichloromethane phase was evaporated. Ehrsson[4] has shown that the esterification is quantitative for octanoic and longer chain acids under similar conditions. The residue was taken up in hexane and loaded on to a silica Sep-Pak (20 × 10 mm I.D. plastic columns from Waters Assoc., Milford, MA, U.S.A.) and the PFB esters were eluted with dichloromethane–hexane (3:17).

Four sets of standard PFB esters were prepared: (a) PFB-18:0; (b) an approximately equimolar mixture of PFB-18:0; PFB-18:1, PFB-18:2 and PFB-18:3; (c) an approximately equimolar mixture of the straight-chain fatty acid PFB esters, C_2, C_3, C_4, C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16} and C_{18}; (d) a mixture containing 2 parts of PFB-*trans*-18:1 and 1 part of PFB-*cis*-18:1. Mixture (d) was prepared as described above whereas the other three required a scaling up of the amounts. Consequently, these three standards were partially purified by application to a Merck Lobar column (size A, 240 × 10 mm I.D.) packed with silica, followed by elution with dichloromethane–hexane mixtures and detection at 254 nm with a Pye Unicam SP-6 spectrophotometer.

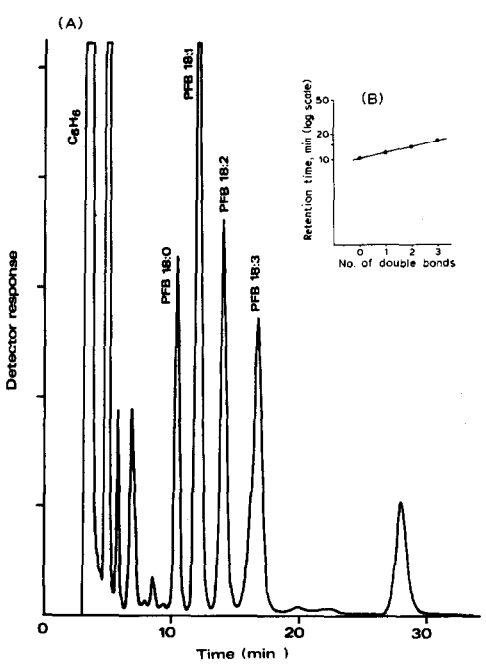
Separations of PFB esters were carried out by normal-phase chromatography on a μPorasil semi-preparative column (300 × 7.8 mm I.D.) (Waters Assoc.) using dry dichloromethane–hexane half-saturated with water (3:17 or 1:9) and by reversed-phase chromatography on a C_{18} μBondapak semi-preparative column (300 × 7.8 mm I.D.) (Waters Assoc.) with methanol–water (19:1).

PFB esters were collected from HPLC separations and identified by comparison with standards by gas chromatography (GC) on a Pye GCV instrument using EGSS-X columns (1.5 m × 4 mm I.D. at 180°C and 20 ml/min and 5.5 m × 4 mm I.D. at 200°C and 20 ml/min), the effluent gas being divided, 24 parts to a flame-ionisation detector and 1 part to an electron-capture detector. In some instances these identifications were confirmed by GC–mass spectrometry (MS) using a Finnigan Model 3200 Chemical Ionization system interfaced to a Finnigan Incos 2300 Data System, with methane as the GC carrier gas (flow-rate 20 ml/min) and chemical ionization (CI) reagent gas (source pressure 0.8 Torr), ion source temperature 130°C and a glass column 1.8 m × 2 mm I.D., 3% OV-17, programmed from 200°C at 10°C/min to 300°C.

Methyl *cis*-9,10-methylenehexadecanoate (Me 16:CH_2) and methyl *cis*-9,10-methyleneoctadecanoate (Me 18:CH_2) were purchased from Science Labs. (State College, PA, U.S.A.). Methyl heptadec-10-enoate (Me 17:1) and methylnonadec-10-enoate (Me 19:1) were purchased from Nu Check Prep (Elysian, MN, U.S.A.). Each methyl ester was converted into the free acid by saponification with 20% potassium hydroxide in methanol for 40 min at 80°C and PFB esters were synthesized as above.

RESULTS AND DISCUSSION

The PFB 18:0 standard was purified by HPLC on the μPorasil column which afforded a sample for a UV spectrum, the wavelength of maximum absorption (hexane) being 263 nm and with $\log \varepsilon = 2.78$. Pentafluorobenzyl esters do not have a



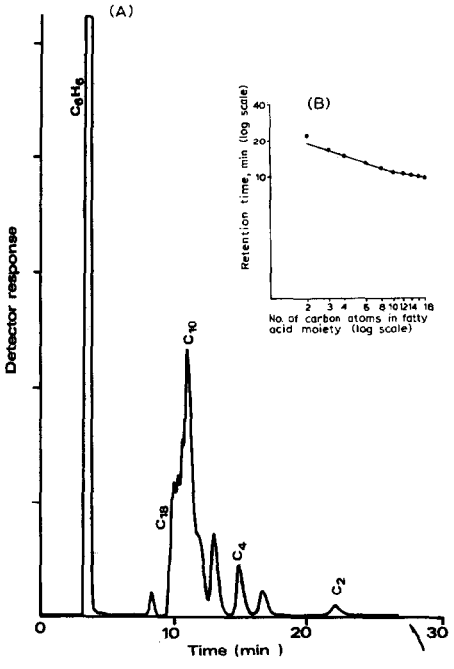


Fig. 1. Normal-phase semi-preparative HPLC separation of PFB-18:0, PFB-18:1, PFB-18:2 and PFB-18:3 (*ca.* 0.1 mg of each). (A) HPLC trace. μPorasil semi-preparative column. Solvent: dry dichloromethane–hexane half-saturated with water (3:17), 4 ml/min. UV detection, 254 nm, 0.1 a.u.f.s. (B) Logarithmic plot of the retention data in (A).

Fig. 2. Normal-phase semi-preparative HPLC separation of the PFB esters of straight-chain saturated fatty acids: C_2, C_3, C_4, C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18} (*ca.* 2.5 mg total). (A) HPLC trace. Conditions as in Fig. 1A; 1.0 a.u.f.s. (B) Log–log plot of the retention data in (A).

high molar absorptivity at 254 nm (log ε in hexane is 2.67). Thus 1 μmol with a retention time of 15 min gives 0.113 a.u.f.s. However, this is adequate for semi-preparative work, particularly as the calibration graph is linear. These derivatives have the additional advantage that aliquots can be taken directly from fractions collected from HPLC for analysis or identification by GC or GC–MS.

TABLE I

RELATIVE INTENSITIES OF THE MAJOR IONS OBSERVED IN THE CHEMICAL IONIZATION (METHANE) MASS SPECTRA OF THE PFB ESTERS OF 18:0, 18:1, 18:2 AND 18:3

PFB ester	*Ion*				
	$[CH_2–C_6F_5]^+$ (*m/z 181*)	$[RCO]^+$	$[RCOO]^+$	$[M–H]^+$	$[M + H]^+$
18:0	50	21	100	38	4
18:1	100	24	9	11	43
18:2	100	73	64	30	39
18:3 (isomer)	75	27	20	15	100
18:3	65	43	32	16	100

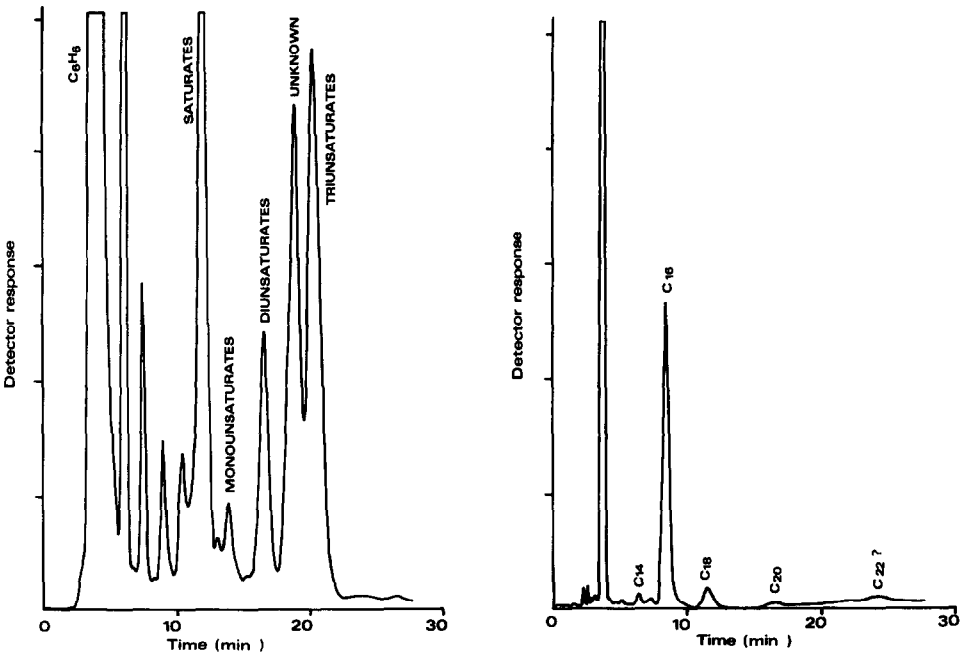


Fig. 3. Normal-phase HPLC separation of the PFB esters from a barley leaf extract. Conditions as in Fig. 1A. Total injected equivalent to *ca.* 1.8 g fresh weight of leaves. PFB esters were synthesized from a saponified extract using the method described in the text.

Fig. 4. Reversed-phase separation of the saturated fraction of the PFB esters from a barley leaf extract. C_{18} μBondapak semi-preparative column. Solvent: methanol–water (19:1). Other conditions as in Fig. 1A. Total injected equivalent to *ca.* 0.2 g fresh weight of leaves.

Fig. 1A shows the separation of the PFB esters of 18:0, 18:1, 18:2 and 18:3 on the μPorasil column. Baseline separation of these compounds is obtained in less than 20 min. The peaks that elute prior to PFB 18:0 (Fig. 1A) appear to be butylated hydroxytoluene, excess of pentafluorobenzyl bromide and perhaps a dimer formed from pentafluorobenzyl bromide during the reaction period. As both unsaturated and shorter PFB esters elute after PFB 18:0, these impurities do not affect the desired separation. Also shown (Fig. 1B) is a plot of log(retention time) against number of double bonds. The resulting straight line suggests that retention due to a double bond is independent of a neighbouring double bond even though the two or three double bonds are only separated by methylene groups. Mass spectra were taken of the isolated peaks from the HPLC separation. The data in Table I show that the proportion of the ion intensities varies considerably for the different degrees of unsaturation. Thus, for example, $[M–H]^+$ is more important than $[M + H]^+$ for PFB 18:0, but the reverse is true for all of the unsaturates. The 18:3 isomer listed in Table I is on the leading edge of PFB-18:3 (Fig. 1A) and in some chromatograms is clearly separated. It appears to be present in the 18:3 free acid as purchased and has not been identified further. We have also demonstrated that the PFB-*trans*-18:1 elutes prior to PFB-*cis*-18:1. However, the resolution was only 1.25 and so the method of choice for this particular separation would be HPLC with silver nitrate-loaded silica columns, which give much higher resolutions[5].

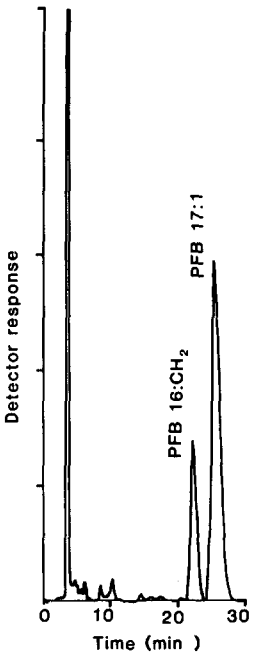


Fig. 5. Normal-phase HPLC separation of PFB-16:CH_2 and PFB-17:1. Conditions as in Fig. 1A. Solvent: dry dichloromethane–hexane half-saturated with water (1:9). 0.2 a.u.f.s. Approximately 0.17 mg of PFB-16:CH_2 and 0.65 mg of PFB-17:1.

The separation of the straight-chain saturated PFB esters is shown in Fig. 2A. Although the longer chain esters elute very close together there is some separation, which increases as the chain lengths shorten, suggesting that this method could be used for separating short-chain fatty acids (say $\leqslant C_6$). Fig. 2B is a plot of log (retention time) against log (number of carbon atoms). Except for PFB-acetate, the plot forms two straight lines with an abrupt change of slope at C_{10}.

It is probably of some relevance here that if the column has been used with isopropanol, it can take as much as 100 column volumes of dry dichloromethane–hexane half-saturated with water (3:17) to equilibrate the column fully. However, this can be markedly reduced if the column is given an intermediate wash with dichloromethane. On the other hand, if completely dry solvents are used to separate PFB esters, the peaks tail badly. In terms of Scott's model[6] for the silica gel surface, these facts suggest that in the system described here the column packing is covered with a bilayer of water surmounted by a monolayer of dichloromethane. Displacement of the dichloromethane by the carbonyl of PFB esters or by double bonds would then lead to separation according to the number of double bonds. Further, the relative displacement of dichloromethane by the carbonyl group in homologous PFB esters might well be reduced with increasing chain length owing to the inductive effect. However, the nature of the interaction that produces the change of slope at C_{10} in the log–log plot (Fig. 2B) is clearly in need of explanation.

Fig. 3 shows the separation of the PFB esters obtained from a barley leaf extract. The four peaks containing saturates, monounsaturates, diunsaturates and

triunsaturates are well separated from each other and can be isolated and separately applied to a reversed-phase column for separation on the basis of chain length. Such a separation, for the saturates isolated as in Fig. 3, is shown in Fig. 4. Taken together, Figs. 3 and 4 give a typical pattern for fatty acids from barley leaves[7].

The separation of PFB esters of 17:1 and 16:CH_2 is shown in Fig. 5. A similar result was obtained using PFB esters of 19:1 and 18:CH_2. Each fraction was collected and subjected to methane CI GC–MS. The CI mass spectra of each isomeric pair were virtually identical, with only minor variations in the intensity of the ions recorded. A similar result was found with the EI and CI mass spectra of the isomeric methyl ester derivatives[3].

Finally, we feel that pentafluorobenzyl esters are well suited for semi-preparative isolations of fatty acids. Unlike the two HPLC systems described in ref. 1 the normal- and reversed-phase systems described here are entirely complementary. Thus a critical pair in the normal-phase system such as palmitic and stearic acids is easily separated in the reversed-phase system. Conversely, critical pairs in the reversed-phase system such as oleic and palmitic acids are readily separated in the normal-phase system. Jordi[1] used two analytical C_{18} μBondapak columns in series with an acetonitrile gradient over 3 h. It is suggested that critical pairs in this system could be separated by a similar gradient on two fatty acid analysis columns. On the analytical scale this is indeed true but on the preparative scale, particularly if columns are not used in series, critical pairs on the first C_{18} column would still be too close to give clean isolations on the second fatty acid analysis column. In short, Jordi's system is sensitive and eminently suitable for analytical work, while we believe our system to be suitable for semi-preparative work.

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REFERENCES

1 H. C. Jordi, *J. Liquid Chromatogr.*, 1 (1978) 215.
2 R. Schwarzenbach, *J. Chromatogr.*, 202 (1980) 397.
3 R. K. Christopher and A. M. Duffield, *Biomed. Mass Spectrom.*, 7 (1980) 429.
4 H. Ehrsson, *Acta Pharm. Suecica*, 8 (1971) 113.
5 R. Battaglia and D. Fröhlich, *Chromatographia*, 13 (1980) 428.
6 R. P. W. Scott, *J. Chromatogr. Sci.*, 18 (1980) 297.
7 J. C. Hawke and P. K. Stumpf, *Plant Physiol.*, 40 (1965) 1023.