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Note

Separation of unsaturated fungal fatty acid methyl esters by reversed-phase liquid chromatography for further evaluation by gas chromatography

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High-performance liquid chromatography (HPLC) is widely employed for the separation and analysis of many biological compounds which traditionally have been analyzed by gas–liquid chromatography (GLC). One great advantage of HPLC is that it allows the recovery of the sample being analyzed.

Fatty acid separations by HPLC often require derivatization for better detection by a spectrophotometer[1–3]. However, when these derivatives are recovered from the mobile phase, they are not always suitable for further analysis by GLC or GLC–mass spectrometry (MS). The objective of this study was to optimize conditions for separaration and recovery of unsaturated C_{16} and C_{18} fatty acid methyl esters. Two reversed-phase columns were tested, a Perkin-Elmer C_8 and a DuPont Zorbax ODS (C_{18}). The latter column was found to be better suited for our particular needs. The optimal system was then used to separate fatty acids extracted from *Athelia bicolor*[4].

EXPERIMENTAL*

Apparatus

The liquid chromatography system consisted of a Perkin-Elmer (PE) Series 3 pump and system controller, a Rheodyne 7125 high-pressure injection valve with a 20-μl sample loop and a PE LC-85 variable-wavelength UV–VIS spectrophotometer and autocontrol. Data were collected and integrated by a PE Sigma 15 data handler. The C_8 column used was a PE Analytical C_8, particle size 10 μm, 25 cm × 4.6 mm I.D., with 6500 plates. The C_{18} column used was a DuPont Zorbax ODS, particle size 5–6 μm, 15 cm × 4.6 mm I.D., with 11,819 plates.

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The gas chromatograph was a Perkin-Elmer Sigma 115 analyzer equipped with flame ionization detectors and data collection/recorder system. The GLC column (6 ft. × 2 mm I.D.) was packed with 15% DEGS on Chromosorb W AW (80–100 mesh).

Materials

All organic solvents were of HPLC grade and were not further pretreated. Water was glass distilled and circulated through a Water I system (Gelman, Ann Arbor, MI, U.S.A.) until a reading of 12.5 Mohms was obtained.

A fatty acid methyl ester standard mixture, AOCS oil reference mixture RM-6, was obtained from Supelco (Bellefonte, PA, U.S.A.). All other fatty acid standards and derivatization reagents were obtained from Sigma (St. Louis, MO, U.S.A.).

Procedures

Methyl esterification of either fatty acid standards or fungal fatty acids was carried out by heating with BCl_3–methanol as described earlier[4]. The final solvent in this derivatization is hexane which is not miscible with the mobile phase. Therefore, methyl esters were dried under a stream of nitrogen and dissolved in carbon tetrachloride for analysis by HPLC. Routinely, 5 μl were injected.

The C_{18} and C_8 columns were routinely eluted with acetonitrile–water, 90:10 and 80:20, respectively, at a rate of 2 ml/min. The methyl esters were fractionated using a Pharmacia Frac-100 collector set for automatic collection of UV-absorbing peaks. The esters were extracted from the mobile phase by vortex shaking with about 1/2 volume of hexane and removing the hexane layer using a pasteur pipette. This was repeated two more times (95% recovery). The pooled hexane extracts for each fraction were dried in nitrogen and dissolved in 100 μl hexane for GLC analysis.

The 15% DEGS column for GLC was programmed at 1°C/min from the initial 165°C to 190°C.

RESULTS AND DISCUSSION

The combined use of HPLC and GLC for the analysis of complex mixtures of fatty acids of biological origin is a powerful and convenient tool. Methyl ester derivatives are ideal for this type of study since they are volatile for GLC analysis and because their solubility makes them suitable for HPLC fractionation. In addition, methyl esters are often the end product of many extraction procedures. However, one disadvantage is that saturated fatty acids have negligible UV absorbance making them difficult to monitor. In trying to develop a system for the analysis of fatty acid methyl esters, especially those having 16–18 carbons in length, we first examined the Perkin-Elmer analytical C_8 column. A typical chromatogram is shown in Fig. 1. Of the various mobile phases which we investigated, the best isocratic elution was achieved with acetonitrile–water (80:20). The flow-rate of 2 ml/min enabled the analysis to be completed in about 18 min. The elution characteristics of various fatty acid standards are shown in Table I. For the RM-6 mixture a reasonable separation was obtained, however *cis–trans* isomers did not separate well. Stearic acid, which was eluted late in the analysis could be eluted more quickly by increasing the acetonitrile to 90%, however, after its elution, additional time would be required to reequilibrate properly the column to 80% acetonitrile.

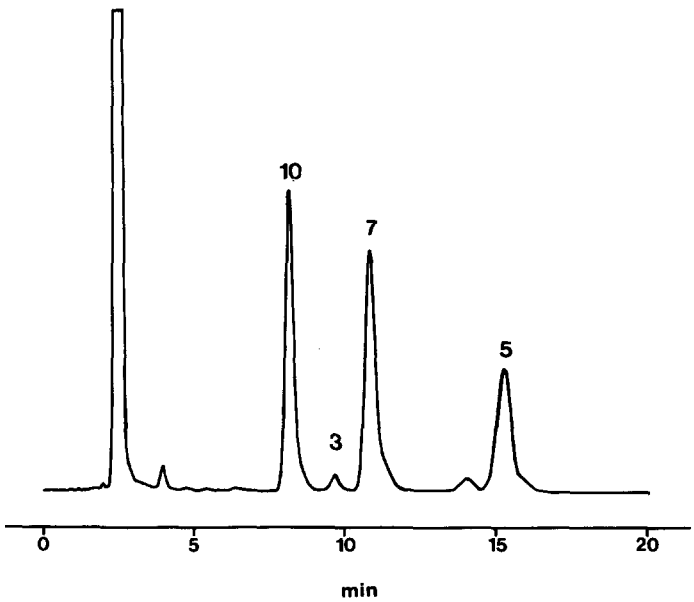

Fig. 1. Elution profile, as monitored at 210 nm, of fatty acid methyl ester standards from a C_8 column using acetonitrile–water (80:20). The numbers correspond to the methyl esters listed in Table I.

Although the C_8 column had been used for much of our work with fungal fatty acid analyses, a DuPont Zorbax ODS column was tested and found to give better resolution. A typical chromatogram is shown in Fig. 2. The mobile phase which gave the best isocratic separation in a reasonable time was acetonitrile–water (90:10) with a flow-rate of 2 ml/min. The RM-6 mixture could be analysed in about

TABLE I

ELUTION CHARACTERISTICS OF METHYL ESTERS ON A PERKIN-ELMER ANALYTICAL C_8 COLUMN WITH ACETONITRILE–WATER (80:20)

Flow-rate: 2 ml/min. Response factors were calculated by dividing the area of an eluted peak (as assigned by the Sigma 15 integrator monitoring absorbance at 210 nm) by the number of μg injected. c = *cis*; t = *trans*.

Methyl ester	*Amount injected (μg)*	*Separation factor relative to methyl oleate*	*Capacity ratio*	*Response factor relative to methyl oleate*
1 Myristic (14:0)	1.4	0.55	6.58	0.0
2 Palmitic (16:0)	18.9	0.91	11.02	0.0
3 Palmitoleic (16:1)	1.9	0.59	7.10	1.08
4 Stearic (18.0)	8.8	1.54	19.60	0.0
5 Oleic (18:1c)	25.6	1.0	12.07	1.00
6 Elaidic (18:1t)	26.5	1.05	12.72	1.31
7 Linoleic (18:2c,c)	4.4	0.69	8.28	7.23
8 Unknown	4.2	0.70	8.40	19.23
9 Linolelaidic (18:2,t,t)	3.9	0.75	9.06	9.38
10 Linoleic (18:3)	1.9	0.48	5.79	15.70

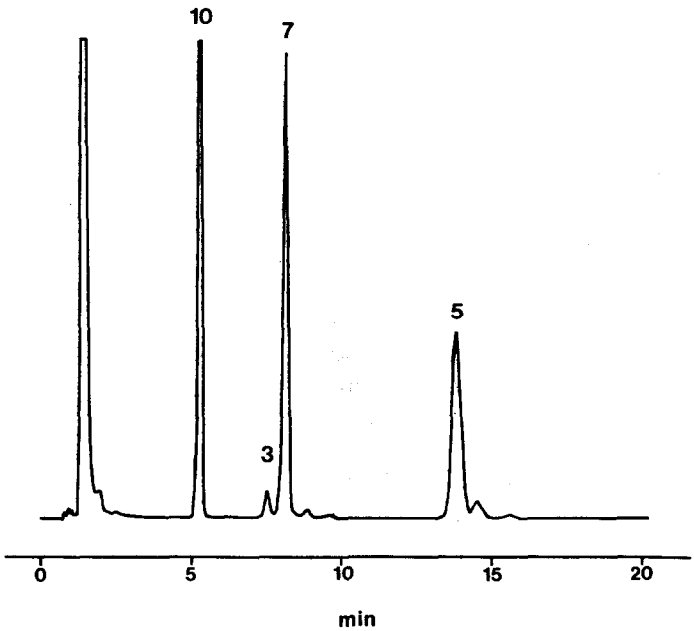

Fig. 2. Elution profile, as monitored at 210 nm, of fatty acid methyl ester standards from a C_{18} (ODS) column using acetonitrile–water (90:10). The numbers correspond to the methyl esters listed in Table II.

18 min, except for stearic acid which was eluted in about 30 min. The elution characteristics for this column are given in Table II. This column gave improved separation of *cis–trans* isomers, however, stearic acid took much longer to elute.

In both of the columns the elution order followed the same basic pattern that has been found with *p*-bromophenacyl esters: (i) retention time increased with chain length; (ii) retention time decreased with increased unsaturation; (iii) the *cis* unsatu-

TABLE II

ELUTION CHARACTERISTICS OF METHYL ESTERS ON A DUPONT ZORBAX ODS COLUMN WITH ACETONITRILE–WATER (90:10)

Flow-rate: 2 ml/min. Response factors calculated as in Table I.

Methyl ester	*Amount injected (μg)*	*Separation factor relative to methyl oleate*	*Capacity ratio*	*Response factor relative to methyl oleate*
1 Myristic (14:0)	1.4	0.52	9.18	0.0
2 Palmitic (16:0)	18.9	1.05	18.43	0.0
3 Palmitoleic (16:1)	1.9	0.52	9.18	1.09
4 Stearic (18:0)	8.8	2.18	38.22	0.0
5 Oleic (18:1c)	25.6	1.00	17.50	1.00
6 Elaidic (18:1t)	26.5	1.12	19.54	1.27
7 Linoleic (18:2c,c)	4.4	0.57	9.92	7.29
8 Unknown	4.2	1.03	10.25	20.64
9 Linolelaidic (18:2t,t)	3.9	0.66	11.58	9.92
10 Linolenic (18:3)	1.9	0.34	6.03	16.63

TABLE III

PEAK RESOLUTION OF UNSATURATED ISOMERS

Isomers	*Peak resolution, R_s*	
	Analytical C_8	*Zorbax ODS*
Oleic–elaidic	1.5	4.3
Linoleic–linolelaidic	1.8	4.8
Oleic–unknown	0.2	1.2

rated isomer was eluted before the *trans* isomer[5]. The only change in elution order between the columns was that methyl palmitate was eluted before methyl oleate in the C_8 column but after in the ODS column.

In order to determine the efficiency at which methyl esters could be recovered after chromatography with these two columns, fractions were quantitatively extracted with hexane and assayed by GLC. The efficiency of recovery was similar for both columns, ranging from 85 to 95% for methyl esters injected in quantities greater than 10 μg, and from 75 to 85% for methyl esters injected in quantities less than 10 μg. The capacity ratios for the C_8 column are lower than those for the ODS column, which is expected from the longer analysis time of the latter. The response factors in regard to detection by the UV monitor at 210 nm (Tables I and II) were calculated by taking the area assigned to each peak by the integrator and dividing it by the amount of the component (in μg) injected into the HPLC. A relative response factor, RRF, was obtained by dividing each response factor by that of methyl oleate. The RRF of methyl esters eluted from both columns were similar. All monounsaturates

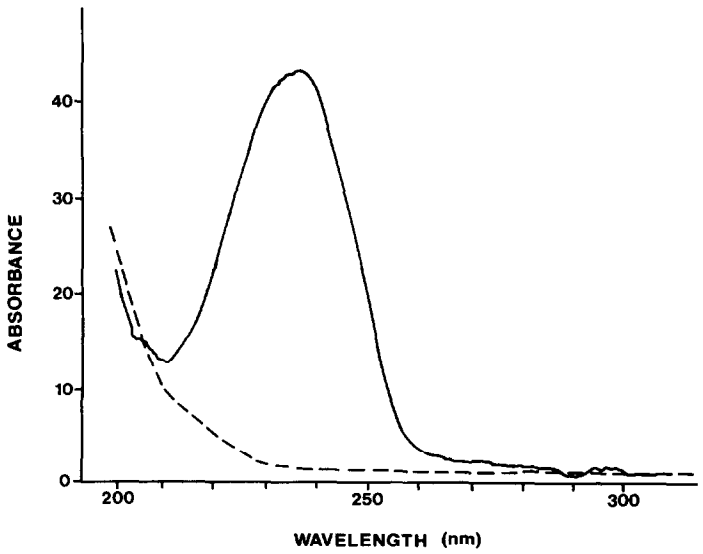

Fig. 3. UV spectra of linoleic 18:2 methyl ester (– – –) and an unidentified fungal 18:2 methyl ester (——).

had RRFs close to 1: diunsaturates had RRFs of about 7 for methyl linoleate and 10 for methyl linelaidate; the triunsaturate, methyl linolenate, had an RRF of about 16. The *trans* isomers tended to have higher RRFs.

As shown in Table III, the Zorbax ODS column was capable of resolving well the *cis–trans* isomers tested. The peak resolution, R_s, was greater than 4, compared to about 1.5 for the C_8 column.

The Zorbax ODS column also gave the best separation of the unknown fungal fatty acid (Tables I and III). Recovery of this unknown and analysis by GLC–MS shows it to be 18:2, however, it behaves very differently from the two standards included in this study. It has a capacity ratio similar to the 18:2 standards, however, the RRF is much greater, suggesting that the propanol is conjugated. The UV-absorbance scan in Fig. 3 shows an absorbance maximum at 236 nm, which is absent from all the non-conjugates standards in this study.

As a result of this study we have found that the DuPont Zorbax ODS column was better suited for analyzing C_{16}–C_{18} unsaturated fatty acid methyl esters. After separation of the fatty acids by HPLC they could be directly analyzed by GLC without further derivatization. This feature is very useful when working with small amounts of material.

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