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## Note

# High-performance liquid chromatography of fatty acids as their *p*-phenylazophenacyl esters

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Although gas–liquid chromatography (GLC) is the method of choice for the analysis of fatty acids, several inherent advantages of high-performance liquid chromatography (HPLC) makes this technique more convenient for the analysis of polymerized acids and oils and of other non-volatile products. However, the lack of chromophoric groups in the fatty acids molecules makes difficult the detection and estimation of these compounds. Therefore, it is of interest to prepare fatty acid derivatives containing chromophoric groups, which can easily be detected in the ultraviolet or visible spectral ranges in which the common HPLC detectors work.

A variety of fatty acids have been analysed as their phenacyl[1–5], *p*-bromophenacyl[6–8], *p*-nitrobenzyl[2], *p*-methylthiobenzyl[10] and 1-chlorophenacyl[11] esters. An alternative approach is based on the preparation of fluorescent derivatives such as the 2-naphthacyl[9,12], 9-diazomethylanthracene[13] and 4-bromoethyl-6,7-dimethoxycoumarine[14] esters.

We have made previously use of the reagent 2-bromo-4′-phenylazoacetophenone (*p*-phenylazophenacyl bromide) for the identification of organic acids[15–19] as well as for their separation by reversed-phase paper and thin-layer chromatography[20]. We now report on the use of this reagent for the preparation of derivatives of fatty acids having absorption at 330 nm [$E^{M}$ (molar extinction coefficient) $\approx$ 26 000 $M^{-1}$ $cm^{-1}$], and their separation and estimation by HPLC. By using these *p*-phenylazophenacyl esters, the fatty acid composition of several oils has been determined, and the results have been compared with those obtained by GLC.

## MATERIALS AND METHODS

### *Materials*

Fatty acids (>95% pure according to GLC) were obtained from Fluka or Merck. The reagent was from Kodak (Rochester, NY, U.S.A.). It was shown by TLC to contain some polar impurities, but was used without purification. Triethylamine (puriss. Fluka) and acetone (Panreac) were distilled before use. Solvents for HPLC were from Merck or Scharlau. The eluents were always degassed and filtered (0.45 μm, Millipore). The seed oils were from commercial sources and the corresponding fatty acids prepared in the usuall way.

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*Preparation of derivatives*

A sample (5–10 mg) of fatty acids was weighed in a 100 × 13 mm PTFE (polytetrafluoroethylene)-lined screw-cap Sovirel test-tube. A 1-ml volume of a 5% triethylamine solution in acetone and then 1.5 ml of acetone containing the equivalent amount of reagent were added. The mixture was shaken for 30 min.

The purification of the derivative was carried out by spotting part or all of the reaction solution, as a row on a TLC plate of silica gel G. If the derivative precipitated from the solution, some drops of benzene were added to disolve it. A standard of a fatty acid derivative was spotted on the same plate. The plate was then developed with benzene. The row having the same $R_F$ value as that of the standard was scrapped off the plate into a small chromatographic column (200 × 5 mm) and eluted with benzene. The resulting solution was taken to dryness with nitrogen and the residue dissolved in an appropriate volume of the same solvent to be used in the HPLC separation.

*HPLC analyses*

These were carried out in a modular apparatus equipped with an ultraviolet and visible detector of variable wavelength (Biotronic, Model BT 3030, fixed at 330 nm), flow cell (capacity, 8 μl), a Biotronic pump Model BT 3020, a reporting-integrator (Hewlett-Packard, Model 3390 A), $C_{18}$ reversed-phase column containing Bio-Sil ODS, 5 μm (250 × 4 mm, Bio-Rad), Rheodyne injector (20 μl) and a pre-column containing ODS-5S (Bio-Rad).

The solvents and flow conditions were as follows: (a) for the derivatives of saturated fatty acids of low molecular weight (up to dodecanoic acid), acetonitrile–water (95:5) at 1 ml/min; (b) for the derivatives of fatty acids of high molecular weight, acetonitrile–water (98:2) at 1 ml/min; (c) for the derivatives of fatty acids from seed oils as well as those from oleic, linoleic and linolenic acids, acetonitrile–water (99:1) at 1.5 ml/min.

*GLC*

Methyl esters of fatty acids were prepared following the method of Schlenk and Gellerman[21], and were analysed in a Perkin-Elmer F17 apparatus under the following conditions: column, 200 × 4 mm, 5% ethylene glycol succinate (EGS) on Supercoport (80–100 mesh), temperatures, oven 165°C, injector and detector 225°C; flow-rates, carrier gas (nitrogen) 25 ml/min, hydrogen 25 ml/min and air 100 ml/min; flow ionization detector; reporting integrator Hewlett-Packard Model 3390 A.

## RESULTS AND DISCUSSION

*Fatty acids*

*Chain length less than twelve carbon atoms.* Fig. 1 shows the separation of the derivatives of fatty acids from formic to undecanoic acid, obtained under the experimental conditions described above. The excess of reagent, if present, has a retention time of 1.89 min.

*Long chain acids (up to 22 carbon atoms).* Fig. 2 shows the separation of the derivatives of fatty acids from decanoic to icosanoic acid.

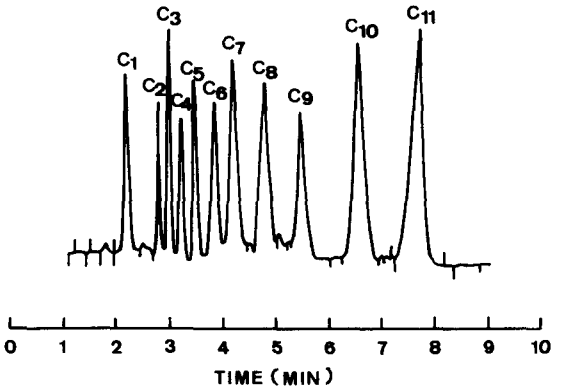



Fig. 1. HPLC separation of the derivatives of fatty acids ($C_1$–$C_{11}$).

*Unsaturated acids (linolenic, linoleic and oleic).*The chromatogram in Fig. 3 shows the separation of the derivatives of these fatty acids having retention times of 4.89, 6.47 and 9.24 min, respectively.

*Geometric isomers.* Oleic (*cis*-9-octadecenoic) acid and elaidic (*trans*-9-octadecenoic) acid, as shown in Fig. 4, were well separated with retention times of 14.78 and 15.63 min, respectively, using acetonitrile–water (98:2) as eluent.

*Sensitivity of method*

The use of the phenylazophenacyl esters affords a high sensitivity of detection. A mixture of fatty acids containing quite different proportions of oleic, linoleic and linolenic acids was derivatized as described above. The final solution contained a total of 0.132 mg of derivatives per ml. A 20-$\mu$l volume of the original solution, and of successively diluted solutions, was injected. The results of the determination are included in Table I. The last injection, corresponding to a total of 0.041 $\mu$g of derivatives, still gave a measurable chromatogram, and we consider this as the minimum amount needed to obtain a reliable analysis. This amount corresponds to some 140 pmol of total fatty acids, so that the detection limit of the method is in the nanomole range.

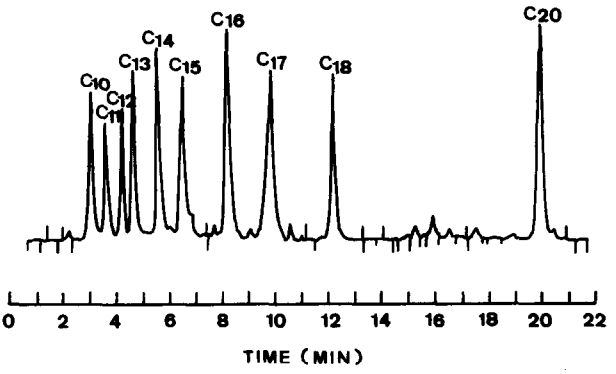
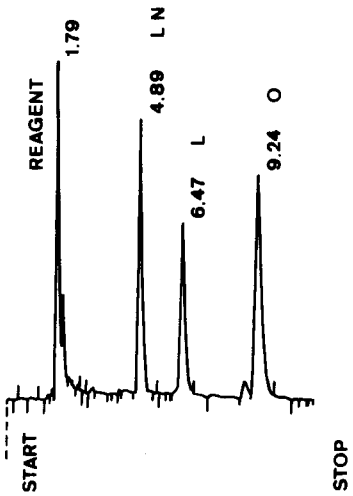



Fig. 2. HPLC separation of the derivatives of fatty acids ($C_{10}$–$C_{20}$).



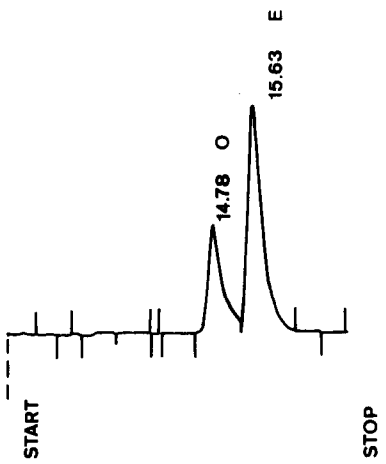




Fig. 3. HPLC separation of derivatives of oleic (O), linoleic (L) and linolenic (LN) acids.

Fig. 4. HPLC of derivatives of the geometric isomers oleic (O) and elaidic (E) acids.

*Quantitation*

Two artificial mixtures of fatty acids containing hexadecanoic, heptadecanoic and octadecanoic acids were prepared and analysed by HPLC. The results obtained, as the averages from three determinations, are shown in Table II.

*Applications*

The fatty acid derivatives of several vegetable oils were prepared and analysed by HPLC. The eluent was acetonitrile–water (99:1) and the flow-rate 1.5 ml/min. Table III shows the results obtained compared with those from GLC analyses. When palmitoleic acid was present in the mixture, as it is the case of olive oil fatty acids, a better eluent for the separation is methanol–acetonitrile–water (89:9:9). This is similar to the one used by Halgunset *et al.*[6] for the separation of the *p*-bromophenacyl esters of fatty acids. The most convenient flow-rate in this case is 0.8 ml/min.

TABLE I

ANALYSES OF A STANDARD MIXTURE OF *p*-PHENYLAZOPHENACYL ESTERS OF FATTY ACIDS

Eluent: acetonitrile–water (99:1); flow-rate, 1.5 ml/min.

| *Injection No.* | *Injected (μg)* | *Composition (%)* | | |
|---|---|---|---|---|
| | | *Oleic* | *Linoleic* | *Linolenic* |
| 1 | 2.640 | 1.9 ± 0.3 | 19.9 ± 0.1 | 78.2 ± 0.6 |
| 2 | 0.660 | 1.9 ± 0.3 | 20.4 ± 0.1 | 77.7 ± 0.5 |
| 3 | 0.165 | 1.9 ± 0.1 | 20.3 ± 0.2 | 77.8 ± 0.6 |
| 4 | 0.083 | 1.9 ± 0.2 | 20.3 ± 0.1 | 77.8 ± 0.5 |
| 5 | 0.041 | 1.9 ± 0.2 | 20.1 ± 0.1 | 78.2 ± 0.5 |

* Mean ± standard deviation.

TABLE II

ANALYSES OF TWO STANDARD MIXTURES OF DERIVATIVES OF FATTY ACIDS

Eluent: acetonitrile–water (98:2); flow-rate, 1 ml/min.

| *Fatty acid* | *Blend A* | | *Blend B* | |
|---|---|---|---|---|
| | *Actual (%)* | *HPLC (%)** | *Actual (%)* | *HPLC (%)** |
| Palmitic | 26.4 | 28.4 | 33.3 | 34.4 |
| "Margaric" | 26.2 | 25.9 | 36.9 | 35.9 |
| Stearic | 47.4 | 45.7 | 29.8 | 29.7 |

* Mean of three determinations.

TABLE III

COMPARISON OF ANALYSES OF THE FATTY ACID COMPOSITION OF VEGETABLE OILS BY HPLC AND GLC

a = GLC; b = HPLC.

| *Fatty acid* | *Coconut* | | *Poppy-seed* | | *Linseed* | | *Sunflower* | | *Lupinus* | | *Sawflower* | | *Maize* | | *Palm* | | *Olive* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *a* | *b* | *a* | *b* | *a* | *b* | *a* | *b* | *a* | *b* | *a* | *b* | *a* | *b* | *a* | *b* | *a* | *b* |
| $C_{8:0}$ | – | 2.8 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| $C_{10:0}$ | 7.2 | 7.6 | – | – | – | – | – | – | – | – | – | – | – | – | 1.2 | – | – | – |
| $C_{12:0}$ | 51.8 | 51.9 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| $C_{14:0}$ | 19.6 | 19.5 | – | – | – | – | – | – | – | – | – | – | – | – | 1.5 | – | – | – |
| $C_{16:0}$ | 9.6 | 9.0 | 25.5 | 24.4 | 7.0 | 7.0 | 9.0 | 9.8 | 10.9 | 11.0 | 7.2 | 7.7 | 13.2 | 14.4 | 46.5 | 49.0 | 11.6 | 11.8 |
| $C_{16:1}$ | – | – | – | – | – | – | – | – | 6.5 | 6.2 | 2.3 | 2.7 | – | – | – | – | 1.0 | 1.0 |
| $C_{18:0}$ | 2.9 | 4.0 | 5.2 | 4.5 | 4.3 | 5.8 | 6.2 | 6.6 | 49.7 | 49.4 | 11.0 | 10.9 | 2.5 | 2.6 | 4.4 | 4.5 | 3.8 | 4.8 |
| $C_{18:1}$ | 7.0 | 5.1 | 30.5 | 27.1 | 18.9 | 21.1 | 28.9 | 28.5 | 30.2 | 30.9 | 79.5 | 78.7 | 28.2 | 28.4 | 37.2 | 35.8 | 77.4 | 77.0 |
| $C_{18:2}$ | 1.8 | – | 45.8 | 44.1 | 17.9 | 14.5 | 55.9 | 55.2 | 2.7 | 2.5 | – | – | 54.1 | 54.5 | 8.3 | 10.7 | 6.3 | 5.8 |
| $C_{18:3}$ | – | – | – | – | 51.9 | 51.1 | – | – | – | – | – | – | 1.8 | – | – | – | – | – |

The method can readily be used to follow the fractional distillation of fatty acids under reduced pressure. A drop of the distillate is taken at intervals and the derivative prepared and analysed by HPLC.

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