IMPROVED SEPARATION OF C_{12}—C_{22} FATTY ACID PHENACYL ESTERS BY REVERSED PHASE COLUMN LIQUID CHROMATOGRAPHY

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Phenacyl esters of C_{12}—C_{22} fatty acids were separated on Separon SGX C18 column, using a gradient elution with methanol–acetonitrile–water. The proposed gradient showed better resolution of the critical pairs $C_{18:3}$–$C_{14:0}$, $C_{16:1}$–$C_{20:4}$, and $C_{16:0}$–$C_{18:1}$ than the gradient elution with methanol–water or acetonitrile–water, or than the isocratic elution with methanol–acetonitrile–water. The optimum volume concentration (83%) of the sum of both methanol and acetonitrile was maintained constant for 35 min; in this period the acetonitrile concentration decreased linearly from the initial 42—60% to 0% while the methanol concentration increased from the initial 41 — 23% to 83% at the same rate. After 35 min the elution was completed with a methanol–water gradient. The whole analysis can be performed within 63 min at a flow rate 1 ml/min.

In recent years a number of methods have been described for the separation of fatty acids by means of HPLC. Several types of derivatives can be made use of to increase the detection sensitivity of these compounds that in their underivatized form exhibit neither distinct UV absorptivity nor fluorescence. Phenacyl[1-6], 2-naphthacyl[5,7,8], *p*-nitrobenzyl[2], naphthylamine[9], *p*-bromophenacyl[10-15], *m*-methoxyphenacyl[16], pentafluorobenzyl[17], 5-dimethylamino-1-napthalensulfonyl-ethanolamine[18], 9-diazomethylanthracene[19,20], *p*-phenacylazophenacyl[21], 2-nitrophenylhydrazine[22] or 4-bromoethyl-7-acetoxycoumarin[23] derivatives can serve as typical examples.

Phenacyl and substituted phenacyl esters are most often used. They are easily prepared with quantitative yields, are well stable and enable quantitative analysis of fatty acids even at nanogram levels[4].

Separations are usually carried out on a reversed phase using acetonitrile–water or methanol–water as mobile phases. The order of elution is ruled by the length of the fatty acid carbon chain and the number of double bonds. The retention time increases with the increasing chain length and decreasing number of the double bonds[3,5,12].

These opposing tendencies lead to the occurence of several pairs of fatty acids which are difficult to separate and the separation of which may be considered as a criterion of the resolution efficiency of an analytical procedure. These critical pairs

are linolenic (18 : 3) and myristic (14 : 0) acids, palmitooleic (16 : 1) and arachidonic (20 : 4) acids and palmitic (16 : 0) and oleic (18 : 1) acids[3,5,12,17,23].

In this contribution we compare the resolution efficiency of various elution systems for $C_{12}-C_{22}$ fatty acid phenacyl esters and suggest an optimized gradient elution which seems to offer better separation within a shorter period of time than previously reported papers[3,5,12,17,23].

EXPERIMENTAL

Standards of fatty acids were purchased from the following sources: lauric (12 : 0), linolenic (18 : 3) and arachidonic (20 : 4) acids were obtained from Serva (Heidelberg, F.R.G.), palmitooleic (16 : 1), palmitic (16 : 0), oleic (18 : 1) and behenic (22 : 0) acids were obtained from Sigma (St. Luis, U.S.A.) and linoleic (18 : 2), stearic (18 : 0) and arachidic (20 : 0) acids were the products of Calbiochem (San Diego, U.S.A.). All these standards were research grade quality.

Acetonitrile, HPLC grade, was obtained from E. Merck (Darmstadt, F.R.G.). Methanol, *p.a.* quality, obtained from Lachema (Brno, Czechoslovakia), was rectified before use. Water, glass-distilled, was further deionized by passing over an ion-exchanger.

α-Bromoacetophenone was purchased from Fluka (Huappauge, U.S.A.) or prepared in the laboratory by bromination of acetophenone and recrystallization from ethanol (m.p. 50°C). Triethylamine and acetone *p.a.* quality, purchased from Lachema, were redistilled before use.

Phenacyl derivatives of fatty acid standards were prepared according to Wood and Lee[5].

Analyses were carried out on a Spectra Physics SP 8100 High Performance Liquid Chromatograph, equipped with SP 8400 UV-VIS Variable-Wavelength Detector and SP 4100 Plotting Integrator (Santa Clara, U.S.A.). Separations were performed on 250 × 4 mm i.d. columns,

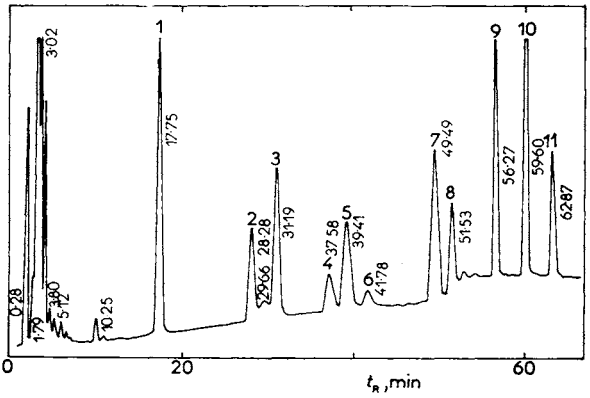

FIG. 1

Chromatogram of a mixture of fatty acid phenacyl esters run at the elution mode C. Column 250 × 4 mm i.d., Separon SGX C18, 7 μm, column temperature 30°C, flow 1 ml/min. Acids: **1** lauric (12 : 0), **2** linolenic (18 : 3), **3** myristic (14 : 0), **4** palmitooleic (16 : 1), **5** arachidonic (20 : 4), **6** linoleic (18 : 2), **7** palmitic (16 : 0), **8** oleic (18 : 1), **9** stearic (18 : 0), **10** arachic (20 : 0), **11** behenic (22 : 0)

TABLE I

Retention times and resolutions of the critical pairs of fatty acid phenacyl esters. Column: 250 × 4 mm i.d., Separon SGX C18, 7 μm, column temperature 30°C

Elution mode[a]	Flow rate ml/min	t_R, min		R_s[b]	t_R, min		R_s[b]	t_R, min		R_s[b]
		$C_{18:3}$	$C_{14:0}$		$C_{16:1}$	$C_{20:4}$		$C_{16:0}$	$C_{18:1}$	
A (CH_3OH–H_2O)	1·0	39·23	39·23	0	47·66	56·18	4·9	69·00	71·22	2·0
B (CH_3CN–H_2O)	1·0	32·03	37·46	2·9	45·83	45·83	0	51·14	52·79	2·0
C (CH_3OH–CH_3CN–H_2O)	1·0	28·28	31·19	2·7	37·58	39·41	1·6	49·49	51·53	1·8
D (CH_3OH–CH_3CN–H_2O)	1·2	26·21	29·90	3·4	36·77	38·60	1·4	48·14	51·02	2·0
E (CH_3OH–CH_3CN–H_2O)	1·2	25·42	28·78	2·8	36·64	38·83	1·3	51·76	53·14	1·6

[a] A — CH_3OH–H_2O — 83–17% (0—35 min), B — CH_3CN–H_2O — 70–30% (0—40 min) — 100–0% (50—60 min), C — CH_3OH–CH_3CN–H_2O — 41–42–17% (0 min) — 86–0–14% (30 min) — 100–0–0% (46—65 min), D — CH_3OH–CH_3CN–H_2O — 23–60–17% (0 min) — 83–0–17% (40 min) — 100–0–0% (50—60 min), E — CH_3OH–CH_3CN–H_2O — 41–41·5–17·5% (0 min) — 83–0–17% (40 min) — 100–0–0% (55—60 min). [b] $R_s = 2\Delta t_R/(Y_{t,1} + Y_{t,2})$, $Y_{t,i}$ — peak width at base.

packed with 7 μm octadecyl-bonded spherical silica Separon SGX C18 (Laboratorní přístroje, Prague, Czechoslovakia).

All solvents were flushed with helium. Samples dissolved in methanol (0·1 mg/ml) were injected through a 10 μl sample loop. The column temperature was maintained at 30°C and all recordings were done at 242 nm.

For composition of mobile phases see Table I.

RESULTS AND DISCUSSION

Gradient elution with methanol–water showed a good resolution of $C_{16:1}-C_{20:4}$ and $C_{16:0}-C_{18:1}$ fatty acid pairs but the pair of $C_{18:3}-C_{14:0}$ fatty acids was not resolved (Table I). On the other hand, acetonitrile–water gradients readily resolved the pairs of $C_{18:3}-C_{14:0}$ and $C_{16:0}-C_{18:1}$ fatty acids but failed to separate $C_{16:1}$ and $C_{20:4}$ fatty acids (Table I).

This behaviour of methanol and acetonitrile based systems indicated the necessity of using a ternary gradient employing both methanol and acetonitrile for elution. In our experiments acetonitrile was shown to be the necessary component of the mobile phase only for the resolution of $C_{18:3}$ and $C_{14:0}$ fatty acids, eluted at the beginning of the chromatographic separation, while all the more retained fatty acids were well resolved with a methanol–water system. The optimum volume concentration of both organic modifiers in the mobile phase was shown to be about 83%. The acetonitrile concentration required at the start of the elution proved to be 42–60%. During elution this concentration decreased to 0% within 35 min following a linear gradient and this decrease was compensated by introducing an increasing methanol gradient at the same rate.

A typical chromatogram of the mixture of $C_{12}-C_{22}$ fatty acid phenacyl esters is presented in the Fig. 1. Retention times of separated fatty acids and their resolutions under various elution conditions are summarized in Table I.

It is known[3,12,23] that the methanol to acetonitrile ratio significantly influences the resolution of fatty acids. The works reported previously used either isocratic conditions, which did not allow fully to exploit all the possible advantages of a ternary system such as that described above, or did not deal with fatty acids longer than C_{18}. The resulting separations were thus incomplete either because of the presence of unresolved peaks or because of the limitations imposed by the chain length of the separated acids. Conditions suggested in our work offer a better resolution of fatty acids $C_{16:1}$ and $C_{20:4}$ and a faster separation of the mixture of $C_{12}-C_{22}$ fatty acids. These fatty acids can be separated within 63 min at a flow rate 1 ml/min.

REFERENCES

1. S. Lam, E. Grushka: J. Chromatogr. Sci. *15*, 234 (1977).
2. E. Grushka, H. D. Durst, E. J. Kikta jr: J. Chromatogr. *112*, 673 (1975).
3. R. F. Borch: Anal. Chem. *47*, 2437 (1975).

4. R. Wood, T. Lee: Fed. Proc., Fed. Am. Soc. Exp. Biol. *41*, 1288 (1982).
5. R. Wood, T. Lee: J. Chromatogr. *254*, 237 (1983).
6. R. Wood: J. Chromatogr. *287*, 202 (1984).
7. W. Distler: J. Chromatogr. *192*, 240 (1980).
8. M. J. Cooper, M. W. Anders: Anal. Chem. *46*, 1849 (1974).
9. M. Ikeda, K. Shimada, T. Sakaguchi: J. Chromatogr. *272*, 251 (1983).
10. H. D. Durst, H. Milano, E. J. Kikta, jr, S. A. Connely, E. Grushka: Anal. Chem. *47*, 1797 (1975).
11. P. T. S. Pei, W. C. Kossa, S. Ramachandran, R. S. Henley: Lipids *11*, 814 (1976).
12. J. Halgunset, E. W. Lund, A. Sunde: J. Chromatogr. *237*, 496 (1982).
13. B. Jaselskis, N. L. Stemm, W. D. Johnston: Talanta *29*, 54 (1982).
14. T. N. Tweeten, D. L. Wetzel: Cereal Chem. *56*, 398 (1979).
15. J. Weatherson, L. M. MacDonald, T. Blake, M. H. Benn, Y. Y. Huang: J. Chromatogr. *161*, 347 (1978).
16. N. E. Bussell, R. A. Miller, J. A. Setterstrom, A. Gross in the book: *Biological/Biomedical Applications of Liquid Chromatography* (G. L. Hawk, Ed.), p. 57. Marcel Dekker, New York 1979.
17. A. G. Netting, A. M. Duffield: J. Chromatogr. *336*, 115 (1984).
18. P. J. Ryan, T. W. Honeyman: J. Chromatogr. *312*, 461 (1984).
19. S. A. Baker, J. E. Monti, S. T. Christian, F. Benington, R. D. Morin: Anal. Biochem., *107*, 116 (1980).
20. Y. Antoku, T. Sakai, H. Iwashita: J. Chromatogr. *342*, 359 (1985).
21. E. Vioque, M. P. Maza, F. Millan: J. Chromatogr. *331*, 187 (1985).
22. H. Miwa, C. Hiyama, M. Yamamoto: J. Chromatogr. *321*, 165 (1985).
23. H. Tsuchiya, T. Hayashi, M. Sato, M. Tatsumi, N. Takagi: J. Chromatogr. *309*, 43 (1984)

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