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Note

Gradient separation of fatty acids (C_{14}–C_{30}) by reversed-phase high-performance liquid chromatography

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Gas chromatography (GC) is routinely employed in the analysis of fatty acid mixtures. During the past ten years high-performance liquid chromatography (HPLC) has become increasingly important for analysis, quantitation and preparative separation of fatty acids[1]. Fatty acids can be separated as their phenacyl and naphthacyl esters, or as esters with 9-diazomethylanthracene that strongly absorbing in the UV region at 254 nm[2–4], and as methyl esters that can be detected via their refraction indexes[5,6] or by UV absorption (203–214 nm)[7]. Most frequently, the stationary phase RP-18 was used[2,3,5–7]; phases RP-8[8], RP-9[9] and RP-30[10] were used only occasionally under stricter elution conditions (acetonitrile, aqueous phosphoric acid). The stationary phase RP-18 was employed for the separation of those methyl esters of fatty acids with up to 22 carbon atoms[2,7], with the exception of highly unsaturated very long-chain fatty acids (*e.g.* $C_{24:4}$ up to $C_{30:5}$)[11]. Owing to their relatively high polarity the retention times of these acids do not exceed 60 min. Fatty acids with up to 32 carbon atoms were rather separated as *m*-methoxyphenacyl esters[12], whose high polarity facilitated the use of phase RP-18.

When the analyses of fatty acids by HPLC and GC are to be correlated it is most useful to prepare methyl esters. These, however, have a lower polarity (especially those of saturated acids and acids with a higher number of carbon atoms) than *e.g.* *m*-methoxyphenacyl esters, and therefore they do not separate on the reversed-phase RP-18. For this reason we used the more polar phase RP-1 as the stationary phase in this work.

EXPERIMENTAL

Fatty acid (even-numbered saturated C_{14} to C_{30} and monoenic C_{16}–C_{20} and C_{24}) were purchased from Fluka (Switzerland) and Sigma (U.S.A.). The other fatty acids (see Table I) were isolated from the green freshwater alga *C. kessleri*[13].

HPLC was carried out with the SP 8000 apparatus (Spectra Physics, U.S.A.) on a 50 cm × 6 mm I.D. column (Separon SI Cl, Laboratory Instruments, Czechoslovakia). The mobile phase linear gradient (2 ml/min) ran from a mixture of methanol–water (50:50) into methanol (30 min) and further methanol (60 min); detection was by a UV detector at 210 nm. The column efficiency was 8700–10 000

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TABLE I

CAPACITY RATIOS (k') OF METHYL ESTERS OF FATTY ACIDS BY MEANS OF REVERSED-PHASE HPLC ON STATIONARY PHASE RP-1

Number	*Methyl ester*	*Apparent k'*	*Number*	*Methyl ester*	*Apparent k'*
1	14:0	0.808	16	20:0	8.616
2	15:0	1.358	17	20:1	7.208
3	15:1	0.566	18	21:0	9.783
4	16:0	2.941	19	22:0	11.200
5	16:1	1.183	20	23:0	12.500
6	16:2	0.566	21	24:0	13.858
7	16:3	0.358	22	24:1	12.591
8	17:0	4.275	23	25:0	15.083
9	17:0*	3.466	24	26:0	16.033
10	17:1	2.733	25	26:1	15.323
11	18:0	5.666	26	28:0	17.483
12	18:1	4.158	27	28:1	17.111
13	18:2	2.791	28	29:0	18.103
14	18:3	2.016	29	30:0	20.410
15	19:1	7.058	30	30:1	20.013

* 2-Methylhexadecanoic acid.

plates, V_0 = 4 ml, and t_R of methyl stearate was 800 sec. Acids present in the natural mixture were identified by gas chromatography on both capillary and packed columns, combined with mass spectrometry using the Hewlett-Packard 5992 B apparatus[13]. The methyl esters of fatty acids were prepared by means of boron trifluoride–methanol[14]. The purity and supplementary identification of the individual peaks from HPLC were verified by GC using the Varian Aerograph 2740 apparatus (Varian, U.S.A.), and a column (180 cm × 2 mm I.D.) packed with 3% XE-60 on Chromosorb AM DMCS 80/100 (Lachema, Czechoslovakia). The carrier gas was nitrogen (flow-rate 25 ml/min), the injector temperature 240°C, the column temperature 180°C, and the detector temperature 250°C.

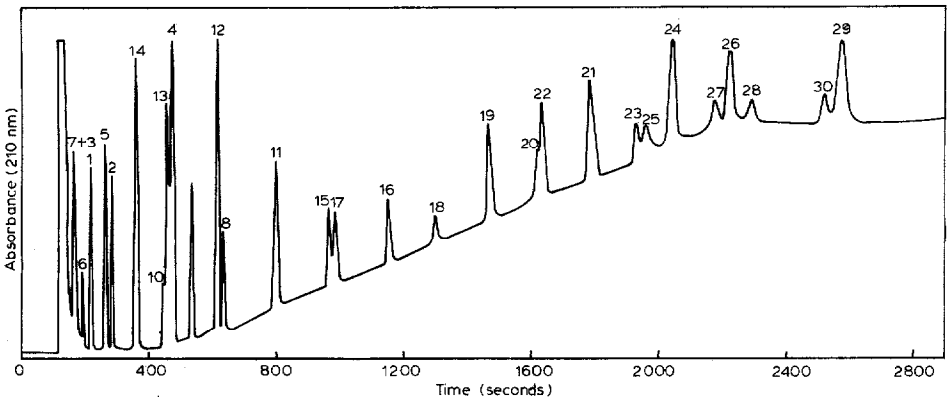

Fig. 1. Chromatogram of a 10-μl sample containing 0.1 mg/μl fatty acid methyl esters (commercial standards and natural mixture from *C. kessleri*). The detector was set at 0.08 a.u.f.s. and at 210 nm. For peak numbers see Table I.

RESULTS AND DISCUSSION

The use of reversed-phase HPLC on Separon SI C1 made it possible to separate saturated and mono unsaturated methyl esters of fatty acids ranging from C_{14} to C_{30} within less than 1 h (Fig. 1). It is thus possible to identify fatty acids by means of HPLC as well as GC.

The disadvantage of decreased sensitivity while detecting methyl esters of fatty acids by UV spectra is counterbalanced by their easy identification.

From our experimental data it follows that the influence of the double bond decreases as the number of carbons in the chain of a given methyl ester increases, *i.e.* the quotient of the capacity ratios for saturated and mono unsaturated methyl esters approaches one.

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