Journal of Chromatography, 472 (1989) 290–295
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 392

Note

Reversed-phase liquid chromatography for enrichment of very-long-chain fatty acids and their identification by gas chromatography–mass spectrometry

T. ŘEZANKA*, T. KOZA and R. KYSILKA

Institute of Microbiology, Czechoslovak Academy of Sciences, Vídeňská 1083, 14220 Prague 4 (Czechoslovakia)

I. VÍDEN

Department of Food Chemistry and Analysis, Institute of Chemical Technology, 16628 Prague 6 (Czechoslovakia)

and

M. WURST

Institute of Microbiology, Czechoslovak Academy of Sciences, Vídeňská 1083, 14220 Prague 4 (Czechoslovakia)

(First received December 27th, 1988; revised manuscript received January 29th, 1989)

Very-long-chain fatty acids (VLCFA) with more than 23 carbon atoms occur more rarely[1] in nature than those with the usual 14–24 carbon atoms in the molecule. They are found in both the plant and animal kingdoms. Two basic types of VLCFA can be distinguished, the saturated fatty acids (FA), with accompanying monounsaturated FA and polyenoic VLCFA. With the exception of, *e.g.*, plant wax, VLCFA are present at trace levels not exceeding 1–2% of the total FA in plants and animals and also in microorganisms. Palmitic and oleic acid represent major acids occurring in living organisms.

For the detection of trace amounts of VLCFA, several methods have been developed. Gas chromatography (GC) has often been used for qualitative and quantitative determinations, but not for the enrichment of a sample with VLCFA. Preparative GC was applied to the isolation of fatty acid methyl esters (FAME) with short and medium-length chains up to C_{18} for about 20 years. The oldest method is based on the interesting phenomenon of the solubility of sodium salts of VLCFA in non-polar solvents, *e.g.*, light petroleum[2]. Other methods are based on chromatographic methods, mainly with separation in the reversed-phase mode[3–5]. The principle of this method, employed by Takayama *et al.*[3], consists in a partial separation of FA according to the chain length on Sephadex LH-20. Two partially separated maxima were thus obtained; the first eluted contained VLCFA in the range C_{35}–C_{55} and the second C_{24}–C_{35} VLCFA.

Reversed-phase high-performance liquid chromatography (RP-HPLC) has been used by many workers, but most of them have employed this method for the separation of the individual FA in either the analytical[6–9] or preparative mode[10,11]. On the basis of our previous experience with semi-preparative RP-HPLC[12,13], we

0021-9673/89/$03.50

have used this method for the rapid enrichment of the VLCFA fraction with minimal losses. To demonstrate the suitability of the method used, FA mixtures containing VLCFA prepared from the fungi of the Basidiomycetes family[14] or rat Harderian gland were utilized. In these samples, the presence of VLCFA had been demonstrated by gas chromatography–mass spectrometry.

EXPERIMENTAL

All solvents were distilled and oxygen was removed before use. Acid standards (18:0, 20:0, 24:0 and 28:0) were purchased from Sigma (St. Louis, MO, U.S.A.). The natural mixture was composed of the methyl esters of the acids that we had isolated from the fungus *Ganoderma applanatum* (Basidiomycetes)[14] and from rat Harderian glands[15].

Separation of VLCFA in the form of salts

A standard mixture of VLCFA (6 mg) composed of the equal masses of acids (18:0, 24:0 and 28:0) was dissolved in 1 ml of a 0.1 *M* solution of a corresponding hydroxide (sodium, potassium, ammonium, calcium) and extracted three times with 1 ml of hexane. The combined extracts were dried by evaporation and converted into methyl esters by using 14% boron trifluoride in methanol.

Thin-layer chromatography (TLC)

A 1-mg amount of a standard mixture (or of the natural mixture) in the form of FAME was placed on TLC plates (silica gel $100F_{254}$, precoated layer thickness 0.25 mm, 20 × 20 cm; Merck, Darmstadt, F.R.G.) and chromatographed by using chloroform–benzene (1:1). The compounds were detected at 254 nm; the corresponding spots were scraped off and eluted with diethyl ether.

Reversed-phase TLC (RP-TLC)

A 1-mg amount of a standard FAME mixture was placed on RP-TLC plates (RP-18 F_{254s}, layer thickness 0.25 mm, 20 × 20 cm; Merck) and chromatographed with acetic acid–acetonitrile–tetrahydrofuran (30:30:40). After the chromatogram had been developed, the spots detected at 254 nm were scraped off and eluted with diethyl ether (R_F 0.52 = FAME 18:0, R_F 0.35 = FAME 24:0, R_F 0.24 = FAME 28:0). With the natural mixtures, a band in the range R_F = 0.40–0.05 was collected by scraping.

Reversed-phase high-performance liquid chromatography

A semi-preparative separation of FAME was carried out by using a Varian 8500 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.). A stainless-steel column (250 mm × 8 mm I.D.; Separon SGX C_{18} sorbent, 7 μm) (Laboratorní přístroje, Prague, Czechoslovakia) was employed. The flow-rate of the mobile phase was 2.0 ml/min. In the time interval 0–60 min the column was eluted with methanol that was later replaced with diethyl ether (15 min). The column was again conditioned with methanol (15 min). The fraction collected up to 17 min was discarded, and that obtained within the interval 17–90 min was used for further analysis (detection at 208 nm).

Gas chromatography

A Sigma 3B apparatus (Perkin-Elmer, Norwalk, CT, U.S.A.) with a fused-silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness) having a chemically bonded SPB-5 stationary phase (Supelco, Gland, Switzerland) was employed. The samples were chromatographed under the following conditions: temperature programme, 230–320°C at 3°C/min; temperature of the injector (splitless) and detector (FID), 340°C; carrier gas, hydrogen at a linear flow-rate of 40 cm/s.

Gas chromatography–mass spectrometry

The mixture of FAME was analysed on a Shimadzu Model QP 1000 quadrupole GC–MS system (Shimadzu, Tokyo, Japan) using an SPB-1 fused-silica capillary column (Supelco, Bellefonte, PA, U.S.A.) (60 m × 0.25 mm I.D.) with a film thickness of 0.25 μm. The injector temperature (splitting ratio 1:20) was 280°C and the oven temperature was increased from 160 to 330°C at 6°C/min.

RESULTS AND DISCUSSION

Four methods as described below were used for the enrichment with VLCFA. Although a loss of VLCFA has been described in the literature[2], we were not able to enrich selectively the light petroleum extract with VLCFA in this way. By using the above-mentioned salts, none of the extractions carried out resulted in any enrichment with 24:0 and/or 28:0. For example, by using sodium salts of the model mixture, 3.1 μg of FA were obtained in three extractions, the proportions of 18:0, 24:0 and 28:0 being 1.00:1.01:1.01, which, from the practical point of view, represented a negligible enrichment of the hexane fraction with VLCFA.

Another method that did not yield the expected results was preparative TLC on silica gel. Again, procedures had been described[16], including the composition of the mobile phase and the sorbent activity, ensuring the separation of homologues exhibiting differences of four and more CH_2 groups. In this case, the separation of 18:0 from 24:0 was good but the separation of 24:0 from 28:0 was poor. However, normal-phase TLC could not be adapted for the chromatographic separation of these complex mixtures.

Reversed-phase chromatographic methods, either RP-TLC or, better, RP-HPLC, were found to be successful. When RP-TLC was used, the proportions in the model mixture were changed from 1:1:1 (18:0, 24:0, 28:0) to 1:10:13, which represented an enrichment with VLCFA by one order of magnitude.

Even though a separation of the individual FAME has been reported in the literature[17,18], we were not able to separate the individual homologues in the natural mixture. Another disadvantage that we also were not able to overcome, in spite of the use of an antioxidant (*tert.*-butylhydroxyanisole, BHA) in the mobile phase, saturation of the solvents with nitrogen and subsequent separation of the compounds in a nitrogen atmosphere was the oxidative degradation of the unsaturated bonds. The loss of 24:1 can be caused by a process other than by peroxidation, *e.g.*, by smearing of the zone during TLC. As for the detection of peroxide(s), they are thought to have a significantly higher molecular weight (by at least two oxygen atoms) and, therefore, exhibit longer elution times and are thermally degradable at the elution temperature. This is why it would be necessary to carry out the derivatization, which would,

however, result in another increase in molecular weight and in the elution temperature and time, and hence the compounds would not be eluted from the column as one peak within a reasonable time. The proportion of 24:1 to 24:0 in the original mixture was 0.87[14], whereas after RP-TLC it was as low as 0.23, which unequivocally indicated a loss of 24:1.

The best results were obtained by using RP-HPLC. Methyl arachidate was chosen as the standard, having a retention time of 17 min under the conditions used. Detection at 208 nm was very difficult, especially during the preparation, and, therefore, the conditions developed for the analytical mode were employed. The methyl ester 20:0 was chosen because major acids up to C_{20} were present in the two natural mixtures. The separation efficiency is readily seen in Fig. 1, where a chromatogram of the original mixture obtained from *Ganoderma applanatum* (a) is shown together with the fraction acquired by RP-HPLC (b), whose retention time was longer than 17 min.

Unfortunately, no UV-absorbing derivative of such long FA can be chromatographed by GC. As far as the commonest, UV-absorbing, compounds are concerned, *viz.*, bromophenacyl esters, their polarity and molecular weight increase so much that during the injection and mainly during a GC run they are degraded and cannot be eluted from the column.

The disadvantage met during RP-TLC, *viz.*, the oxidative degradation of monoenoic FAME, was minimized by using RP-HPLC (the proportion of 24:1 to 24:0 was 0.87 in the original mixture and changed only slightly to 0.84 after RP-HPLC). As a result of the enrichment of the individual fractions with VLCFA, so far unknown homologues were identified (Fig. 1b). A mass spectrum of the highest homologue found (34:0 FAME) is shown in Fig. 2, indicating the presence of these acids in the sample.

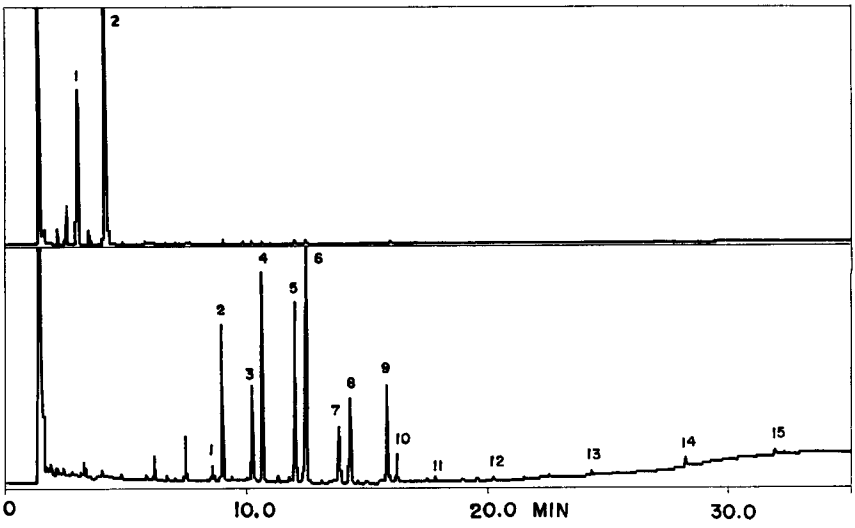

Fig. 1. GC of FAME of fungus *Ganoderma applanatum*. (a) (upper trace), total FAME. Peaks: 1 = 16:0; 2 = 18:1. (b) (lower trace), FAME after preparative RP-HPLC. Peaks:1 = 22:1; 2 = 22:0; 3 = 23:1; 4 = 23:0; 5 = 24:1; 6 = 24:0; 7 = 25:1; 8 = 25:0; 9 = 26:1; 10 = 26:0; 11 = 27:0; 12 = 28:0; 13 = 30:0; 14 = 32:0; 15 = 34:0. Only major FAME are shown.

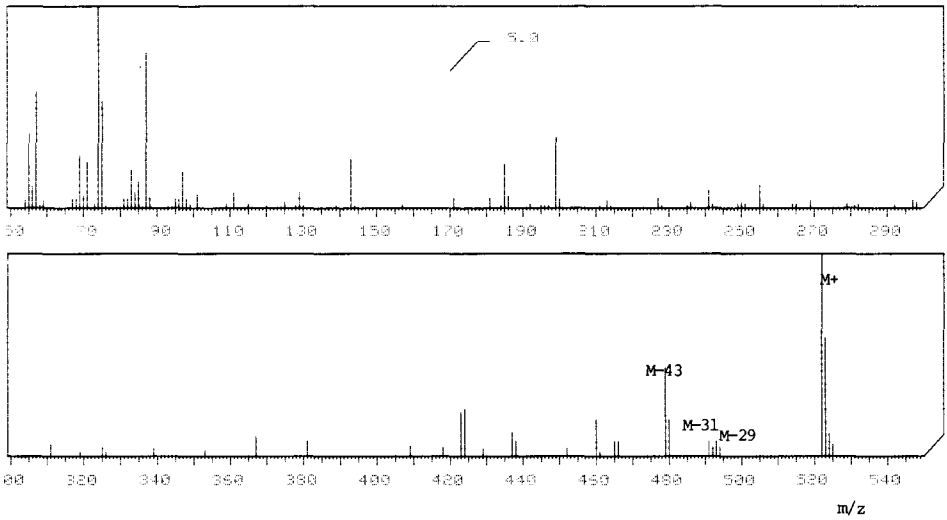

Fig. 2. Electron impact mass spectrum of 34:0 FAME [see peak 15 in Fig. 1b (lower)]. M^+ = molecular ion.

For the saturated FAME, the significant M^+ ions are $M-29$, $M-31$ and $M-43$, which were ubiquitous (see Fig. 2 for FAME 34:0); for the monoenic acids, M^+ together with significant ions $M-32$ and $M-74$ were present. All FAME show the presence of the ions of the general type, *viz.*, $(CH_2)_nCOOCH_3$, where n = 2,6,10,14,..., which corresponds to m/z values of 87, 143, 199 and 255, respectively. These ions (n = 2, m/z 87; n = 6, m/z 143; n = 10, m/z 199; n = 14, m/z 255; n = 18, m/z 311; n = 22, m/z 367; n = 26, m/z 423; n = 30, m/z 479) were also present in FAME 34:0 (see Fig. 2). It is concluded that the only suitable method for the enrichment of the samples with VLCFA is RP-HPLC. This method provides excellent yields and no degration of FAME takes place, in contrast to RP-TLC. The latter method therefore seems to be suitable only for saturated FAME.

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