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Rapid method for the enrichment of very long-chain fatty acids from microorganisms

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ABSTRACT

A method for the enrichment very long-chain fatty acids (VLCFAs) from total fatty acid-containing samples is described. The method is based on the use of reversed-phase high-performance liquid chromatography and/or solid-phase extraction cartridges with subsequent identification of VLCFAs by capillary gas chromatography–mass spectrometry. By combining the two techniques, unusual VLCFAs (higher than C_{22}) were identified in newly isolated oligotrophic bacteria. VLCFAs have already been described in sulphate-reducing bacteria and in some wood-decaying fungi.

INTRODUCTION

Except for mycobacteria, in which very long-chain fatty acids (VLCFAs) were detected a long time ago [1], we have very little knowledge about these unusual acids. Therefore, this topic has attracted much attention during recent years [2,3].

One of the ways of identifying VLCFAs (having more than 22 carbon atoms in the molecule) in microorganisms, in which their content is very low and highly variable, is to enrich the sample. Our task was to develop such an enrichment method. This point has been neglected very often, and few papers deal with a complex study of enrichment of VLCFA-containing samples.

One of the two following methods may be chosen. The first one involves separation of individual lipid classes and identification of VLCFAs in particulate molecular species (*e.g.* sphingomyelin [4] or phosphatidylcholine [5]). The second method includes selective enrichment of VLCFA-containing mixtures of total fatty acids (FAs) by chromatography. The latter procedure is preferable for the analysis of microbial lipids as some of them are unusual. In addition, this method is less labour-intensive and allows the isolation of as much as milligrams of VLCFAs within several hours.

Good results were obtained by Takayama *et al.* [6], who studied mycobacterial VLCFAs. The method was based on classical column chromatography with lipophilic Sephadex LH-20. Identification was performed by further TLC and RP-HPLC with MS.

Excellent resolutions of standards was obtained by TLC on silica gel, but separation of a natural mixture resulted in total failure [7] (*i.e.* degradation of unsaturated fatty acids, insuffici-

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ent plate numbers and very difficult detection with some problems with non-destructive stains on the Ag^+ layer). Similar results were reported by Quereshi *et al.* [8], who succeeded in TLC separation of VLCFAs from mycobacteria as *p*-bromophenacyl esters and obtained four fractions. The subsequent RP-HPLC showed that almost all peaks were present in all four TLC fractions, even if at different concentrations.

The best results were obtained if the reversed phase was used, either in TLC or in HPLC [7]. Nevertheless, some disadvantages of TLC were observed, *e.g.* capacity of the plate, partial degradation of unsaturated FAs and, also, non-separation of homologues in a natural mixture that differed by one CH_2 group. The best method of all turned out to be RP-HPLC, which could be used not only for separation of the individual compounds but also for enrichment of the total FAs with VLCFAs.

As early as 1984 a method [9] was developed enabling the enrichment of total FAs with VLCFAs by using HPLC (RP-1) at the semi-preparative scale. The same method was successfully used in another study, with the difference that the authors used a C_{18} reversed-phase column [10].

Having already faced the problem of identification of VLCFAs in microbial cells several times [11–13], we developed a simple and rapid technique of enrichment of VLCFA-containing samples. This method is based on separation of the total FAs, by using either RP-HPLC or solid-phase extraction (SPE) cartridges packed with an octadecyl-bonded silica.

EXPERIMENTAL

Preparations of standards

Stearic (1704 mg, 6 mmol) and cerotic (1188 mg, 3 mmol) (Sigma, St. Louis, MO, USA) acids were derivatized by reacting the free acids with boron fluoride–methanol [13]. Methyl esters of behenic acid were obtained in the same way. Two standard mixtures of fatty acid methyl esters (FAMEs) from 18:0 and 26:0 FAMEs were prepared in chloroform (standard 1 = 908 mg; molar ratio 1:1; standard 2 = 888 mg, molar ratio 99:1).

The mixture of natural FAMEs was obtained from:

(1) Soil oligotrophic bacteria *Renobacter vacuolatum* NP-300-G [11].

(2) Fungi (*Ganoderma applanatum*) of the Basidiomycetes family [12].

(3) Sulphate-reducing bacterium *Desulfotomaculum* sp. str. 43 [13].

Derivatization and GC–MS identification

The oxazolines were prepared using a modified method, as described before [14]: 5 mg of dicyclohexylcarbodiimide were added to a solution of 5 mg of FAs in 1 ml of dichloromethane. After stirring for 10 min, 5 mg of 2-amino-2-methylpropanol were added (20°C, 4 h). Evaporated mixture was dissolved in 1 ml of diethyl ether and treated with 0.5 ml of thionylchloride ($SOCl_2$) (20°C, 1 h), then washed with ice-cold water and eluted through a column with anhydrous sodium sulphate and silica gel.

High-performance liquid chromatography

HPLC was performed in the Gradient LC System G-I (Shimadzu, Kyoto, Japan) with two LC-6A pumps (0.5 ml/min), an SCL-6A system controller, an SPD ultraviolet detector (207 nm), an SIL-1A sample injector and a C-R3A data processor. A semipreparative column Separon SGX C_{18} (250 mm × 8 mm I.D.) (Tessek, Prague, Czechoslovakia) with 7-μm particles were used.

The column was first eluted with methanol for 60 min, then with diethyl ether for 15 min and finally with methanol for 15 min. The fractions collected within more than 9 min were used for further analysis (for 22:0 retention time is 8.2 min).

Solid-phase extraction

The Separon SGX C_{18} silica-cart plastic cartridge system (1-ml tubes) (Tessek) was used. A total amount of 1–2 mg of FAMEs was applied to the column in a small volume of dichlormethane. Methanol–isopropanol (98:2) was allowed to flow under slight syringe pressure at a rate of approximately 0.5 ml/min; 0.1-ml fractions were collected manually. Better results are obtained with detectors but the price of detec-

tor(s) is higher. The solvent mixture was then evaporated in a vacuum and each fraction was analysed by GC–MS. The retention volume for FAME 22:0 was 2.1 ml.

For identification of FAMEs we used a Shimadzu QP-1000 GC–MS system (Shimadzu) with a 60 m × 0.32 mm I.D. (0.25 μm particle size)/SPB-1 fused-silica capillary column (Supelco, Gland, Switzerland), a split/splitless injector and helium as a carrier gas. The oven temperature was programmed from 100 to 320°C at the rate of 4°C/min. Ionization energy was 70 eV and electron multiplier voltage was 2.5 kV.

Oxazolines were identified under similar conditions as methyl esters, with the only difference being in the column temperature. In this case the oven was programmed from 150 to 330°C at the rate of 5°C/min.

RESULTS AND DISCUSSION

The capacity of RP-HPLC was between 100 and 200 mg of the injected mixture (standard No. 1) as shown in Table I. By using SPE, the injected amount decreases to about 10 mg. In both cases, a ratio (18:0 to 26:0) of 98–99:2–1 was taken as a satisfactory enrichment. The proportion of the injected mixture to the weight of the sorbent remained constant (1:100). Based on these facts, the two methods RP-HPLC and SPE are considered to be adequate.

In this paper we used methyl esters, as they are more suitable for GC–MS. UV-absorbing or fluorescent derivatives cannot be used for GC–MS as they are usually not sufficiently volatile. In general, lower organisms (microorganisms) contain very unusual types of complex lipids and their identification is very difficult.

Unfortunately, when the molar ratio of 18:0 to 26:0 was 99:1 (standard No. 2), which was quite a common value in the natural material, the results changed significantly. In particular, a decrease in the capacities of the column and cartridge of as much as one order of magnitude was observed. More important was the mass proportion of 18:0 and 26:0 in the eluate. For example, if the injection was 1 mg per SPE column, the enriched eluate contained 5.74 μg of 18:0 FAME and 16.44 μg of 26:0 FAME. So, if a natural mixture with C_{18} content a hundred times more than that of C_{26} acids was analysed, the resulting content of the two compounds reached almost 1:3. Excluding this limitation, the method seemed to be suitable for a preseparation at the VLCFA enrichment step.

To find out how far the theoretical assumptions derived from the analysis of two simple component mixtures could be extrapolated to the natural mixture, three sources were used: standards 1 and 2 (mentioned above) and the natural mixture.

In our experiments the natural mixture revealed almost the same patterns as standard 2. Some complications could be observed only during the elution of monoenoic FAMEs, where the saturated homologue shorter by two carbon

TABLE I

DEPENDENCE OF THE SAMPLE ENRICHMENT WITH VLCFAs ON THE TYPE OF SEPARATION (RP-HPLC, SOLID-PHASE EXTRACTION), COLUMN CAPACITY AND THE FAME MOLAR RATIO

FAME	Molar ratio	Type of separation (amount of injected mixture, mg)											
		LC–SPE (1)		LC–SPE (10)		LC–SPE (50)		LC–SPE (100)		LC–SPE (200)		LC–SPE (500)	
18:0	50:50[a]	0.1[b]	0.3	0.2	1.4	0.4	12.1	0.8	45.7	3.8	49.9	25.7	50.0
26:0		99.9	99.7	99.8	98.6	99.6	87.9	99.2	54.3	96.2	50.1	74.3	50.0
18:0	99:1	0.5	1.4	1.3	19.6	7.8	44.2	32.1	99.0	99.0	99.0	99.0	99.0
26:0		95.5	98.6	98.7	80.4	92.2	55.8	67.9	50.0	1.0	1.0	1.0	1.0

[a] Molar ratio of 18:0 and 26:0 FAMEs in injected mixture.
[b] Molar ratio of 18:0 and 26:0 FAMEs in eluted mixture.

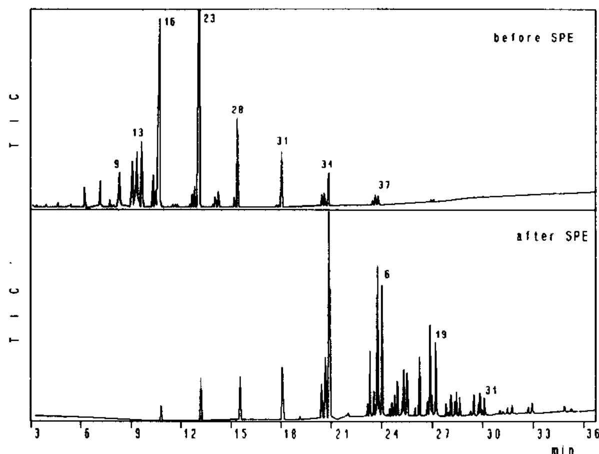

Fig. 1. Gas chromatography of FAMEs from soil oligotrophic bacteria *Renobacter vacuolatum* NP-300-G. Upper trace, total FAME = before SPE. For peak numbers, see Table II. Lower trace, methyl esters of VLCFAs after SPE (fraction above 2.2 ml was collected; for details, see Experimental section). For peak numbers, see Table III.

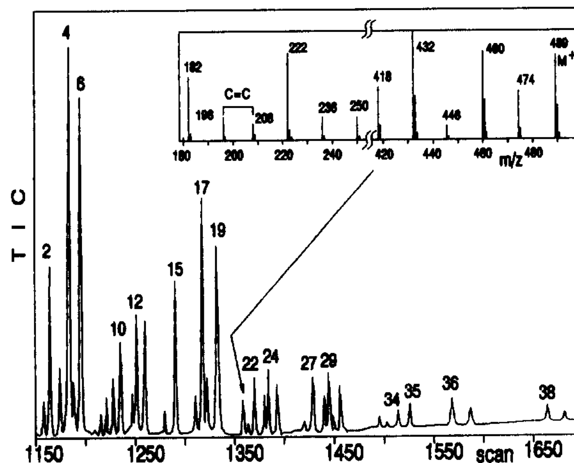

Fig. 2. GC–MS of oxazolines, prepared from VLCFA enrichment. The identities of peaks are listed in Table III. In the upper right corner the mass spectrum of ai-20–29:1 oxazoline is presented.

TABLE II

COMPOSITION OF FATTY ACIDS FROM OLIGOTROPHIC BACTERIA *RENOBACTER VACUOLATUM* NP-300-G (BEFORE ENRICHMENT)

i = Iso; ai = *ante*-iso.

Peak number	Fatty acid	Percentage	Peak number	Fatty acid	Percentage
1	8:0	0.18	20	9–18:1	0.28
2	9:0	0.25	21	i-18:0	0.95
3	10:0	0.37	22	11–18:1	1.47
4	11:0	0.18	23	18:0	22.15
5	12:0	1.69	24	i-19:0	0.16
6	13:0	2.17	25	ai-19:0	0.95
7	i-14:0	0.66	26	19:0	1.43
8	7–14:1	0.18	27	11–20:1	0.77
9	14:0	2.75	28	20:0	9.23
10	i-15:0	3.24	29	i-22:0	0.57
11	ai-15:0	4.56	30	13–22:1	0.34
12	8–15:1	0.44	31	22:0	6.70
13	15:0	5.37	32	i-24:0	0.95
14	i-16:0	3.61	33	15–24:1	1.19
15	7–16:1	1.47	34	24:0	3.29
16	16:0	19.49	35	i-26:0	0.38
17	i-17:0	0.32	36	26:1	0.89
18	10–17:1	0.41	37	26:0	0.79
19	17:0	0.18			

atoms was co-eluted. This rule of critical pairs is well known [15].

In addition to the four originally identified peaks (24:1, 24:0, 26:1 and 26:0) and another two tentative ones (28:1 and 28:0), we identified a total of 33 new peaks in the natural mixture (Fig. 1). The content of 18:0 and 26:0 became almost 1:3 compared with the standard mixtures. Fig. 1 reveals enrichment in other acids as well. C_{24} acids were found to be greatly enriched and, thus, 24:0 turned out to be a major acid. Fig. 2 depicts a chromatogram of oxazolines starting from C_{26} acids. If Figs. 1 (bottom) and 2 are compared, no significant differences in either separation or proportion of the compounds are observed. So, we could suggest that methyl esters and oxazolines of VLCFAs behave in a similar way.

When FAMEs from a fungus and a sulphate-reducing bacterium were analysed, similar results were obtained.

Fig. 2 shows a mass spectrum of ai-20–29:1 oxazoline. The mass spectra of VLCFA methylesters have been described previously [12,13]. Methyl esters have the best chromatographic properties but, unfortunately, the information obtained from their mass spectra is not satisfactory. It is impossible to determine the position of double bond(s) and very difficult to identify the substitution of the main chain with a methyl group. Using these derivatives, first described by Yu *et al.* [14,16], we were able to identify the monoenoic and branched acids even in such a complex mixture as was found in oligotrophic bacteria. The elution temperature of oxazolines was higher than that of methyl esters by 5°C only.

The mass spectrum of ai-20–29:1 oxazoline possesses two interesting regions. The first of them comprises the values of m/z in the neighbourhood of the molecular ion and, thus, enabled us to identify the branching of the chain easily [14]. In oxazolines, the splitting of the individual fragments was more evident than in methyl esters. Therefore, the ions m/z 489 [M^+], 474 [$M-CH_3$], 460 [$M-C_2H_5$] and 432 [$M-C_4H_9$] were more abundant in the mass spectrum. In contrast, the ion m/z 446 [$M-C_3H_7$],

TABLE III

VLCFAs FROM SOIL OLIGOTROPHIC BACTERIA *RENOBACTER VACUOLATUM* NP-300-G (AFTER ENRICHMENT BY SPE)

Peak number	Fatty acids	Percentage	Peak number	Fatty acids	Percentage
1	i-17–26:1	0.83	21	i-29:0	0.45
2	i-26:0	6.50	22	18–29:1	1.85
3	15–16:1	1.97	23	ai-29:0	1.15
4	17–26:1	14.97	24	20–29:1	1.97
5	19–26:1	1.59	25	29:0	1.59
6	26:0	14.14	26	i-21–30:1	0.51
7	i-19–27:1	0.64	27	i-30:0	1.53
8	ai-19–27:1	0.96	28	19–30:1	1.15
9	i-27:0	1.72	29	21–30:1	1.85
10	15–27:1	3.18	30	23–30:1	0.76
11	ai-27:0	1.59	31	30:0	1.46
12	19–27:1	3.89	32	ai-22–31:1	0.45
13	27:0	3.57	33	ai-31:0	0.32
14	i-19–28:1	0.96	34	22–31:1	0.57
15	i-28:0	4.59	35	31:0	0.76
16	17–28:1	1.72	36	23–32:1	0.89
17	19–28:1	9.11	37	32:0	0.51
18	21–28:1	2.17	38	25–34:1	0.45
19	28:0	6.37	39	34:0	0.25
20	ai-20–29:1	1.08			

corresponding to the structure of the iso-acid, was almost absent. The ions having lower *m/z* values, *e.g.* 418 $[M-C_5H_{11}]$, 404 $[M-C_6H_{13}]$, etc., exhibited normal abundances and their intensity was similar to that of saturated acids. The other region important for the determination of the molecular structure was the region of the ions that originate from the double bonds. The major ions in this region were the peaks of *m/z* 182 and 222, which were formed by the allylic cleavage on both sides of the double bond. Naturally, the difference of 12 a.m.u. between the ions *m/z* 196 and 208 totally confirmed this fact. Therefore, from the MS and chromatographic behaviour observed, we suggest the structure of ai-20–29:1 to be a novel one, never described before.

In conclusion, we would like to mention that a simple calculation could be carried out, showing that SPE is much cheaper than RP-HPLC. The price of one cartridge is about US$ 3, whereas that of a C_{18} semipreparative column is about US$ 800. In these calculations we do not include the prices of instruments, but only the customer products.

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