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Identification of very long chain fatty acids by atmospheric pressure chemical ionization liquid chromatography-mass spectroscopy from green alga *Chlorella kessleri*

A method is described for the enrichment of very long chain fatty acids (VLCFAs) from total fatty acids of heterotrophically cultivated green freshwater alga *Chlorella kessleri* and their identification as picolinyl esters by means of liquid chromatography-mass spectrometry with atmospheric pressure chemical ionization (LC-MS with APCI). The method is based on the use of preparative reversed phase HPLC of hundred-milligram amounts and their subsequent identification by microbore APCI LC-MS. A combination of these two techniques was used to identify unusual VLCFAs up to C_{47}, both saturated and monounsaturated, with two positional isomers (ω-9 and ω-26).

Key Words: VLCFA; APCI LC-MS; RP-HPLC; Green freshwater alga; *Chlorella kessleri*

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1 Introduction

VLCFAs with more than 24 carbon atoms occur more rarely in nature than those with the usual 14–24 carbon atoms in the molecule. They are found in both the plant and the animal kingdoms [1]. Two basic types of VLCFAs can be distinguished: saturated acids with accompanying monounsaturated FAs, and polyenoic VLCFAs.

The main problem in the identification of VLCFAs in natural materials, i.e. in plants, animals, and also in microorganisms is their low concentration [2]. With few exceptions (e.g. plant wax) [3], it usually does not exceed tenths or hundredths of a percent.

In a previous paper, we published methods for enriching mixtures of natural fatty acids [4]. These methods are based predominantly on chromatographic techniques, i.e. on thin-layer chromatography or HPLC, both in reversed phase mode. The method of choice turned out to be RP-HPLC [5], which could be used not only for enrichment of total FAs with VLCFAs but also for separation of individual compounds.

Detection of the eluted components is complicated as fatty acids hardly absorb in the UV region. It is therefore necessary either to use the absorption of the terminal carboxylic group of the double bond(s) or to derivatize this carboxylic group. Picolinyl esters are the only derivatives that can later be used for both gas-chromatography mass spectrometry (GC-MS) and/or for separation by means of RP-HPLC [6, 7].

The individual components were resolved by mobile phases based on methanol with a small amount of pyridine in water [6]. In our previous paper [5], the unwanted interactions between the picolinyl moiety and the stationary phase were eliminated by triethylamine. The column with phases based on C8/C18 multi-alkyl bonding and exhaustive endcapping minimizes the use of organic modifiers. It was also recently used successfully by Christie [7], with pure acetonitrile serving as mobile phase.

Gas chromatography has recently been used for the separation of VLCFA [1, 8]. This method is suitable for the analysis of compounds which are volatile as such or after derivatization. As is known from the literature [9], the discrimination of higher homologues increases with increasing number of carbons and double bonds. The technique used in this paper, i.e. APCI, avoids this shortcoming (see also Rezanka [10, 11]).

Based on our previous experience with semi-preparative RP-HPLC [5] we have now used its preparative mode for the rapid enrichment of the VLCFA fraction with minimal losses. FA mixture containing VLCFAs prepared from the green alga *C. kessleri* was utilized to demonstrate the suitability of the method. The presence of VLCFAs in this sample was demonstrated by a newly developed method, i.e. identification of picolinyl esters of the VLCFAs by means of the LC-MS with APCI.

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2 Experimental

2.1 Standards and isolation

Fatty acid standard (octacosanoic) was purchased from Sigma (Prague, Czech Republic). All solvents were redistilled and degassed before use. The natural mixture of FAs was obtained from lyophilized fresh-water green alga *C.kessleri* cultivated heterotrophically according to a Czech patent [12]. From 100 g of lyophilized alga a fatty acid fraction of 1.85 g was obtained. This fraction was derivatized to the corresponding picolinyl derivatives giving after purification 901 mg of derivatized fatty acids, they were submitted to preparative LC to obtain 8.41 mg of picolinyl derivatives of $C_{29}-C_{47}$ fatty acids. This fraction was then analyzed by LC-MS. The picolinyl esters were prepared according to Christie [13].

The free fatty acids (1.85 g) were dissolved in diethyl ether (100 mL) and converted into the mixed anhydride derivatives by reaction with trifluoroacetic anhydride (10 mL) at 50°C for 2 h. The excess reagent was evaporated and a 10% solution of nicotinyl alcohol in tetrahydrofuran (25 mL) was added and the mixture was left at 50°C for 2 h. Diethyl ether (100 mL) and hexane (20 mL) were added and the mixture was washed with water (20 mL), 1 M HCl (20 mL, three times) and water (20 mL, three times) and dried with anhydrous sodium sulfate. The solvents were evaporated under reduced pressure and picolinyl esters were purified by silica gel chromatography with the mixture hexane-diethyl ether (1:1 *v/v*) as the eluting agent [13], yield 901 mg.

2.2 Preparative chromatography

A Chromatospac Prep 100 preparative chromatograph (Jobin-Yvon, Longjumeau, France) with axial compression of the chromatographic bed (61.2 × 8 cm ID) was used for the isolation of enrichment fraction (picolinyl esters from $C_{29}-C_{47}$). The column efficiency was 6510 theoretical plates, V_0 1135 mL and t_R of the picolinyl ester of octacosanoic acid (28:0) was 36.4 min, the mobile phase flow-rate was 47 mL/min^{-1}. The mean particle diameter of the Separon C18 reversed-phase packing was 15 μm (formerly Laboratorní prístroje, Prague, Czechoslovakia). A Holochrome H/MD variable-wavelength detector (Gilson, France) with a 40-μL flow-through cell was set at 235 nm. All experiments were carried out at room temperature.

The 901 mg picolinyl esters (see above) were dissolved in 25 mL of the mobile phase (injection volume) (methanol-isopropanol-triethylamine, 85:15:0.05). In the time interval 0–40 min the column was eluted with mobile phase that was later replaced with diethyl ether (30 min). The column was again conditioned with mobile phase for 20 min. The fraction up to 38.5 min was discarded and that obtained within interval 38.5–70 min was used for further analysis. After evaporation of the mobile phase, the total yield of a mixture of fatty acids with the chain longer than 28 carbon atoms was 8.41 mg. These were separated and identified by LC-MS.

2.3 LC-MS with APCI

The HPLC set-up consisted of a 1090 Win system, a PV5 ternary pump, and an automatic injector (HP 1090 series, Hewlett Packard, USA) and two columns in series. Hichrom columns, i.e. HIRPB-250AM 250 × 2.1 mm ID, 5 μm phase particle. A column efficiency of approximately 53000 plates/50 cm was obtained. A quadruple mass spectrometer system Navigator (Finnigan MAT, San Jose, CA, USA) was used which was fitted with an atmospheric pressure chemical ionization source (vaporizer temperature 400°C, capillary heater temperature 220°C, corona current 5 μA, sheath gas high-purity nitrogen, pressure ca. 380 kPa, and auxiliary gas (also nitrogen) flow rate 15 mL/min. Positively charged ions with *m/z* 200–900 were scanned with a scan time of 0.5 s. The whole HPLC flow (0.37 mL/min) was introduced into the APCI source without any splitting. Fatty acid picolinyl esters were separated using a gradient solvent program with acetonitrile (ACN), dichloromethane (DCM), and propionitrile (EtCN) as follows: initial ACN/EtCN/DCM (60:30:10, *v/v/v*); linear from 10 min to 40 min ACN/EtCN/DCM 30:40:30, *v/v/v*); held until 60.5 min; the composition was returned to the initial conditions over 8 min. A peak threshold of 0.3% intensity was applied to the mass spectra. Data acquisition and analyses were performed using PC with MassLab 2.0 for Windows NT 4.0 applications/operating software.

3 Results and discussion

Fatty acids isolated from the fresh water green alga *Chlorella kessleri* have already been identified and quantified using a GC-MS method [14, 15]. As mentioned above, long chain fatty acids are extremely sensitive to thermal degradation, i.e. to conditions under which they are eluted from the GLC column. To minimize the possibility of thermal degradation, we used two HPLC steps; the FA derivatives were first concentrated by preparative RP-HPLC (**Figure 1**) and individual molecular types of VLCFA derivatives were separated, quantified, and identified by LC-MS.

The difficulty of identifying the VLCFA in the whole complex of fatty acids was eliminated by separating lower fatty acids (up to C_{28}) by HPLC, i.e. enriching higher FA. Although the proportion of VLCFA drops dramatically (i.e.

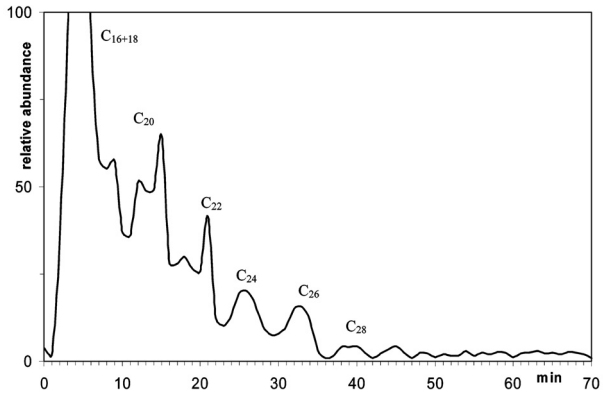

Figure 1. Preparative RP-HPLC chromatogram of FA derivatives from the green freshwater alga *Chlorella kessleri*. Peak identification: C_{16+18} – derivatives of the FA with chain lengths 16 and 18 carbon atoms, C_{20} – FA derivatives with 20 atoms, etc.

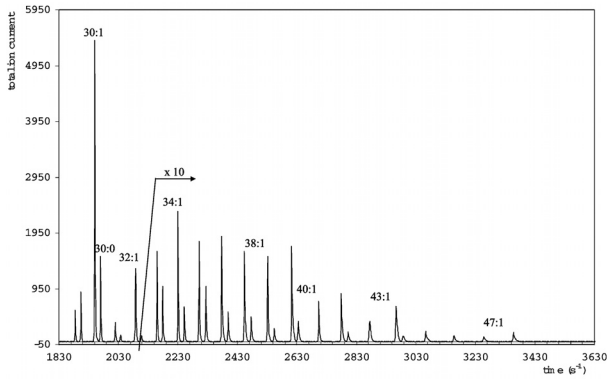

Figure 2. LC-MS APCI chromatogram of enriched fraction (after preparative RP-HPLC, see Figure 1). Peak identification: 30:1 – picolinyl ester of 9-triacontenoic acid, 30:0 picolinyl ester of triacontanoic acid; for further explanation, see Table 1.

the formation of higher homologues of VLCFA during their biosynthesis), the C_{47} is also detectable even though it constitutes only 10^{-2}% of total fatty acids. The total ion current was applied for quantification, as shown also in **Figure 2**.

The enrichment of the individual fractions with VLCFA enabled us to identify as yet unknown homologues (Figure 2).

Table 1 shows the percent representation of VLCFA in the whole complex of fatty acids of *C. kessleri.* The fatty acids were saturated and monounsaturated from C_{29} up to C_{47}, odd and even numbered, most of them with a straight chain. The ratio of saturated to unsaturated fatty acids changes with the number of carbons; in the C_{29} acids, the saturated fatty acids predominate, in the C_{30} acids, the unsaturated acid predominates, and in the higher homologues (above 43 carbons), the saturated fatty acids are missing.

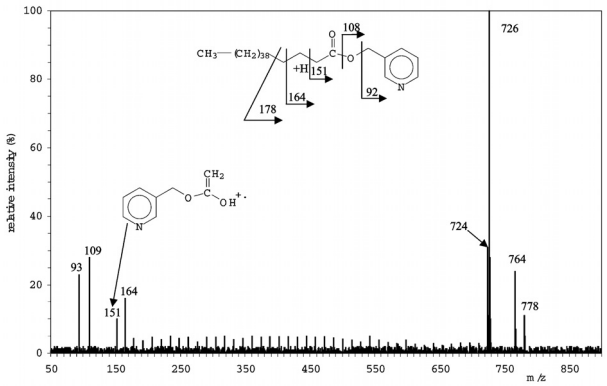

Figure 3. Mass spectrum of picolinyl ester of 43:0 acid, the structures of major ions are shown; for an explanation of values, see text and Table 1.

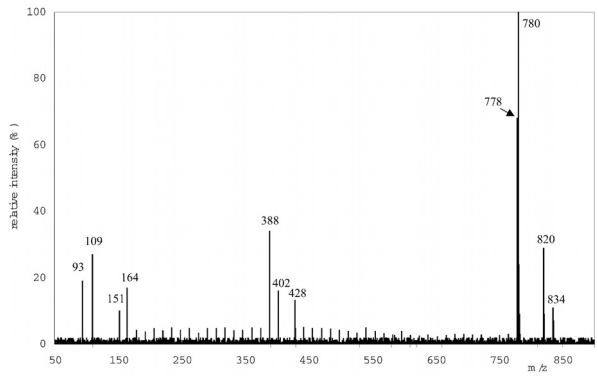

Figure 4. Mass spectrum of picolinyl ester of 47:1 acid, for an explanation of values, see above.

In contrast to EI ionization [16] where the mass spectra of the picolinyl esters of straight chain saturated fatty acids contain ions from the lower part of mass spectra, i.e. ions *m/z* 92, 108, 151, and 164, these ions are in the minority in APCI, as shown in **Figure 3** (e.g. 43:0). Even-numbered ions differing by 14 amu and representing cleavage between methylene groups also have low intensity. A distinct situation can be found in the M^+ region. Here we can observe the formation of two abundant ions $(M-1)^+$ and $(M+1)^+$ including the generation of adducts with the mobile phase such as $(M+40)^+$ $(M+C_2H_2N)$ and $(M+54)^+$ $(M+C_3H_4N)$. In all investigated VLCFA spectra, the ion $(M+H)^+$ is the most abundant one.

In the case of monoenoic derivatives (47:1, see **Figure 4**), one can detect in the EI-MS spectrum a minority group of ions *m/z* 92, 108, 151, and 164. The same spectrum features ions that differ from the others by a value of 14 amu (at 47:1, to *m/z* = 388), as well as other abundant ions with a gap of 26 or 40 amu (i.e. ions at *m/z* 388, 414, and 428). In comparison with EI-MS [16], these ions are

Table 1. Fatty acid composition in *C. kessleri.*

No	Acid	% of total FA[a)]	% of ω-9[b)]	% of ω-26[b)]	M+H[c)]	A	B	C	A′	B′	C′
1	29:1	6.3	100	0	528	414	388	374			
2	29:0	9.9			530						
3	30:1	60.0	100	0	542	428	402	388			
4	30:0	17.0			544						
5	31:1	3.9	–	–[d)]	556	442	416	402	204	178	164
6	31:0	1.3			558						
7	32:1	14.6	90	10	570	456	430	416	218	192	178
8	32:0	1.2			572						
9	33:1	1.8	40	60	584	470	444	430	232	206	192
10	33:0	1.1			586						
11	34:1	2.6	80	20	598	484	458	444	246	220	206
12	34:0	0.7			600						
13	35:1	2.0	40	60	612	498	472	458	260	234	220
14	35:0	1.1			614						
15	36:1	2.1	70	30	626	512	486	472	274	248	234
16	36:0	0.6			628						
17	37:1	1.8	30	70	640	526	500	486	288	262	248
18	37:0	0.5			642						
19	38:1	1.7	50	50	654	540	514	500	302	276	262
20	38:0	0.2			656						
21	39:1	1.9	20	80	668	554	528	514	316	290	276
22	39:0	0.4			668						
23	40:1	0.8	40	60	682	568	542	528	330	304	290
24	41:1	1.1	10	90	696	582	556	542	344	318	304
25	41:0	0.2			698						
26	42:1	0.4	20	80	710	596	570	556	358	332	318
27	43:1	0.7	10	90	724	610	584	570	372	346	332
28	43:0	0.1			726						
29	44:1	0.2	10	90	738	624	612	584	386	360	346
30	45:1	0.4	0	100	752				400	374	360
31	46:1	0.1	10	90	766	652	626	612	414	388	374
32	47:1	0.2	0	100	780				428	402	388

a) $\times 10^{-2}$ of total FA.

b) Based on abundance of diagnostic ions A, B, and C (ω-9), respective A′, B′, and C′ (ω-26).

c) Base peak for all homologues, both saturated and unsaturated.

d) Because the ion C′ has a value of *m/z* 164 for Δ^5, it can be determined due to higher observed intensity that can be attributed to the position of C═C bond.

more abundant in the APCI spectrum. Unfortunately, under conditions in which our LC-MS operated, adducts of a mobile phase on the double bond, which were described in the literature [17, 18], were not determined. The ions $(M-H)^+$, $(M+H)^+$, $(M+40)^+$, and $(M+54)^+$ were also very abundant in the M^+ region. A large difference in ion distribution was found with the $(M-H)^+$ ion, which exhibited a 3-fold higher intensity in monoenoic VLCFA than in saturated VLCFA. The peak no. 19 (Figure 2; 38:1), whose mass spectrum is shown in **Figure 5**, represents a mixture of two positional isomers in a ratio of approximately 1:1. Detection of ions at *m/z* 262, 276, 302 and 500, 514, 540 pointed to the presence of a mixture of two positional isomers ω-9 and ω-26. Semiquantification of both positional isomers was based on counting the total abundance of all above-mentioned ions (see also Table 1). Their ratio

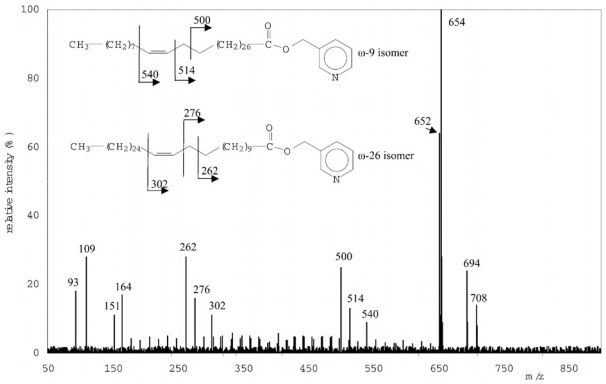

Figure 5. Mass spectrum of picolinyl ester of 38:1 acid mixture, for an explanation of values, the structures of major ions are shown, see above.

was 1:1. A similar procedure was used to evaluate the proportion between other isomers of ω-9 and ω-26 type.

However, although the separation efficiency of the two columns (2 × 25 cm) was estimated at about 100 000 theoretical plates per meter, it was insufficient for the separation of positional isomers of monoenoic VLCFA.

The combination of preparative RP-HPLC and microbore LC-MS thus extends the analytical possibilities of both methods and contributes to the acquisition of new information on biological materials.

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