Analysis of Polyunsaturated Fatty Acids Using High Performance Liquid Chromatography – Atmospheric Pressure Chemical Ionization Mass Spectrometry

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Summary

Analysis of polyunsaturated fatty acids using high performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry is described. The standard fatty acid methyl esters from 16 to 22 carbons were analyzed by LC-MS with APCI. The effect of orifice voltage and total carbon atoms *versus* number of double bonds in each homologue on the mass spectra is discussed. The correction coefficients for homologues from saturated fatty acids to hexaenoic acid are also mentioned.

1 Introduction

Gas chromatography with flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS) are the methods most widely used for analysis and identification of fatty acids in natural materials. However, their application often causes problems which may be solved, for example, by using a short capillary column for analysis of very long chain fatty acids [1], or using lower elution temperature for thermally unstable usually polyunsaturated fatty acids (PUFA). Nitrogen derivatives are very often used for localizing double bonds, most commonly pyrrolidides, oxazolines, picolinyl esters [2]; dimethyl disulfides are also used for this purpose [3–6]. Moreover, the thermal stability of PUFA decreases with increasing number of double bonds in a molecule. For these reasons high performance liquid chromatography (HPLC) has been increasingly used for fatty acids analysis in recent years. Numerous derivatives have been used in HPLC analysis [7, 8]. Unfortunately, their identification is almost always based on comparison of the retention characteristics of single peaks in the sample with the retention characteristics of standards. There have recently been great improvements in liquid chromatography-mass spectrometry (LC-MS), as illustrated by the analysis of triacylglycerols [9–11]. However, only few publications are concerned with fatty acids analysis [12, 13]. This publication describes the first LC-MS analysis with atmospheric chemical ionization (APCI) of fatty acid methyl ester standards as well as a real-life natural sample of methyl esters (plant oil).

2 Experimental

2.1 Instrumentation

The HPLC set-up consisted of a 1090 Win system and PV5 ternary pump which includes HP 1090 HPLC with ternary PV5 pump and automatic injector (HP 1090 series, Hewlett Packard, USA) and two columns in series: Supelcosil narrow-bore columns, *i.e.* Supelcosil LC-18 250 × 2.1 mm i.d., 3 μm particles, 120 Å pore size. A Navigator quadruple mass spectrometer system (Finnigan MAT, San Jose, CA, USA) was used which was fitted with an atmospheric pressure chemical ionization source (vaporizer temperature at 400 °C, capillary heater temperature 220 °C, corona current 5 μA, sheath gas – high purity of nitrogen – pressure 55 psi, and auxiliary gas (also nitrogen) flow are 15 mL/min. Positively charged ions with *m/z* 70–800 were scanned at a scan time of 0.5 s. The entire HPLC flow (0.45 mL/min) was introduced into the APCI source without splitting. The detection limit for 16:0 was 9 μM (signal-to-noise = 3) under the optimized conditions. Fatty acid methyl esters were separated using a gradient solvent program with acetonitrile (ACN), dichloromethane (DCM), and propionitrile (PrCN) as follows: initial ACN/PrCN/DCM (60:20:20, *v/v/v*); linear from 600 s to 960 s ACN/PrCN/DCM (50:30:20, *v/v/v*); held until 1560 s; linear from 1560 s to 2100 s ACN/PrCN/DCM 30:40:30, *v/v/v*); held until 2700 s; the composition was returned to the initial conditions over 480 s. 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.

2.2 Gas Chromatography-Mass Spectrometry

The total FAMEs were separated and identified using a Finnigan MAT 1020 B (Finnigan MAT, San Jose, California). A 60 m × 0.25 mm Supelcowax 10 column with a film thickness of 0.25 μm (Supelco, Gland, Switzerland) was used. Injection temperature was 100 °C. The temperature program was as follows: 100 °C for 1 min, then 100 °C to 230 C over 7 min, 230 °C to 280 °C over 25 min, and 280 °C for 10 min. The carrier gas was helium at a linear flow rate 70 cm/s. The spectra at 70 eV were scanned within *m/z* 60–700.

2.3 Materials and Methods

All PUFA, see **Table 1** were obtained from Sigma-Aldrich s.r.o., Prague, Czech Republic. The linseed oil was obtained from the local market. All HPLC grade solvents (acetonitrile,

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Table 1. Polyunsaturated fatty acid methyl esters from Sigma-Aldrich and Supelco.

7 Methyl ester of acid	No.	Short name	Composition by molar %	Purity (%)	Correction factor	Key fragment (m/z)
hexadecanoic	1	16:0[a, b)]	11.250	99	1.000	–
9-hexadecenoic	2	16:1w-7	3.125	99	1.002	–
octadecanoic	3	18:0[a, b)]	11.250	99	0.997	–
9-octadecenoic	4	18:1w-9[a)]	5.000	99	0.999	–
9,12-octadecadienoic	5	18:2w-6[a)]	5.000	99	1.003	150
6,9,12-octadecatrienoic	6	18:3w-6[a)]	5.000	99	1.009	150
9,12,15-octadecatrienoic	7	18:3w-3	3.125	99	1.009	108
6,9,12,15-octadecatetraenoic	8	18:4w-3	3.125	90	1.017	108
eicosanaoic	9	20:0[b)]	6.250	99	0.995	–
11-eicosenoic	10	20:1w-9	3.125	99	0.997	–
11,14-eicosadienoic	11	20:2w-6	3.125	98	1.001	150
5,8,11-eicosatrienoic	12	20:3w-9	3.125	98	1.007	192
8,11,14-eicosatrienoic	13	20:3w-6	3.125	99	1.007	150
11,14,17-eicosatrienoic	14	20:3w-3	3.125	97	1.007	108
8,11,14,17-eicosatetraenoic	15	20:4w-6	3.125	99	1.015	150
5,8,11,14,17-eicosapentaenoic	16	20:5w-3	3.125	99	1.025	108
docosanoic	17	22:0[b)]	6.250	99	0.992	–
13-docosenoic	18	22:1w-9	3.125	99	0.994	–
13,16-docosadienoic	19	22:2w-6	3.125	99	0.998	150
13,16,19-docosatrienoic	20	22:3w-3	3.125	98	1.004	108
7,10,13,16-docosatetraenoic	21	22:4w-6	3.125	99	1.012	150
7,10,13,16,19-docosapentaenoic	22	22:5w-3	3.125	97	1.023	108
4,7,10,13,16,19-docosahexaenoic	23	22:6w-3	3.125	99	1.035	108

[a)] Quantitative mixture GLC-10 and [b)]quantitative mixture GLC-40, both from Supelco, were combined.

propionitrile, and dichloromethane) were obtained also from Sigma. Fatty acid methyl esters were prepared as described previously [14].

Correction factors can be calculated as follows: A standard solution of compounds from Table 1 is prepared and produces chromatogram shown in **Figure 2**. The weights W injected are known. The areas A are measured as the absolute abundance of each ion in each mass spectrum which is summed, and that sum is regarded as a function of scan number (or retention time). The total ion current plot or reconstructed ion chromatogram is obtained, see Figure 2. The ratio A/W is calculated for each peak. The correction factor F is calculated by dividing the A/W of each peak by the value of A/W for methyl palmitate. These factors are relative to methyl palmitate, *i.e.*, the methyl palmitate factor is arbitrarily set equal to 1.00. Under the same detector conditions, these factors can be used time and time again to calculate the weight percent of other esters relative to methyl palmitate. From these results the weight of an unknown ester can be calculated:

$$W_b = \frac{W_a \cdot A_b}{F_b \cdot A_a}$$

where: W_b = weight of component **b**; W_a = weight of standard **a** (methyl palmitate); A_a = measured area of standard **a** (methyl palmitate); A_b = measured area of component **b**, F_b = correction factor of compound "**b**" relative to compound "**a**" at equal weights.

The response of a mass spectrometer is independent of temperature, mobile phase, and flow rate. This makes it well suited for quantitative analysis, where it is possibly the best detector available.

3 Results and Discussion

Commercially available methyl esters of common acids, *i.e.* those widespread in nature, are listed in Table 1. Two quantitative mixtures – GLC 10 and GLC 40 (Supelco) – were used as standards together with standards of single methyl esters (Sigma), see Section 2. Unfortunately, the range of commercially available PUFA is very limited, being restricted to PUFA with 18, 20, and 22 carbon atoms.

In atmospheric-pressure ionization mass spectrometry, fragmentation can easily be induced in one of the higher-pressure regions of the ion passage from the source into the mass analyzer. Acceleration of ions between nozzle (tube) and skimmer by ΔV(N–S) or between skimmer and RF-only multipole by ΔV(S–Q) results in collisions of ions with gas in which ions are entrained in their flow into the vacuum. Sometimes this process is called "in-source CID" (collision-induced dissociation) which is clearly a misnomer. Names of this process

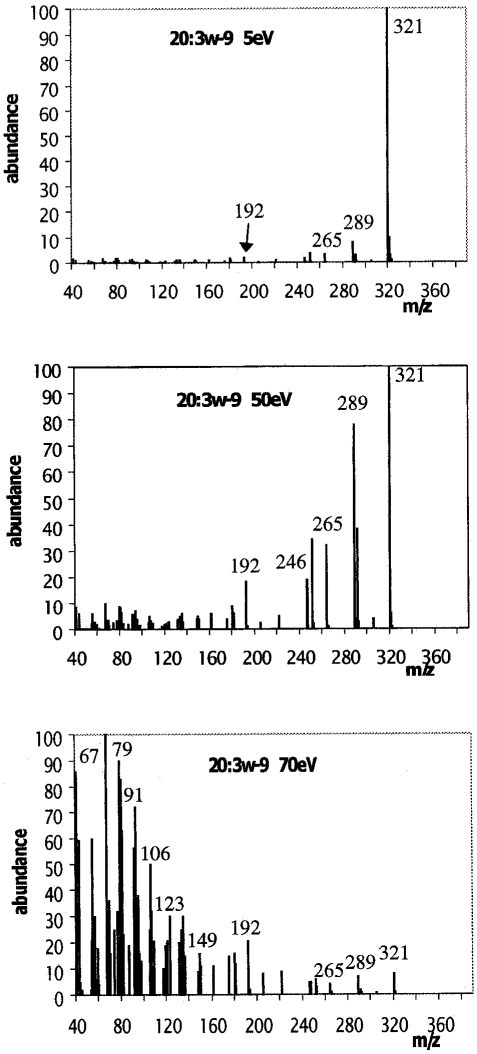

Figure 1. The CID mass spectrum of 20:3w-9 methyl esters at different cone voltages.

include: nozzle-skimmer fragmentation, cone-voltage fragmentation, high-orifice potential fragmentation, and octapole fragmentation, *etc.*

Fragment ions produced by collision-induced dissociation are very efficiently transported into the mass analyzer. The major advantage of this MS method is its simplicity: only one voltage has to be increased, no switching and adjustment of collision gas or retuning of ion optics is necessary. Of course, no

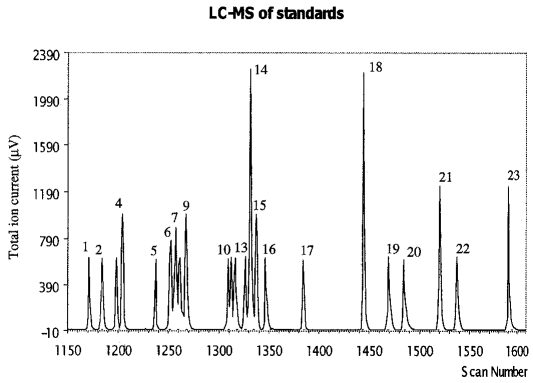

Figure 2. Total ion currents of fatty acid methyl esters (standards), numbering of peaks, see Table 1.

parent ion selection is possible. In LC-MS one can perform one LC run without CID and one with CID to obtain fragments of all components. An elegant application of up-front CID in LC-MS is provided by stepping the CID (orifice) voltage during the course of each analysis. A high voltage is used to generate and detect low-mass fragments, while in second analysis the voltage is reduced, in order to collect unfragmented parent ions.

In a well-designed API mass spectrometer, the abundance of a sample ion at *m/z* 200–400 decreases if ΔV(N-S) is reduced from 50 eV down to a few volts. Good test compounds for unwanted CID are, for example, our derivatives of fatty acids. At low collision energy, the positive-ion ES spectrum shows the $[M+H]^+$ ion at *m/z* 321 practically as the only one peak. It should be easy to adjust ΔV(N–S) and ΔV(S–Q) to obtained a spectrum free of fragment ions. Even at a moderate collision energy, where most sample ions would survive, the $[M+H]^+$ ion is converted into fragments, *i.e.* M–29 $(M-C_2H_5)^+$, M–31 $(M-CH_3O)^+$, M–56 $(M-C_4H_8)^{+\bullet}$, and M–69 $(M-C_5H_9)^+$, at *m/z* 292, *m/z* 289, *m/z* 265, and *m/z* 252, respective, with high peaks. The largest number of fragments is observed at the highest collision energy (70 eV), especially peaks in the low *m/z* region, *i.e.* at the *m/z* 67, 79, 93, 95, 108, 121, *etc.*, see also **Figure 1**.

The reconstructed ion chromatogram (RIC) of the separated FAME standards is shown in Figure 2. The single peaks were identified on the basis of their mass spectra. Although it is commonly accepted that APCI-MS gives very little fragmentation, it is possible to obtain very rich spectra by changing the cone voltage, see Figure 1. **Figure 3** shows mass spectra recorded at 50 eV of all three commercially available methyl esters of eicosatrienoic acid, see also Table 1. On the basis of the well known rule first published by Poulos [1]: "Methyl esters of a number of different fatty acids of the *n*–3 family

are reported to give a characteristic fragment at $m/z = 108$,

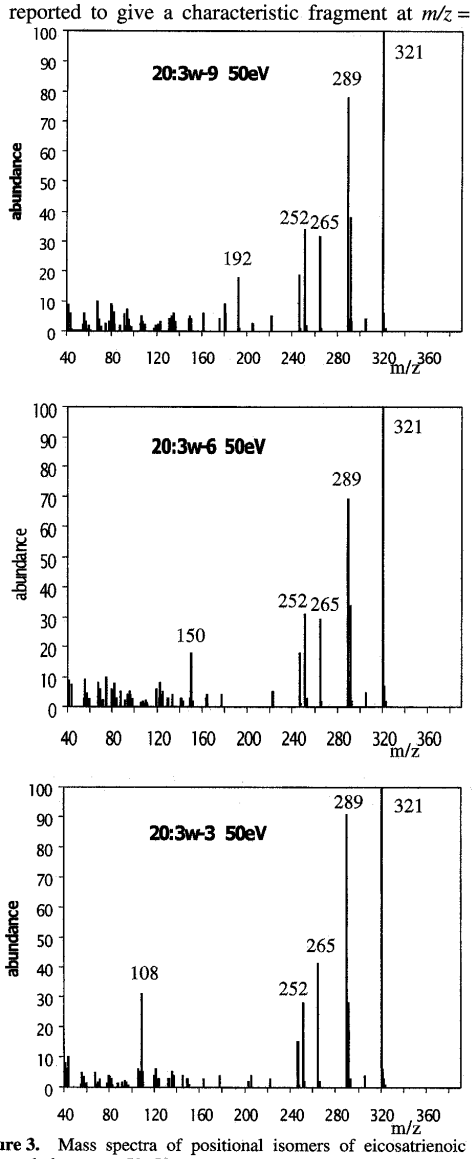

Figure 3. Mass spectra of positional isomers of eicosatrienoic fatty acid methyl esters at 50 eV.

while those of the $n-6$ series give a prominent ion at $m/z = 150$." Fatty acid methyl esters of the $n-9$ family would therefore be expected to have an abundant ion at $m/z = 192$. We are able to determine the positions of double bonds in methyl esters of PUFA from the abundance of mentioned

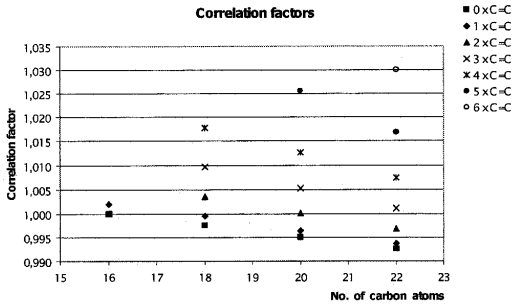

Figure 4. Correction factors for standards, their dependence on number on carbon atoms and double bonds.

ions in their mass spectra. The other standards were identified by the same method.

Determination of the quantitative abundance of methyl esters of PUFA is a far more important problem than their qualitative assay. Table 1 lists the amounts of methyl esters of standards (in molar ratios), their purity according to supplier, and also correction factor. However, it is clear from Table 1 that the abundance of single peaks can be easily used as the correction factor is close to 1. Two basic trends can be observed, see also **Figure 4**. First, together with increased molecular weight, or better with increased number of carbon atoms in the molecule of acid, the correction factor decreases from 1 (in 16:0 PUFA) to 0.992 (22:0). The second trend, clearly seen in unsaturated FAMEs is the increasing factor in the lane from 0 to 6 double bonds. It increased from 0.992 (22:0) up to 1.035 (22:6). In contrast, the position of double bonds does not affect the correction factor, which is the same in all isomers; see for example methyl ester of linoleic acid and methyl ester of γ-linoleic acid (1.009). The same situation is found in methyl esters of all three isomers of eicosatrienoic acids see Figure 3. Detailed consideration of the data in Table 1 as well as Figure 4, clearly shows that the single lines (saturated, mono-, di-, tri-, tetra-, and penta-unsaturated) do not create parallels, but they add to each other with increasing molecular weight.

The above-mentioned analysis does not differentiate between fatty acid methyl esters such as 5,9,12–18:3 and 6,9,12–18:3 (18:3w-6). The mass spectra do not indicate the position of the first double bond. The key fragments indicate only the fatty acid family (omega 9, 6, or 3), *i.e.* non-interrupted methylene. Only common fatty acids with methylene-interrupted double bonds may be identified correctly. Otherwise, derivatization is necessary as already mentioned in the introduction.

The comparison of correction factors of fatty acids in various papers on LC-MS is unfortunately impossible. However, this has been done several times for GC [15]. The results published for GC of fatty acids of methyl esters exhibit decreas-

Table 2. Difference in linseed oil measured by GC-MS and LC-MS.

Fatty acid	GC-MS mol%	LC-MS mol%
16:0	7.0 ± 0.9	7.0 ± 0.7
18:0	5.0 ± 0.4	5.0 ± 0.3
18:1w-9	18.0 ± 1.1	18.0 ± 0.6
18:2w-6	36.0 ± 1.4	36.0 ± 1.2
18:3w-3	34.0 ± 1.2	34.0 ± 1.1

Mean values ± standard deviation of 5 separate analyses.

ing correction factors with increasing length of the chain. In contrast, the correlation factor decreases with increasing number of unsaturated bonds (in our LC-MS method the factor increases). Based on all the data mentioned above we can state that LC-MS with APCI is a convenient method not only for qualitative, but also for quantitative assay of fatty acids as methyl esters. To confirm our theoretical result we used the LC-MS method with APCI for analysis of natural plant oil. The results of analysis of linseed oil are summarized in **Table 2**. We achieved excellent separation of single peaks including separation of stereoisomers of unsaturated acids with 18 C atoms. Table 2 also shows the quantitative comparison of two chromatographic methods. The measured values indicate that the differences between GC-MS and LC-MS are insignificant. The single methyl esters were usually baseline separated, with the exception of some peaks which were probably geometrical isomers. In conclusion, we can say that LC-MS with APCI gave very good results in the analysis of methyl esters of fatty acids and it is at least as suitable as GC-MS for both qualitative abundance of single fatty acids including their mass spectra and their quantitative abundance. Based on our measurements of single correction factors we can also define the quantitative abundance of fatty acid methyl esters.

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