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Application of Ethyl Esters and d_3-Methyl Esters as Internal Standards for the Gas Chromatographic Quantification of Transesterified Fatty Acid Methyl Esters in Food

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Ethyl esters (FAEE) and trideuterium-labeled methyl esters (d_3-FAME) of fatty acids were prepared and investigated regarding their suitability as internal standards (IS) for the determination of fatty acids as methyl esters (FAME). On CP-Sil 88, ethyl esters of odd-numbered fatty acids eluted ~0.5 min after the respective FAME, and only coelutions with minor FAME were observed. Depending on the problem, one or even many FAEE can be added as IS for the quantification of FAME by both GC-FID and GC-MS. By contrast, d_3-FAME coeluted with FAME on the polar GC column, and the use of the former as IS requires application of GC-MS. In the SIM mode, *m*/*z* 77 and 90 are suggested for d_3-methyl esters of saturated fatty acids, whereas *m*/*z* 88 and 101 are recommended for ethyl esters of saturated fatty acids. These *m*/*z* values give either no or very low response for FAME and can thus be used for the analysis of FAME in food by GC-MS in the SIM mode. Fatty acids in sunflower oil and mozzarella cheese were quantified using five saturated FAEE as IS. Gravimetric studies showed that the transesterification procedure could be carried out without of loss of fatty acids. GC-EI/MS full scan analysis was suitable for the quantitative determination of all unsaturated fatty acids in both food samples, whereas GC-EI/MS in the SIM mode was particularly valuable for quantifying minor fatty acids. The novel GC-EI/MS/SIM method using fatty acid ethyl esters as internal standards can be used to quantify individual fatty acids only, that is, without determination of all fatty acids (the common 100% method), although this is present. This was demonstrated by the exclusive quantification of selected fatty acids including methyl-branched fatty acids, erucic acid (18:1n-9*trans*), and polyunsaturated fatty acids in cod liver oil and goat's milk fat.

KEYWORDS: Fatty acid methyl esters; fatty acid ethyl esters; sunflower oil; mozzarella cheese; erucic acid; cod liver oil; goat's milk fat; GC-EI/MS-SIM

INTRODUCTION

Determination of fatty acids is a routine method in food chemistry and food control. Typically, the lipid phase of food, which mostly consists of triacylglycerides, is extracted with organic solvents. The fatty acid profile is then determined after transesterification into fatty acid methyl esters (FAME) followed by GC-FID determination (*1*). GC-FID is the method of choice for the determination of the relative contributions of individual fatty acids to the fatty acid pattern because of relative constant response factors irrespective of the chain length and number of double bonds. However, the sensitivity and selectivity of GC-FID are relatively low, and some applications require the use of GC-MS for confirmatory measurements. For quantitative determination of individual fatty acids in food samples, methyl esters of fatty acids not found at remarkable concentrations in food have been applied as internal standards (IS) (*2*). Typical examples for IS are methyl esters of 17:0 (17:0-ME) or other fatty acids with odd carbon numbers such as 7:0-ME, 9:0-ME, 13:0-ME, 19:0-ME, 21:0-ME, and 23:0-ME as well as 26:0-ME and 21:1-ME (*2*−*7*). However, even these rare fatty acids are often present in food samples. Furthermore, coelutions with other fatty acids can lead to inaccurate results. On the other hand, using several odd-chain fatty acids as IS for the determination of even-chain fatty acids proved to be superior to the use of a single IS (*2*). There are two strategies for the use of IS: (i) spiking a certain fatty acid into the lipid matrix before extraction (*5*, *8*) or (ii) addition of one or more esterified fatty acids after conversions of lipid fatty acids into FAME (*3*, *9*, *10*). In the latter case, the IS must not necessarily be a FAME.

In the present study we have explored under which conditions fatty acid ethyl esters (FAEE) or d_3-methyl esters of fatty acids (d_3-FAME) can be useful IS for the determination of FAME. Both GC-FID and GC-MS in the full scan mode require that the IS do not elute at the retention time of relevant FAME in food or other samples. Recently, we have presented a GC-EI/

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MS method in the selected ion monitoring (SIM) mode that enables the determination of food-relevant fatty acids as methyl esters (*11*). This novel GC-EI/MS/SIM method is based on four low mass fragment ions (*m*/*z* 74, 79, 81, and 87) and combines high sensitivity with high selectivity (*11*). We thus tested whether the respective SIM masses enable the use of internal standards even if the IS would coelute with the native FAME derivatives as derived from food samples. The resulting method was used for the quantification of fatty acids in food samples.

MATERIALS AND METHODS

Chemicals and Samples. Cyclohexane (purest, VWR, Darmstadt, Germany) and ethyl acetate (purest, Acros Organics, Geel, Belgium) were combined (1:1, v/v) and distilled to obtain the azeotropic mixture (54:46, v/v). *n*-Hexane (HPLC gradient grade) and methanol (HPLC gradient grade) were from Fluka (Taufkirchen, Germany). Isooctane (analytical reagent grade) was from Fisher Scientific (Ulm, Germany), and isolute-HM-N was from Separtis (Grenzlach-Wyhlen, Germany). Boron trifluoride−methanol complex solution (13−15% BF_3 in methanol) was from Riedel-de-Haën (Taufkirchen, Germany). BF_3 ethyl etherate (purum, dist.) and ethanolic BF_3 (∼10%, ∼1.3 M, purris) were from Fluka.

A Supelco 37 component FAME mix (37c-FAME mix, Sigma-Aldrich, Taufkirchen, Germany) as well as additional standards of free fatty acids and FAME (Larodan, Malmö, Sweden) were used. Addition retention times were derived from fatty acids in the following food samples: sunflower oil (Heess, Stuttgart, Germany), goat's milk (Andechser Creamery, Andechs, Germany), buffalo mozzarella cheese (Padania Alimenti, Casalmaggiore, Italy), and cod liver oil (Rügen Fisch, Sassnitz, Germany).

Sample Preparation. Food samples except oils were lyophilized prior to extraction. Lipids were gained by accelerated solvent extraction (ASE, Dionex, Idstein, Germany) with ethyl acetate/cyclohexane (54:46, v/v, see above) as the solvent (*11*, *12*). After removal of the solvent, the lipid phase (namely, the fatty acid glycerides) was transesterified. For this purpose 2−10 mg of fat or oil and 0.5 mL of alcoholic KOH (0.5 M) were heated for 5 min at 80 °C. After cooling, 1 mL of BF_3 solution (see below) was added and heated for an additional 5 min at 80 °C. Two milliliters of saturated sodium chloride solution and 2 mL of *n*-hexane were added to the cooled solution (ice bath). The esters were extracted and analyzed by GC-MS in full scan and SIM mode (*11*). For preparation of FAME we used 0.5 mL of methanolic KOH and 1 mL of methanolic BF_3, for d_3-FAME we used KOH in d_4-methanol and BF_3 ethyl etherate, and for FAEE we used ethanolic KOH and ethanolic BF_3. Esters from standards of free fatty acids were prepared by treatment with BF_3 (in diethyl ether) and either ethanol or the NMR solvent d_4-methanol (CD_3OD).

Gas Chromatography Coupled to Electron Ionization Mass Spectrometry (GC-EI/MS). Analyses were performed with a Hewlett-Packard 5890 series II gas chromatograph interfaced to a 5971A mass selective detector. One microliter of sample dissolved in *n*-hexane was injected with a 7673A autosampler (splitless mode, split opened after 2 min). The injector and transfer line temperatures were kept at 250 and 280 °C. The temperature of the ion source was 165 °C. Helium (purity 5.0) was used as the carrier gas at a constant flow rate of 1 mL/min. A 50 m × 0.25 mm i.d., 0.20 μm d_f fused-silica capillary column coated with CP-Sil 88 (Chrompack, Middelburg, The Netherlands) was installed in the GC oven. The GC oven program was the following: after 5 min at 45 °C, the oven was heated at 7 °C/min to 180 °C, at 3 °C/min to 200 °C (hold time 1 min), and finally at 3 °C/min to 220 °C (hold time of 10 min). The total run time was 51.62 min. In the full scan mode *m*/*z* 50−450 were recorded after a solvent delay of 8 min. In the SIM mode, the six to eight fragment ions were determined including *m*/*z* 74 and 87 for FAME, *m*/*z* 88 and 101 for FAEE, and *m*/*z* 77 and 90 for d_3-FAME as well as *m*/*z* 81 and 79 for all esters of PUFA.

Quantification of Fatty Acids. Free fatty acids of 12:0, 14:0, 16:0, 17:0, and 18:0 were accurately weighed (7−15 mg) in triplicates and ethylated as shown above. The yields of the FAEE were 96.6 ± 2.0. Between 21.6 and 27.3 μg/mL of the five FAEE were 1:1 (v/v) combined with a 1:10 diluted 37c-FAME mix solution to determine relative response factors of FAME and FAEE (see Results and Discussion). A similar mix was prepared from methyl-branched fatty acids for the determination of their responses.

For the quantification of fatty acids, 50−100 μL of transesterified oil or fat and 20 μL of the IS mix were added in a calibrated vial and filled to 500 μL with *n*-hexane. In the GC-EI/MS scan mode, individual areas of the selected ion traces of FAME were determined and corrected by the known concentration and response factors of the five IS (see above). FAME without respective FAEE as IS were quantified using the response of 16:0-EE. In the GC-EI/MS SIM mode, methyl esters of saturated fatty acids were determined with *m*/*z* 87, those of monoenoic fatty acids with *m*/*z* 74, and those of PUFA with the sum of *m*/*z* 79 and 81 (*11*). The FAEE used as IS were determined with *m*/*z* 101. Ratios of FAME (*m*/*z* 88) to FAEE (*m*/*z* 101), $n = 4$, were 0.64 ± 0.03 for 12:0, 0.67 ± 0.03 for 14:0, 0.70 ± 0.03 for 16:0, 0.72 ± 0.03 for 17:0, and 0.75 ± 0.03 for 18:0. All further fatty acids in the 37c-FAME mix were determined using the response of the quantification ion relative to 16:0-ME, whereas other fatty acids were determined with the average response factor of the class of fatty acid (saturated, monoenoic, or polyenoic fatty acid).

RESULTS AND DISCUSSION

Gas Chromatographic Feature of Methyl, Ethyl, and d_3-Methyl Esters of Fatty Acids. The alternative esters could be successfully prepared as was found for FAME (see also below). Moreover, the proposed reaction scheme for derivatization (see Materials and Methods) resulted in virtually identical GC peak patterns for all three classes of esters.

Use of GC-MS clarified that none of the investigated food samples (goat's milk, sunflower oil, mozzarella cheese, fish oil, and suet) contained FAEE or d_3-FAME after conversion of the lipid fraction into FAME; likewise, the alternatively transesterified samples were free of FAME.

As anticipated, FAEE eluted after the respective FAME from the GC column. The difference in the (netto) retention times ($\Delta t'_R$) was ∼0.5 min for early eluting fatty acids (**Table 1**). Owing to the longer retention times, the separation factors of the corresponding alkyl esters subsequently decreased with increasing chain length (**Table 1**). FAEE of saturated fatty acids abundant in food (16:0 and 18:0) eluted about one-third between the respective FAME and the FAME with one more carbon (**Figure 1**). In this time window, only low abundant monoenoic FAME isomers were observed. However, no coelution of any saturated FAEE with relevant FAME was observed (**Table 1**). In the case of particular interests in the determination of certain FAME, suitable FAEE can be selected accordingly.

Deuterium-labeled FAME were prepared using the readily available NMR solvent d_4-methanol. Three of the heavy hydrogen isotopes are found on the resulting esters, whereas the deuterium originally attached to the oxygen was lost due to the reaction scheme. Smith and Schewe used a similar technique for the preparation of five individual d_3-FAME (*13*). FAME and d_3-FAME were only partly resolved, with the d_3-FAME eluting slightly prior to the native FAME ($\Delta t_R \sim 2$ s, **Table 1**) due to the higher volatility of the labeled compounds (*14*). GC separation of native and deuterium-labeled compounds is mainly due to the slightly higher vapor pressures of the latter, and this effect is most pronounced on nonpolar stationary GC phases (*15*). Thus, peak resolution of unlabeled and d_3-labeled FAME on the polar CP-Sil 88 column was very unlikely and, indeed, was not observed at any condition applied. When we changed to a nonpolar CP-Sil 8 (equivalent to DB-5) column, a partial resolution of unlabeled and labeled was obtained (see Supporting Information). Note that the full resolution of d_3-labeled and

Table 1. Gas Chromatographic Retention Times (in Rising Order) of the Methyl, d_3-Methyl, and Ethyl Esters of 29 Fatty Acids Determined on CP-Sil 88[a]

fatty acid	FAME (t'_r)[b]	d_3-FAME (t'_r)	FAEE (t'_r)	α[c]
4:0	6.29 ± 0.01	6.25 ± 0.01	7.05 ± 0.01	1.080
6:0	10.75 ± 0.00	10.73 ± 0.00	11.24 ± 0.01	1.036
8:0	14.03 ± 0.00	14.01 ± 0.00	14.67 ± 0.00	1.037
10:0	17.01 ± 0.01	17.00 ± 0.02	17.53 ± 0.00	1.026
11:0	18.47 ± 0.01	18.46 ± 0.01	18.92 ± 0.00	1.021
12:0	19.63 ± 0.01	19.61 ± 0.02	20.08 ± 0.00	1.020
13:0	20.89 ± 0.01	20.87 ± 0.02	21.29 ± 0.01	1.017
i14:0[d]	21.44 ± 0.01	21.42 ± 0.02	21.85 ± 0.00	1.017
14:0	21.98 ± 0.01	21.96 ± 0.01	22.41 ± 0.00	1.017
i15:0	22.61 ± 0.01	22.59 ± 0.01	23.04 ± 0.01	1.017
a15:0[d]	22.84 ± 0.00	22.83 ± 0.01	23.28 ± 0.01	1.017
14:1n-5	22.91 ± 0.00	22.90 ± 0.01	23.32 ± 0.01	1.016
15:0	23.20 ± 0.01	23.18 ± 0.00	23.64 ± 0.01	1.017
i16:0	23.83 ± 0.00	23.82 ± 0.01	24.29 ± 0.00	1.017
16:0	24.47 ± 0.00	24.44 ± 0.01	24.96 ± 0.01	1.017
i17:0	25.15 ± 0.00	25.13 ± 0.00	25.60 ± 0.00	1.016
16:1n-7	25.30 ± 0.00	25.28 ± 0.01	25.72 ± 0.00	1.015
a17:0	25.41 ± 0.00	25.39 ± 0.01	25.86 ± 0.00	1.016
17:0	25.79 ± 0.00	25.77 ± 0.00	26.27 ± 0.01	1.016
18:0	27.17 ± 0.00	27.15 ± 0.03	27.70 ± 0.00	1.017
18:1n-9*trans*	27.78 ± 0.01	27.78 ± 0.01	28.28 ± 0.01	1.016
18:1n-9	27.97 ± 0.00	27.93 ± 0.01	28.44 ± 0.00	1.015
18:1n-7	28.07 ± 0.00	28.04 ± 0.00	28.56 ± 0.01	1.016
19:0	28.98 ± 0.00	28.95 ± 0.00	29.41 ± 0.00	1.013
18:2n–6	29.28 ± 0.00	29.24 ± 0.01	29.69 ± 0.01	1.013
18:3n-3	30.90 ± 0.01	30.85 ± 0.01	31.28 ± 0.01	1.013
20:2n-6	32.54 ± 0.01	32.54 ± 0.01	33.01 ± 0.00	1.014
20:5n-3	36.53 ± 0.00	36.48 ± 0.00	36.77 ± 0.01	1.006
22:6n-3	41.29 ± 0.00	41.22 ± 0.00	41.48 ± 0.00	1.004

[a] Retention times were established from standard compounds as well as fatty acids in food samples (suet, cod liver oil, sunflower oil, and goat's milk fat). [b] Netto retention time (dead time = 3.17 min, based on *n*-hexane); mean value and standard deviation ($n = 3$). [c] Separation factor of FAEE to FAME. [d] Letters i and a indicate iso-fatty acids (fatty acids with a methyl branch on the second carbon counted from the tail) and anteiso fatty acids (fatty acids with a methyl branch on the third carbon counted from the tail).

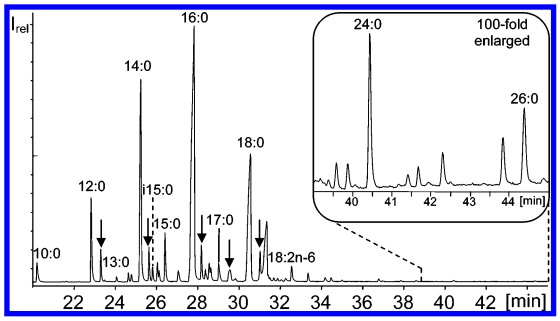

Figure 1. GC-MS/SIM determination (CP-Sil 88 column) of the fatty acid pattern of buffalo mozzarella cheese derived after transesterification into methyl esters with five ethyl esters added as internal standards. Arrows identify added FAEE of 12:0, 14:0, 16:0, 17:0, and 18:0. (Inset) Enlarged part of the late eluting fatty acids

native FAME would have required about five hydrogens substituted with deuterium. Whereas d_3-FAME have no advantage over native FAME when GC-FID is used for the determination, they might be suitable for GC-MS determination in the case of different mass fragment ions.

Mass Spectrometric Feature of Methyl, Ethyl, and d_3-Methyl Esters of Fatty Acids. The pattern, that is, the relative intensity of key fragment ions of FAME, d_3-FAME, and FAEE, was virtually identical for all saturated fatty acids (see Supporting Information for an example). The most abundant fragment ions in the mass spectra of saturated FAME, that is,

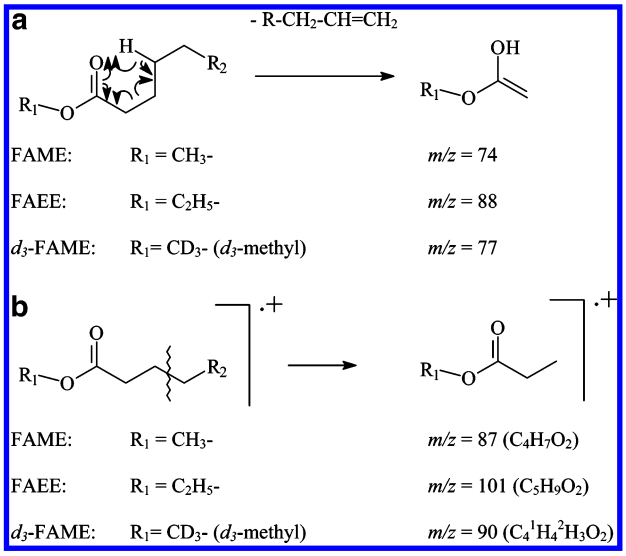

Figure 2. Mechanism of the formation of (**a**) the McLafferty ion and (**b**) *m/z* 87 as well as the respective masses for the three ester derivatives

the McLafferty ion at *m/z* 74 and the fragment ion at *m/z* 87, also dominated in the mass spectra of 17:0-EE and 17:0-d_3ME, but they were shifted Δ14u or Δ3u toward higher mass. The proposed mechanism (**Figure 2**) illustrates that both fragment ions contain the ester group, which explains the different masses observed. For instance, the McLafferty ion of FAME (*m/z* 74) is *m/z* 77 for d_3-FAME and *m/z* 88 for FAEE (**Figure 2a**). Likewise, *m/z* 90 (d_3-FAME) and *m/z* 101 (FAEE) correspond to *m/z* 87 in FAME (**Figure 2b**). The same mass shifts were also observed for the respective molecular ions. Likewise, the fragment ions at *m/z* 241 and 143 in the GC-EI/MS of 17:0-ME were shifted in the same way, but *m/z* 253 was present in the mass spectra of all alkyl esters of 17:0 (see Supporting Information). These examples illustrate that the diverse esters may be used for a simple classification of fragment ions in the mass spectra of FAME. Those fragment ions that still bear the ester group will have different *m/z* values in the case of FAME, d_3-FAME, and FAEE, whereas fragment ions formed after elimination of the ester group will have identical masses. Thus, the fragment ion at *m/z* 253 ([284 u − 31 u]$^-$) in 17:0-ME must arise from an α-cleavage next to the carbonyl group ([M − OR]$^+$), which corresponds to [287 u − 34 u] and [298 u − 45 u] in the cases of d_3-FAME and FAEE. Owing to the different molecular masses, this fragment ion will vary with the carbon chain length. However, [M − OR]$^+$ along with *m/z* 55 ([C_4H_7]$^+$), 97, and 111 were the only important fragment ions with identical *m/z* values in the GC-EI/MS spectra of the three classes of alkyl esters.

The situation was similar for monoenoic fatty acids but completely different for PUFA. Most fragment ions from esters of PUFAs are formed after elimination of the (uncharged) headgroup, so that the respective ions are found in the mass spectra of all three classes of esters of PUFAs. Thus, the mass spectra of the three alkyl esters of EPA (20:5n-3) and other PUFA looked virtually identical in the mass range below *m/z* 150 (**Figure 3**). In other words, none of these fragment ions contained the ester group. This is in agreement with the observation that the major ions in saturated and monoenoic fatty acids (*m/z* 74 and 87 in the case of FAME) do not play any role in the mass spectra of PUFA (*11*). Because the molecular ion was barely detectable in the case of the simple esters of PUFA, GC-MS was not well-suited for the identification of different simple esters of PUFAs.

Table 2. Amounts of Fatty Acids in Sunflower Oil ($n = 3$) and Mozzarella Cheese ($n = 3$)

		sunflower oil ($n = 3$)		buffalo mozzarella ($n = 3$)	
FAME	response[a] ($n = 4$)	SIM amount (g/100 g)	scan amount (g/100 g)	SIM amount (g/100 g)	scan amount (g/100 g)
4:0	0.94 ± 0.08	nd	nd	0.21 ± 0.02	0.26 ± 0.04
6:0	0.94 ± 0.08	nd	nd	0.33 ± 0.02	0.30 ± 0.03
8:0	0.94 ± 0.08	nd	nd	0.26 ± 0.03	0.24 ± 0.05
10:0	0.94 ± 0.08	0.003 ± 0.0027[c]	nd	0.71 ± 0.06	0.69 ± 0.01
11:0	0.89 ± 0.00	nd	nd	0.03 ± 0.00	nd
12:0[d]	0.98 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	2.95 ± 0.22	2.65 ± 0.03
i13:0[e]	0.75 ± 0.02	nd	nd	0.05 ± 0.00	nd
13:1	3.43 ± 0.05	nd	nd	0.07 ± 0.01	nd
a13:0[e]	0.75 ± 0.02	nd	nd	0.03 ± 0.00	nd
13:0	0.84 ± 0.01	nd	nd	0.15 ± 0.01	0.12 ± 0.00
i14:0	0.75 ± 0.02	nd	nd	0.22 ± 0.02	0.22 ± 0.00
14:0[d]	0.92 ± 0.00	0.14 ± 0.00	0.10 ± 0.01	9.43 ± 0.43	9.88 ± 0.19
i15:0	0.75 ± 0.02	nd	nd	0.40 ± 0.06	0.51 ± 0.01
a15:0	0.75 ± 0.02	nd	nd	0.51 ± 0.04	0.65 ± 0.02
14:1n-5	3.43 ± 0.05	nd	nd	1.01 ± 0.03	0.96 ± 0.01
15:0	0.82 ± 0.01	0.02 ± 0.00	nd	1.56 ± 0.12	1.58 ± 0.02
i16:0	0.75 ± 0.02	nd	nd	0.49 ± 0.05	0.52 ± 0.00
16:0[d]	0.70 ± 0.04	8.21 ± 0.07	8.29 ± 0.05	23.6 ± 0.31	24.9 ± 0.12
16:1[f]	3.80 ± 0.08	nd	nd	0.24 ± 0.08	0.11 ± 0.02
i17:0	0.75 ± 0.02	nd	nd	0.34 ± 0.03	0.38 ± 0.01
16:1n-9	3.80 ± 0.08	0.06 ± 0.00	nd	0.54 ± 0.13	0.41 ± 0.04
16:1n-7	3.80 ± 0.08	0.13 ± 0.00	0.08 ± 0.01	1.99 ± 0.09	2.10 ± 0.03
a17:0	0.75 ± 0.02	nd	nd	0.52 ± 0.04	0.75 ± 0.02
17:0[d]	1.09 ± 0.01	0.05 ± 0.00	nd	0.99 ± 0.13	0.94 ± 0.08
17:1n-7	3.80 ± 0.09	nd	nd	0.39 ± 0.07	0.38 ± 0.01
i18:0	0.75 ± 0.02	nd	nd	0.05 ± 0.02	0.03 ± 0.00
18:0[d]	0.90 ± 0.00	5.69 ± 0.05	5.13 ± 0.02	11.7 ± 0.65	12.1 ± 0.24
18:1n-9*trans*	3.84 ± 0.04	0.32 ± 0.02	nd	1.04 ± 0.14	21.3 ± 0.38[g]
18:1n-9	4.39 ± 0.07	28.7 ± 0.25	26.7 ± 0.16	18.2 ± 0.69	
18:1n-7	4.39 ± 0.07	1.04 ± 0.09	0.72 ± 0.02	0.81 ± 0.10	0.62 ± 0.02
18:1[f]	4.39 ± 0.07	nd	nd	0.58 ± 0.17	0.45 ± 0.02
18:1[f]	4.39 ± 0.07	nd	nd	0.68 ± 0.09	0.47 ± 0.03
19:0	0.92 ± 0.02	nd	nd	0.11 ± 0.08	0.12 ± 0.06
18:2n-6	0.06 ± 0.00	53.9 ± 0.24	57.3 ± 0.90	1.82 ± 0.03	2.21 ± 0.14
20:0	0.94 ± 0.02	0.40 ± 0.01	0.31 ± 0.01	0.35 ± 0.05	0.33 ± 0.03
18:3n-3	0.05 ± 0.00	nd	nd	0.36 ± 0.05	0.44 ± 0.03
20:1n-9	4.04 ± 0.04	nd	nd	0.12 ± 0.04	0.11 ± 0.08
21:0	0.86 ± 0.00	0.01 ± 0.00	nd	0.08 ± 0.03	0.08 ± 0.01
22:0	0.92 ± 0.02	0.93 ± 0.01	0.78 ± 0.00	0.13 ± 0.02	0.13 ± 0.01
20:3n-6	0.05 ± 0.00	nd	nd	0.07 ± 0.01	0.09 ± 0.01
23:0	0.91 ± 0.00	0.03 ± 0.00	nd	0.08 ± 0.04	0.06 ± 0.00
24:0	0.97 ± 0.00	0.28 ± 0.00	0.23 ± 0.01	0.08 ± 0.02	0.07 ± 0.01
26:0	0.97 ± 0.00	nd	nd	0.03 ± 0.01	0.05 ± 0.01
sum		**99.8**	**99.7**	**83.2**	**87.1**

[a] Response factor relative to 16:0-EE; multiplication factor of the area of the quantification ion compared to 16:0-EE equal amounts. [b] Not detected (detection limit, <0.003 g/100 g based on S/N < 10). [c] Mean value and standard deviation. [d] Calculated with the response of the respective FAEE. All other fatty acids were determined with the relative response of 16:0-EE. [e] Letters i and a indicate iso-fatty acids (fatty acids with a methyl branch on the second carbon counted from the tail) and anteiso fatty acids (fatty acids with a methyl branch on the third carbon counted from the tail). [f] Position of double bond not determined, owing to the elution order <n-9. [g] Coelution of 18:1n-9*trans* and 18:1n-9*cis*.

SIM Masses of Simple Esters of Fatty Acids. Our recent GC-MS/SIM method is based on the determination of *m*/*z* 87 and 74 for saturated and monoenoic FAME (both fragment ions contain the ester group), whereas di- to hexaenoic acids were determined by the sum of *m*/*z* 79 and 81, which arise from $C_6H_7^{\bullet+}$ and $C_6H_9^{\bullet+}$ radical cations (*11*). The two fragment ions suggested for PUFA were thus identical for all three classes of esters (see **Figure 3**). Due to the coelution of FAME and d_3-FAME, d_3-FAME of naturally occurring PUFA are not suitable IS for the SIM determination of FAME, whereas FAEE may be used in the case of noninterfered elution of FAME using *m*/*z* 79 and 81 (see below). By contrast, saturated FAEE and d_3-FAME appeared to be well-suited IS for the determination of FAME, particularly when GC-MS is applied. FAME showed virtually no response for the suggested SIM masses *m*/*z* 90 (<0.02% of *m*/*z* 87) and very little response for *m*/*z* 77 (~0.25% of *m*/*z* 74) of d_3-FAME. Moreover, *m*/*z* 74 and 87 were not present in the mass spectra of saturated d_3-FAME.

FAEE did not produce any significant amount of *m*/*z* 74 and 87 so that FAEE could even be used in case of coelutions when GC-EI/MS is applied in the SIM mode. This was verified by the analysis of mixes of FAME and FAEE (data not shown). Vice versa, a small response of the SIM masses for saturated FAEE, *m*/*z* 88 and 101, was observed in the mass spectra of FAME. Because no coelution of FAEE with saturated or monoenoic FAME was observed, this did not play a role in CP-Sil 88. However, partial coelution of 18:0-EE and 18:1n-9-ME was observed on the SP-2331 column recently used (*11*). Therefore, this issue should be checked when other GC capillary columns are applied. In our case, no interference was observed and the ethyl ester of any saturated or monoenoic fatty acid can be used as IS for the GC-MS/SIM determination of FAME.

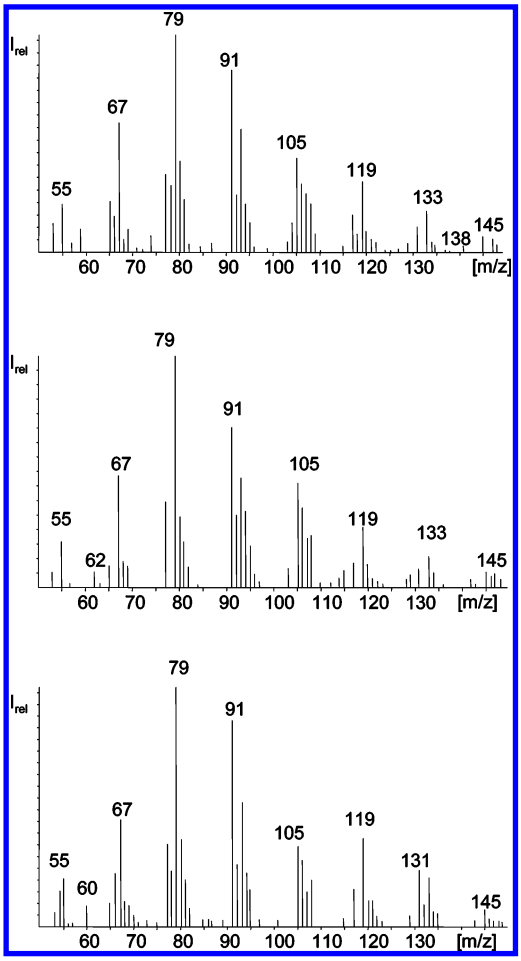

Figure 3. GC-EI/MS mass spectra (excerpt *m*/*z* 50–150) of the (top) methyl ester, (center) d_3-methyl ester, and (bottom) ethyl ester of eicosapentaenoic acid (EPA, 20:5n-3).

Quantification of Fatty Acids in Food Samples as FAME Using a Series of FAEE as IS. The method of quantification of FAME by GC-MS using five FAEE as IS was carried out with sunflower oil and mozzarella cheese (**Figure 1**). Because the IS can be added only after conversion of the fatty acids into FAME, we had to verify that the transesterification procedure could be carried out quantitatively. For this purpose, the extracted lipids were accurately weighed and transesterified. Sunflower oil contains fatty acids as triacylglycerides, and the sample weight of the transesterified FAME differs by only 4 Da from that of triacylglycerides because glycerol is not found in the FAME fraction. Transferred to three fatty acids with a mean chain length of 16 carbons, the error would be ~0.5%, and this was considered to be negligible, so no molar correction was performed. The recovery rate of the transesterified and purified sunflower oil was gravimetrically determined to be 97.4 ± 2.5 ($n = 6$). Therefore, the transesterification procedure could be carried out without significant loss of fatty acids. This was confirmed by the conversion of free fatty acids into FAEE (see Materials and Methods).

The methylated fatty acids of sunflower oil were combined with five internal FAEE standards and determined by GC-MS in the scan and SIM modes (**Table 2**). In the scan mode, 11 fatty acids were detected ranging from 0.08 to 57 g/100 g of lipids. More than 99% of the sample weight could be quantified in any of the determinations (**Table 2**). This is in very good agreement with the gravimetric determination shown above. Because of the higher sensitivity, GC-EI/MS/SIM allowed 18 fatty acids to be quantified in the range of 0.003−54 g per 100 g of lipids in the sunflower oil (**Table 2**). Furthermore, these 18 FAME include 4 of the 8 FAME currently used as IS (see Introduction). However, some variations occurred for unsaturated fatty acids between the SIM and scan modes. The higher value for 18:1n-9 and the lower value for 18:2n-6 in the SIM mode (**Table 2**) are in agreement with previous reports (*11*). Although still in an acceptable range, we recommend that quantification of unsaturated fatty acids in the SIM mode should be carried out with unsaturated FAEE as IS. On the other hand, the good precision in repetitive analysis of minor fatty acids such as 15:0 which was not detected in the scan mode makes the SIM method particularly valuable for low abundant fatty acids in food. Whereas branched-chain fatty acids were not present in the sunflower oil, nine branched-chain fatty acids could be quantified in buffalo mozzarella (**Table 2**). The lower amount quantified (~85%) must be due to the presence of phospholipids, which contain a lower amount of fatty acids and nonfatty acid lipid components. Thus, the yield after transesterification was only 91.0 ± 3.1% ($n = 4$) of the sample weight after extraction. The results of scan and SIM analyses agreed well except for the coelution of oleic and elaidic acids in the scan mode, which was the reason for the difference in the fatty acid composition (**Table 2**). However, the better resolution in the SIM mode allowed for the correct determination of this *trans*-fatty acid. Because our method is not based on the conventional 100% method, it is not necessary to analyze all fatty acids to determine the concentration of specific fatty acids in a sample. For instance, the concentration of ~1 g of elaidic acid per 100 g of lipids of mozzarella cheese (**Table 2**) can directly be determined without determination of the 40 additional fatty acids found in this mozzarella sample. This technique was applied to cod liver oil and goat's milk fat (**Table 3**). Note that only the fatty acids shown in **Table 3** have been quantified. No determination of the relative contribution to all fatty acids (100% method) has been carried out, which is currently the standard technique of FAME determination by GC-FID. As can be seen from the low standard deviations, the method provides good reproducibility even for complex lipids. Only for low abundant fatty acids were the variations between the replicates slightly higher but still acceptable. Hence, the method allows the determination of not only minor fatty acids such as odd-chain fatty acids, methyl-branched fatty acids, and *trans*-fatty acids but also major fatty acids such as palmitic acid or eicosapentaenoic acid (**Table 3**).

Table 3. Quantitative Determination of Selected Fatty Acids in Cod Liver Oil and Goat's Milk Fat

FAME	cod liver oil ($n = 3$) (g/100 g)	goat's milk fat ($n = 3$) (g/100 g)
i15:0	0.34 ± 0.04	0.21 ± 0.01
a15:0	0.05 ± 0.01	0.28 ± 0.03
14:1n-5	0.10 ± 0.01	0.28 ± 0.00
15:0	0.56 ± 0.06	0.95 ± 0.01
i16:0	0.10 ± 0.08	0.14 ± 0.01
16:0	12.3 ± 0.26	20.4 ± 0.28
16:1n-9	0.87 ± 0.02	0.65 ± 0.08
16:1n-7	4.93 ± 0.31	0.73 ± 0.08
17:0	0.53 ± 0.02	0.87 ± 0.03
18:1n-9*trans*	0.15 ± 0.01	2.03 ± 0.13
18:1n-9	18.3 ± 0.63	13.6 ± 0.05
18:1n-7	4.29 ± 0.21	0.49 ± 0.06
18:2n-6	3.43 ± 0.09	1.27 ± 0.04
20:5n-3	12.4 ± 0.24	nd[a]
22:6n-3	17.5 ± 0.72	nd

[a] nd, not detected (limit of detection 0.003 g/100 g of lipids).

Although not exclusively shown, d_3-FAME will also be suitable IS for quantification purposes so that both classes of alternative esters are real substitutes for saturated odd-numbered FAME, which are currently most widely used as IS. When GC-MS is used, an almost unlimited number of IS (FAEE and d_3-FAME) can be applied for the determination of FAME. The alternative esters (d_3-FAME and FAEE) are readily prepared from standard chemicals so that the standards can be produced at low costs in any laboratory. Note, however, that our concept is applicable only when the internal standard is added after transesterification of food lipids (*6*, *9*, *10*). Nevertheless, we are convinced that the proposed method will be helpful for both quality assessment and quantitative determination of FAME as was illustrated in this study.

Supporting Information Available: Separation of FAME and d_3-FAME on a Factor Four CP-Sil8MS column and GC-EI/MS full scan spectra of alkyl esters of 17:0. This material is available free of charge via the Internet at http://pubs.acs.org.

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