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Gas Chromatography/Electron-Capture Negative Ion Mass Spectrometry for the Quantitative Determination of 2- and 3-Hydroxy Fatty Acids in Bovine Milk Fat

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2- and 3-hydroxy fatty acids (2- and 3-OH-FAs) are bioactive substances reported in sphingolipids and bacteria. Little is known of their occurrence in food. For this reason, a method suitable for the determination of OH-FAs at trace levels in bovine milk fat was developed. OH-FAs (and conventional fatty acids in samples) were converted into methyl esters and the hydroxyl group was derivatized with pentafluorobenzoyl (PFBO) chloride to give PFBO-*O*-FA methyl esters. These derivatives with strong electron affinity were determined by gas chromatography interfaced to mass spectrometry using electron-capture negative ion in the selected ion monitoring mode (GC/ECNI-MS-SIM). This method proved to be highly sensitive and selective for PFBO-*O*-FA methyl esters. For the analysis of samples, two internal standards were used. For this purpose, 9,10-dideutero-2-OH-18:0 methyl ester (ISTD-1) from 2-OH-18:1(9*c*) methyl ester as well as the ethyl ester of 3-PFBO-*O*-12:0 (ISTD-2) was synthesized. ISTD-1 served as a recovery standard whereas ISTD-2 was used for GC/MS measurements. The whole-sample cleanup consisted of accelerated solvent extraction of dry bovine milk, addition of ISTD 1, saponification, conversion of fatty acids into methyl esters by use of boron trifluoride, separation of the methyl esters of OH-FAs from nonsubstituted FAs on activated silica, conversion of OH-FAs methyl esters into PFBO-*O*-FA methyl esters, addition of ISTD-2, and measurement by GC/ECNI-MS-SIM. By this method, ten OH-FAs were quantified in bovine milk fat with high precision in the range from 0.02 ± 0.00 to 4.49 ± 0.29 mg/100 g of milk fat.

KEYWORDS: α-Hydroxy fatty acids; β-hydroxy fatty acids; milk fat; quantification; GC/ECNI-MS-SIM

INTRODUCTION

Fatty acids are principal constituents of food and biological samples. They consist of a carboxyl group and usually a long hydrophobic alkyl chain with 4−26 carbons. In addition to these standard fatty acids, several substituted fatty acids, such as 2- and 3- (or α- and β-) hydroxy fatty acids (2- and 3-OH-FAs), are naturally occurring in lipids. For instance, 2-OH-FAs are constituents of sphingolipids in the lipid membranes of plants and animals (*1–5*). In addition, 2- and 3-OH-FAs are characteristic compounds of Lipid A, the lipid component of the lipopolysaccharides (LPS), which are located in the outer membrane of Gram-negative bacteria, whereas 3-OH-FAs were used as chemical markers for the determination of endotoxin in clinical and environmental samples (*6–11*). While many studies have dealt with this subject matter, little data existed on the occurrence and quantities of 2- and 3-OH-FAs in food (*12, 13*).

Quantitative determinations of 2- and 3-OH-FAs usually require the conversion of these fatty acids into derivatives accessible to gas chromatography (GC) with mass spectrometry (MS). In many cases, the OH-FAs are converted into the corresponding methyl esters either without derivatization of the hydroxyl group or by the additional conversion of the hydroxyl group into trimethylsilyl (TMS) ethers, which are measured by electron ionization mass spectrometry (EI-MS) in the selected ion monitoring (SIM) mode or by MS-MS (*6, 10–12, 14–16*). In addition, methyl/pentafluorobenzoyl (ME/PFBO) derivatives have been analyzed by GC/MS in the electron-capture negative ion (ECNI) mode (*8, 9*). In order to increase both the selectivity and sensitivity of analyses, PFBO derivatives have been measured in the SIM mode by using the molecular ion $[M]^-$ that specifies the corresponding OH-FA (*7, 9*).

The aim of the present study was to develop a highly selective and sensitive method for the quantitative determination of 2- and 3-OH-FAs in bovine milk fat in the submilligram/100 g range. Given the low amounts of OH-FAs to be detected, the method included (i) separation of the methyl esters of hydroxylated fatty acids from those of nonhydroxylated fatty acids, followed by (ii) conversion of OH-FAMEs into the corresponding PFBO derivatives (PFBO-*O*-FAMEs). Due to their electron-capturing properties, the PFBO derivatives were ana-

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lyzed by GC/ECNI-MS in the SIM mode. In order to meet quality control standards, two internal standards were synthesized: a dideuterated hydroxy fatty acid for use as recovery standard for the entire sample cleanup and a second internal standard to even out variations in the performance of the mass spectrometer.

MATERIALS AND METHODS

Materials and Chemicals. Bovine milk (lipid content 3.5%, Weihenstephan, Freising, Germany) was used as food sample. 2-OH- and 3-OH-FA standards were obtained from the following commercial sources: 2-hydroxyoctanoic acid (2-OH-8:0), 2-hydroxydecanoic acid (2-OH-10:0), 2-hydroxytetradecanoic acid (2-OH-14:0), γ-decalactone, and γ-dodecalactone were from Sigma−Aldrich (Steinheim, Germany); 2-hydroxydodecanoic acid (2-OH-12:0), 2-hydroxyhexadecanoic acid (2-OH-16:0), 2-hydroxyoctadecanoic acid (2-OH-18:0), 2-hydroxyeicosanoic acid (2-OH-20:0), 3-hydroxyoctanoic acid (3-OH-8:0), 3-hydroxydecanoic acid (3-OH-10:0), 3-hydroxydodecanoic acid (3-OH-12:0), 3-hydroxytetradecanoic acid (3-OH-14:0), 3-hydroxyhexadecanoic acid (3-OH-16:0), 3-hydroxyoctadecanoic acid (3-OH-18:0), and 2-hydroxy-(9*c*)-octadecenoic acid methyl ester [3-OH-18:1(9*c*)-ME] were from Larodan (Malmö, Sweden).

Cyclohexane (purest; VWR, Darmstadt, Germany) and ethyl acetate (purest; Acros Organics, Geel, Belgium) were combined (1:1 v/v) and distilled to obtain an azeotropic mixture (54:46 v/v). *n*-Hexane (HPLC gradient grade) and methanol (HPLC gradient grade) were purchased from Fisher Scientific (Ulm, Germany); diethyl ether, ethanol (absolute), and acetonitrile (HPLC gradient grade) were from Roth (Karlsruhe, Germany); and benzene and triethylamine (TEA) were from Merck (Darmstadt, Germany). All other solvents used in this study were distilled before use.

Deuterium (99.9%) was obtained from Isotec (Miamisburg, OH); Wilkinson's catalyst [$(Ph_3P)_3RhCl(I)$] and pentafluorobenzoyl chloride (99%, PFBO-Cl) were from Sigma-Aldrich (Steinheim, Germany); diatomaceous earth (isolute-HM-N) was from Separtis (Grenzlach-Wyhlen, Germany); discovery silver-ion SPE cartridges (750 mg/6 mL) were from Supelco (Bellefonte, PA); and potassium hydroxide (KOH) and sodium chloride (NaCl) were from Roth (Karlsruhe, Germany). Boron trifluoride−methanol complex solution (13−15% BF_3 in methanol) was from Riedel-de-Haën (Seelze, Germany), and ethanolic BF_3 (∼10%, ∼1.3 M, purris) and silica gel G60 were from Fluka (Steinheim, Germany). Prior to use, silica gel G60 was dried (activated) overnight at 120 °C.

Synthesis of 9,10-Dideutero-2-hydroxyoctadecanoic Acid Methyl Ester. 2-OH-18:1(9*c*)-ME (5 mg) was dissolved in sodium-dried and distilled benzene with a 50-mL pear-shaped flask as reaction vessel. To deoxygenate the reaction mixture, nitrogen was introduced and the solution was stirred vigorously for 2 min. This process was repeated three times with nitrogen and twice with deuterium. Then Wilkinson's catalyst (4 mg) was added, and the flask was flushed three times with deuterium. The reaction mixture was stirred at room temperature for 8 h. Afterward, the solvent was evaporated under reduced pressure, and diethyl ether (20 mL) was added to the residue. The colored ethereal solution was subjected to adsorption chromatography (1 cm i.d. glass column filled with 5 g of dried silica gel) for purification (*17*). Due to the fact that the deuteration did not proceed completely, the saturated 2-OH-18:0-ME-9,10-d_2 was separated from the monounsaturated 2-OH-18:1(9*c*)-ME by means of discovery silver-ion SPE cartridges. For this purpose, an aliquot [4 mL, containing both 2-OH-18:0-ME-9,10-d_2 and 2-OH-18:1(9*c*)-ME] of the ethereal solution was evaporated under a stream of nitrogen, redissolved in 1 mL of *n*-hexane, and applied onto a silver-ion SPE cartridge previously conditioned with 4 mL of distilled acetone and equilibrated with 4 mL of *n*-hexane. The saturated 2-OH-18:0-ME-9,10-d_2 was eluted with 10 mL of a *n*-hexane−acetone mixture (9:1 v/v). The monoenoic 2-OH-18:1(9*c*)-ME was collected in a separate fraction eluted with 10 mL of *n*-hexane−acetone (1:1 v/v). This separation step was repeated five times. The combined fractions containing about 1.15 mg of 2-OH-18:0-ME-9,10-d_2 (yield ∼ 23%) were evaporated to dryness, redissolved in 4 mL of *n*-hexane, and used as recovery standard (internal standard 1, ISTD 1).

Standard Solutions. Esterification of free OH-FAs was performed according to the official standard procedure (Standard Method of the German Society for Fat Science) (*18*). A mixture containing approximately 5 μg each of 2- and 3-OH-FA and 25 μL of ISTD 1 solution was treated (5 min/80 °C) with 0.5 mL of 0.5 M methanolic KOH. After cooling, 1 mL of methanolic BF_3 was added and the mixture was heated for an additional 5 min at 80 °C. Furthermore, approximately 100 μg of 3-OH-12:0 was treated with 0.5 M ethanolic KOH and ethanolic BF_3 in the same way.

After the reactions, vials were cooled in an ice bath, successively 2 mL of *n*-hexane and 2 mL of saturated sodium chloride solution were added, and the organic phase including the methylated (or ethylated) FAs (FAMEs or FAEEs) was separated, evaporated to dryness under a gentle stream of nitrogen at room temperature, and subjected to the PFBO derivatization procedure as described below. After conversion, the corresponding 3-PFBO-*O*-12:0-EE was used as internal standard for GC/MS measurements (ISTD 2).

Sample Preparation. The bovine milk sample was lyophilized prior to extraction. Lipids were extracted by accelerated solvent extraction (ASE, Dionex, Idstein, Germany) by using 11 mL extraction cells filled with approximately 2.0 g of diatomaceous earth (isolute-HM-N). The azeotropic mixture of cyclohexane and ethyl acetate was used as solvent (*19, 20*). After removal of the solvent, the lipid phase was transesterified according to the official standard procedure (*18*). After addition of 25 μL of ISTD 1 solution, a portion (15−20 mg) of the isolated lipids was treated as described above.

The residue was dissolved in 1 mL of an *n*-hexane−ethyl acetate mixture (98:2 v/v). This solution was transferred onto a silica gel column (0.8 g of dried silica gel in a Pasteur pipet) equilibrated with 3 mL of *n*-hexane−ethyl acetate (98:2, v/v). The column was washed with 10 mL of the same solvent mixture to eliminate the non-OH-FAMEs. OH-FAMEs were eluted into a separate fraction by rinsing the column with 6 mL of ethyl acetate. The residue obtained after the removal of the solvent was subjected to the PFBO derivatization procedure (see next section).

PFBO Derivatization. The PFBO derivatives were formed by adding 150 μL of 35% PFBO-Cl solution and 100 μL of 2% TEA solution (both in acetonitrile) to the dried samples and then heating for 1 h at 100 °C (*8*). After the sample was cooled to room temperature, distilled water (2 mL) and *n*-hexane (3.5 mL) were added, and the products were separated with the organic phase. Prior to GC/ECNI-MS analyses, 50 μL of ISTD 2 (3- PFBO-*O*-12:0-EE) was added to 950 μL of the organic phase including the OH-FAs as their ME/PFBO derivatives.

Gas Chromatography with Electron-Capture Negative Ion Mass Spectrometry. GC/MS measurements were performed with a CP-3800 GC coupled to a 1200 triple-quadrupole mass spectrometer (Varian, Darmstadt, Germany). Helium (purity 5.0) was used as carrier gas. The injector and transfer-line temperatures were set at 250 and 280 °C, respectively. A scan rate of 2 cycles/s was applied, and the filament emission current was set at 50 μA. GC analyses were performed with a Factor Four VF-5 ms column (30 m, 0.25 mm i.d., 0.25 μm d_f; Varian). The oven temperature program started at 60 °C (hold time 1.5 min), which then was raised at 40 °C/min to 180 °C (hold time 2 min), at 2 °C/min to 230 °C (hold time 9 min), and finally, at 10 °C/min to 300 °C (hold time 7.5 min). The total run time was 55 min. Injections were performed in splitless mode and the injection volume was 1 μL. A constant flow rate of 1 mL/min was used throughout the measurements. A solvent delay of 5 min was applied. The ion source temperature was kept at 150 °C. Methane (purity 5.0) was used as the reagent gas at approximately 8.8 Torr. In the full-scan mode, *m/z* 50−600 was recorded throughout the run. In the SIM mode, PFBO-*O*-FAMEs were determined as follows: *m/z* 368/369 for 2- and 3-PFBO-*O*-8:0-ME was measured from 5 to 13 min; *m/z* 396/397 for 2- and 3-PFBO-*O*-10:0-ME was measured from 13 to 18 min; *m/z* 424/425 for 2- and 3-PFBO-*O*-12:0-ME was measured from 18 to 21.5 min; *m/z* 438/439 for ISTD 2 (3-PFBO-*O*-12:0-EE) was measured from 21.5 to 25 min; *m/z* 452/453 for 2- and 3-PFBO-*O*-14:0-ME was measured from 25 to 32 min; *m/z* 480/481 for 2- and 3-PFBO-*O*-16:0-ME was measured from 32 to 41 min; *m/z* 508/509 for 2- and 3-PFBO-*O*-18:0-ME and *m/z* 510/511 for ISTD 1 (2-PFBO-*O*-18:0-ME-9,10-d_2) was

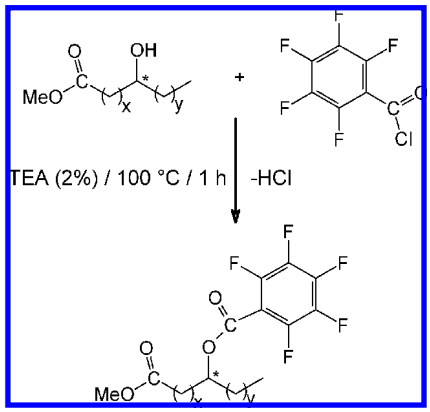

Figure 1. Derivatization of methyl esters of 2-OH-FAs ($x = 0$, $y = 5-17$) or 3-OH-FAs ($x = 1$, $y = 4-14$) with PFBO-Cl.

measured from 41 to 45 min; and finally, *m/z* 536/537 for 2- and 3-PFBO-*O*-20:0-ME was measured from 45 to 55 min.

RESULTS AND DISCUSSION

GC/ECNI-MS Capability of PFBO Derivatives. Because 2- and 3-OH-FAs are only minor constituents in foodstuffs, it is imperative to utilize a very sensitive method for quantification. Consequently, OH-FAs were converted into ME/PFBO derivatives (**Figure 1**) and analyzed by GC/ECNI-MS-SIM (*7*). The results of our own experiments concerning the influence of the reaction temperature on the PFBO derivatization aligned with previous reports (*7, 8*).

Under the present GC conditions, separation of the 2- and 3-PFBO-*O*-FAME isomers was achieved. In addition, it was evident from the GC/ECNI-MS-SIM chromatograms that the 2- and 3-PFBO-*O*-FAMEs gave a different response compared to ISTD 2 (**Table 1**). In general, 3-PFBO-*O*-FAMEs showed a better response than the corresponding 2-PFBO-*O*-FAMEs. Furthermore, the detector response of ME/PFBO derivatives decreased with increasing chain length. This is explainable by the decreasing ratio of the part with electron-capturing properties to the rest of the molecule (i.e., the alkyl chain). However, due to the varying response factors of individual OH-FAs, the application of external standards was essential for quantification.

Representative GC/ECNI-MS mass spectra of 2- and 3-PFBO-*O*-FAMEs are shown in **Figure 2**. Both GC/ECNI-MS spectra of the PFBO-*O*-18:0-ME isomers showed the base peak for the molecular ion at *m/z* 508 and little fragmentation. In addition to $[M]^-$, only three relevant fragment ions—*m/z* 167 (C_6F_5), *m/z* 196 (C_6F_5HCO), and *m/z* 211 ($C_6F_5CO_2$)—were observed in the GC/ECNI-MS spectrum of 2-PFBO-*O*-18:0-ME (**Figure 2A**). These fragment ions were much less abundant in the mass spectrum of 3-PFBO-*O*-18:0-ME (**Figure 2B**). The lower fragmentation of 3-PFBO-*O*-FAMEs is rooted in the higher temperature stability of these compounds. Mielniczuk et al. (*8*) have shown that the relative intensities of the ions in the GC/ECNI-MS mass spectra of 2-PFBO-*O*-FAMEs were strongly dependent on the temperature of the ion source. While they did not observe any molecular ion at an ion source temperature of 150 °C (*8*), on our GC/MS system, $[M]^-$ was the base peak in the ECNI mass spectra of all investigated 2- and 3-PFBO-*O*-FAMEs. The stronger fragmentation of the 2-PFBO-*O*-FAMEs compared to 3-PFBO-*O*-FAMEs was accompanied by worse detection limits when molecular ions were used in the SIM mode (**Table 1**). In addition, the ^{13}C isotopic peak was used as confirmatory ion for verification (**Table 1**). 2- and 3-PFBO-*O*-FAMEs were positively identified when the experimental ratio of the $^{12}C/^{13}C$ signal did not differ more than 10% from the theoretical isotope ratio.

Under the above-mentioned GC/ECNI-MS-SIM conditions, the detection limit was 0.010−0.105 mg/100 g for 2-PFBO-*O*-FAMEs and 0.003−0.026 mg/100 g for 3-PFBO-*O*-FAMEs (depending on the chain length; for individual detection limits, see **Table 1**). This method is not only very selective for the determination of PFBO-*O*-FAMEs but also very sensitive and, thus, was suitable for the determination of trace amounts of 2- and 3-OH-FAs in foodstuffs.

Sample Preparation for Bovine Milk Fat Samples. Despite the high sensitivity and selectivity of the determination method, it should be noted that OH-FAMEs are only minor constituents of the lipids of foodstuffs. After transfer of OH-FAs into the corresponding PFBO-*O*-FAMEs, conventional FAMEs are also found in the extract. Thus, the direct injection of small amounts of PFBO-*O*-FAMEs would be accompanied with the coinjection of high amounts of FAMEs onto the GC column. In order to prevent an overload of the GC column, the bulk of non-OH-FAMEs was separated from the OH-FAMEs prior to PFBO derivatization. For this purpose, adsorption chromatography on dried silica was applied. In order to avoid hazardous halogenated solvents, a solvent mixture made of *n*-hexane and ethyl acetate (98:2) for the elution of non-OH-FAMEs into a first fraction (which was discarded) was chosen. After this step, OH-FAMEs were eluted with 100% ethyl acetate. This eluent selection occurred according to Youhnovski et al. (*21*), although they used cyclohexane instead of *n*-hexane. It turned out that this sample preparation step could be accomplished faster with *n*-hexane.

Sample preparation for the determination of OH-FAMEs in bovine milk consisted of several steps. Thus, the loss of OH-FAs during the sample preparation (accompanied with the falsification of the results) cannot be excluded. Therefore, there was a need for a recovery standard (ISTD 1) to be added to the foodstuff samples before the transesterification step (see next section). Another problem in GC/ECNI-MS is the instability of the instrument from day to day, sometimes even from injection to injection. Repeated analyses of samples by GC/ECNI-MS measurements are subject to unavoidable variations in the total area (uniform for all compounds). To even out variations between standards and samples in subsequent GC runs, an internal standard (ISTD 2) was required to calibrate the GC/ECNI-MS performance.

Selection of Internal Standards. The ideal standards ISTD 1 and ISTD 2 should not be present in foodstuff and they should not coelute with any relevant fatty acid with the same SIM mass. In addition, the recovery standard should run through all sample preparation steps in an analogous manner as the OH-FAs contained in foodstuffs.

In previous studies, odd-numbered OH-FAs (*7–9*) or a perdeuterated nonhydroxy fatty acid (*10*) were used as internal standards. However, odd-numbered OH-FAs did not come into consideration, given that it could not be excluded that these OH-FAs do occur in the food samples (*12, 22, 23*). Deuterated non-hydroxy fatty acids could not be used either, since their physicochemical properties are different from those of OH-FAs. Hence, they do not run through all sample preparation steps in the same way as OH-FAs. However, deuterium-labeled OH-FAs appeared to be a suitable recovery standard (ISTD 1). Since deuterated 2- and 3-OH-FAs are not commercially available, 2-OH-18:0-ME-9,10-d_2 was synthesized from the unsaturated 2-OH-18:1(9*c*)-ME following the protocol for the deuteration of unsaturated (nonhydroxy) methyl fatty esters (*17*). While the

Table 1. Gas Chromatographic Retention Times, Relative Response Factors, Quantification Ions, and Detection Limits and Amounts of 2- and 3-PFBO-*O*-FAMEs in Bovine Milk Fat

no.	PFBO derivative of	retention time, min	RRF[a]	quantification (qualification) ion, *m/z*	detection limits,[b] mg/100 g	bovine milk fat ($n = 5$), mg/100 g
1	2-OH-8:0-ME	10.03	1.109	368 (369)	0.010	4.49 ± 0.29
2	3-OH-8:0-ME	10.21	3.780	368 (369)	0.003	0.79 ± 0.09
3	2-OH-10:0-ME	14.29	0.886	396 (397)	0.014	0.05 ± 0.00
4	3-OH-10:0-ME	14.47	2.944	396 (397)	0.004	3.19 ± 0.25
5	2-OH-12:0-ME	20.05	0.477	424 (425)	0.019	0.02 ± 0.00
6	3-OH-12:0-ME	20.23	2.242	424 (425)	0.005	3.41 ± 0.12
7	3-OH-12:0-EE	22.11	1.000	438 (441)		(ISTD 2)
8	2-OH-14:0-ME	26.67	0.356	452 (455)	0.047	0.49 ± 0.02
9	3-OH-14:0-ME	26.85	1.630	452 (455)	0.007	2.47 ± 0.16
10	2-OH-16:0-ME	33.76	0.224	480 (483)	0.066	1.05 ± 0.01
11	3-OH-16:0-ME	33.96	0.828	480 (483)	0.013	4.20 ± 0.10
12	2-OH-18:0-ME-9,10-d_2	42.76	0.061	510 (511)		(ISTD 1)
13	2-OH-18:0-ME	42.79	0.093	508 (509)	0.105	nd[c]
14	3-OH-18:0-ME	42.92	0.202	508 (509)	0.026	nd
15	2-OH-20:0-ME	46.31	0.092	536 (537)	0.105	nd

[a] Relative response factors of 2- and 3-PFBO-*O*-FAMEs related to 3-PFBO-*O*-12:0-EE (based on the peak areas of the GC/ECNI-MS-SIM chromatogram). [b] Detection limit based on 20 mg sample purified and made up in 1 mL of solvent. The detection limit of standards ranged from 300 fg to 2 pg of 2-OH-PFBO-*O*-FAMEs and from 50 to 500 fg of 3-PFBO-*O*-FAMEs. [c] Not detectable.

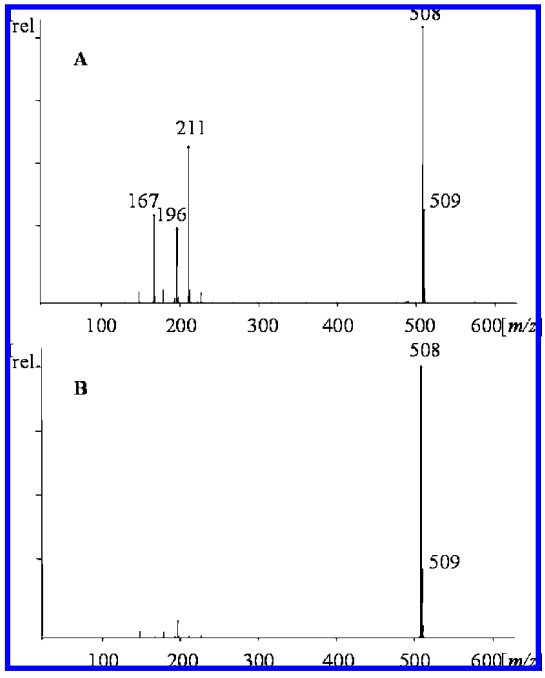

Figure 2. GC/ECNI-MS full-scan mass spectra of (**A**) 2-PFBO-*O*-18:0-ME (**13**) and (**B**) 3-PFBO-*O*-18:0-ME (**14**).

purified 2-PFBO-*O*-18:0-ME-9,10-d_2 (see Materials and Methods) was not fully GC-separated from the native 2-PFBO-*O*-18:0-ME (compare **Table 1**), the molecular ions ($[M]^-$) of both compounds differ by 2 atom mass units. Consequently, 2-OH-18:0-ME-9,10-d_2 was suitable as recovery standard (ISTD 1) for GC/ECNI-MS-SIM.

For the correction of GC/MS instabilities (see above), the PFBO derivative of 3-OH-12:0 ethyl ester (3-PFBO-*O*-12:0-EE) was selected. This compound was added as ISTD 2 to sample solutions directly before the GC/MS measurements. Compared to the methyl ester, the retention time of the ethyl ether was shifted by ~1.9 min to higher retention times (**Table 1**). The efficiency of ISTD 2 was tested in a mixture with ISTD 1 (**Table 2**). Three injections provided variations in the peak areas of ISTD 2 by up to 15%. However, ISTD 1 showed exactly the same behavior. This can be seen from the fact that the ratio of the peak areas differed only in the fourth digit (**Table 2**).

Table 2. Variations in GC/ECNI-MS-SIM Areas of ISTD 1 and ISTD 2 and Ratios of Both Compounds in Three Injections

injection	area of ISTD 1 [kcts] (*m/z* 510)	area of ISTD 2 [kcts] (*m/z* 438)	ratio of ISTD1:ISTD 2
1	8830 (1.000)[a]	495 100 (1.000)	0.01783
2	7746 (0.877)	433 800 (0.876)	0.01786
3	7545 (0.854)	423 600 (0.856)	0.01782
maximum deviation[b]	14.6%	14.4%	0.2%

[a] Values in parentheses are normalized to the value of injection 1, which was set at 1.0. [b] Lowest area divided by the highest area (%).

Thus, the inclusion of ISTD 2 was essential for precise determination of hydroxylated fatty acids as PFBO-*O*-FA methyl esters by GC/ECNI-MS-SIM.

Application of the Method to Bovine Milk Fat. Ten OH-FAs (even-numbered 2- and 3-OH-FAs with chain lengths from 8 to 16 carbons) were identified by means of the correct retention time and the correct ratio of the $^{12}C/^{13}C$ signal by GC/ECNI-MS-SIM (see **Figure 3** and **Table 1**). These results expand the range of compounds described in bovine milk by Parks (*12*). This author identified C_6-C_{16} 3-OH-FAs after conversion into the corresponding TMS/ME derivatives using GC/MS (*12*).

The amounts of 2- and 3-OH-FAs in the isolated bovine milk fat, listed in **Table 1**, are in each case a mean value of five determinations ($n = 5$, five times the complete sample cleanup of bovine milk fat). The recovery of ISTD 1 was 93.4% ± 6.5%. The amounts of 2-OH-FAs in 100 g of bovine milk fat were 0.02−4.5 mg, and the amounts of 3-OH-FAs ranged from 0.79 to 4.2 mg. Except for the abundant 2-OH-8:0, the corresponding 3-OH-FAs were 4-fold to >100-fold more abundant than the corresponding 2-OH-FAs. Even at very low concentrations of <0.1 mg/100 g, as observed for 2-OH-10:0 and 2-OH-12:0, the standard deviation proved to be excellent (**Table 1**). Thus, it is demonstrated that our method is suitable for quantification of traces of 2- and 3-OH-FAs in foodstuffs.

In addition, other constituents of bovine milk fat ascertainable with this method were detected (see **Figure 3**). Since these additional compounds (marked **A**−**E** in **Figure 3**) showed the correct ratio of quantification ion (^{12}C signal) to verification ion (^{13}C signal) in GC/ECNI-MS-SIM, they must be isomers of 2- and 3-OH-FAs. A multitude of γ- and δ-lactones and their precursors (4- and 5-hydroxy fatty acids bound to glycerides or phospholipids) from C8 to C16 exist in bovine milk

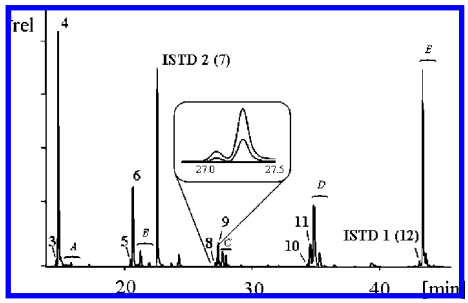

Figure 3. GC/ECNI-MS-SIM chromatogram (Factor Four VF-5 ms column) of bovine milk fat after the sample preparation procedure described above. Compound index: **3**, 2-PFBO-*O*-10:0-ME; **4**, 3-PFBO-*O*-10:0-ME; **5**, 2-PFBO-*O*-12:0-ME; **6**, 3-PFBO-*O*-12:0-ME; **8**, 2-PFBO-*O*-14:0-ME; **9**, 3-PFBO-*O*-14:0-ME; **10**, 2-PFBO-*O*-16:0-ME; **11**, 3-PFBO-*O*-16:0-ME. For amounts of 2- and 3-OH-FAs, see **Table 1**. An enlarged excerpt of the ME/PFBO derivatives of the ^{12}C- (higher abundance peaks) and ^{13}C- (lower abundance peaks) ion traces of 2- (left peak) and 3-OH-14:0 is shown as an inset. Isomers of 2- and 3-OH-FAs are marked as follows: **A**, PFBO-*O*-10:0-ME isomers; **B**, PFBO-*O*-12:0-ME isomers; **C**, PFBO-*O*-14:0-ME isomers; **D**, PFBO-*O*-16:0-ME isomers; **E**, PFBO-*O*-18:0-ME isomers.

fat (*24, 26*). Note that the lactones (inner esters of 4- and 5-OH-FAs) are converted into 4- and 5-OH-FAMEs in the course of the transesterification and subsequently derivatized into the corresponding PFBO-*O*-FAMEs by the procedure used in our study. This was tested with γ-decalactone (inner ester of 4-OH-10:0) and γ-dodecalactone (inner ester of 4-OH-12:0) (see Figure S1, Supporting Information). Consequently, our method is not suited to distinguish lactones from their precursors since both compound classes will be converted into the same derivative. In either case, it was found that a peak at the retention time of 4-PFBO-*O*-10:0-ME was detected in the bovine milk sample (14.97 min), but the retention time of 4-PFBO-*O*-12:0-ME (20.80 min) was shorter than the peak detected in the sample (21.01 min). Both results are in agreement with data of Wyatt et al. (*25*), who identified 4-OH-10:0 but not 4-OH-12:0 in bovine milk. Since δ-lactones and their precursors, the 5-OH-fatty acids, are more common in bovine milk, some of the isomeric peaks detected in this study may originate from 5-PFBO-*O*-FAMEs. Given the fact that additional standards of γ- and δ-lactones or their precursors were not available to us, additional unknown peaks in the chromatograms could not be identified. Future investigations will have to be carried out to give exact evidence concerning this matter. It appears that the present GC/ECNI-MS-SIM method will be suited for exploring this topic.

The present method already proved to be suitable for quantitative determination of 2- and 3-OH-FAs in complex food matrices like bovine milk. The preseparation of non-OH-FAMEs followed by the conversion of the OH-FAMEs into the corresponding PFBO derivatives allowed GC/ECNI-MS-SIM determination of 2- and 3-OH-FAs with high sensitivity and selectivity. Application of a recovery standard and a measuring standard enabled a determination with high precision and good quality assurance, as can be seen from the high recovery rate of the internal standard. Thus, significant losses of 2- and 3-OH-FAs during sample preparation could be excluded. Since bovine milk is rather a complex foodstuff sample, it appears to be warranted that this novel method can be applied as well to other matrices for establishing the content of 2- and 3-OH-FAs in foodstuffs.

Supporting Information Available: GC-ECNI-MS-SIM chromatogram of ME/PFBO derivatives of lactones. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Bohn, M.; Heinz, E.; Lüthje, S. Lipid composition and fluidity of plasma membranes isolated from corn (*Zea mays* L.) roots. *Arch. Biochem. Biophys.* **2001**, *387*, 35–40.

(2) Smith, C. R.; Wolff, I. A. Characterization of naturally occurring α-hydroxylinolenic acid. *Lipids* **1969**, *4*, 9–14.

(3) Hirono, H. Lipids of myelin, white matter and gray matter in a case of generalized deficiency of cytochrome b_5 reductase in congenital methemoglobinemia with mental retardation. *Lipids* **1979**, *15*, 272–275.

(4) Niederer, M.; Bollhalder, R. Identification of species specific central nervous tissue by gas chromatography-mass spectrometry (GC-MS)—a possible method for supervision of meat products and cosmetics. *Mitt. Lebensmittelunters. Hyg.* **2001**, *92*, 133–144.

(5) Biedermann, W.; Lucker, E.; Hensel, A. Detection of tissues of the central nervous system (CNS) as specified risk material (SRM) in meat products by means of gas chromatography-mass spectrometry (GC-MS). *Berl. Muench. Tieraerztl. Wochenschr.* **2002**, *115*, 131–133.

(6) Szponar, B.; Norin, E.; Midtvedt, T.; Larsson, L. Limitations in the use of 3-hydroxy fatty acid analysis to determine endotoxin in mammalian samples. *J. Microbiol. Methods* **2002**, *50*, 283–289.

(7) Mielniczuk, Z.; Alugupalli, S.; Mielniczuk, E.; Larsson, L. Gas chromatography-mass spectrometry of lipopolysaccharide 3-hydroxy fatty acids: comparison of pentafluorobenzoyl and trimethylsilyl methyl ester derivatives. *J. Chromatogr.* **1992**, *623*, 115–122.

(8) Mielniczuk, Z.; Mielniczuk, E.; Larsson, L. Gas chromatography-mass spectrometry methods for analysis of 2- and 3-hydroxylated fatty acids: application for endotoxin measurements. *J. Microbiol. Methods* **1993**, *17*, 91–102.

(9) Sonesson, A.; Larsson, L.; Schütz, A.; Hagmar, L.; Hallberg, T. Comparison of the *Limulus* amebocyte lysate test and gas chromatography-mass spectrometry for measuring lipopolysaccharides (endotoxins) in airborne dust from poultry-processing industries. *Appl. Environ. Microbiol.* **1990**, *56*, 1271–1278.

(10) Lee, A. K. Y.; Chan, C. K.; Fang, M.; Lau, A. P. S. The 3-hydroxy fatty acids as biomarkers for quantification and characterization of endotoxins and gram-negative bacteria in atmospheric aerosols in Hong Kong. *Atmos. Environ.* **2004**, *38*, 6307–6317.

(11) Keinänen, M. M.; Korhonen, L. K.; Martikainen, P. J.; Vartiainen, T.; Miettinen, I. T.; Lehtola, M. J.; Nenonen, K.; Pajunen, H.; Kontro, M. H. Gas chromatographic-mass spectrometric detection of 2- and 3-hydroxy fatty acids as methyl esters from soil, sediment and biofilm. *J. Chromatogr. B* **2003**, *783*, 443–451.

(12) Parks, O. W. Isolation and characterization of nonesterified 3-hydroxy acids in milk. *J. Diary Sci.* **1977**, *60*, 718–720.

(13) Chance, D. L.; Gerhardt, K. O.; Mawhinney, T. P. Gas-liquid chromatography-mass spectrometry of hydroxyl fatty acids as their methyl ester *tert*-butyldimethylsilyl ethers. *J. Chromatogr. A* **1998**, *793*, 91–98.

(14) Özen, H. C.; Bashan, M.; Keskin, C.; Toker, Z. Fatty acid and 3-hydroxy fatty acid composition of two *Hypericum* species from Turkey. *Eur. J. Lipid Sci. Technol.* **2004**, *106*, 68–70.

(15) Ferrando, R.; Szponar, B.; Sanchez, A.; Larsson, L.; Valero-Guillen, P. L. 3-Hydroxy fatty acids in saliva as diagnostic markers in chronic periodontitis. *J. Microbiol. Methods* **2005**, *62*, 285–291.

(16) Wakeham, S. G.; Pease, T. K.; Benner, R. Hydroxy fatty acids in marine dissolved organic matter as indicators of bacterial membrane material. *Org. Geochem.* **2003**, *34*, 857–868.

(17) Lie Ken Jie, M. C. F.; Choi, Y. C. Mass spectral studies of deuterium-labelled picolinyl fatty esters in the determination of double-bond position. *J. Am. Oil Chem. Soc.* **1992**, *69*, 1245–1247.

(18) Standard Method of the German Society for Fat Science (DGF) 1998;C-VI 11d.
(19) Thurnhofer, S.; Vetter, W. A gas chromatography/electron ionization-mass spectrometry-selected ion monitoring method for determining the fatty acid pattern in food after formation of fatty acid methyl ester. *J. Agric. Food Chem.* **2005**, *53*, 8896–8903.
(20) Weichbrodt, M.; Vetter, W.; Luckas, B. Microwave-assisted extraction and accelerated solvent extraction with the solvent mixture ethyl acetate/cyclohexane (1:1, v:v) in view of quantitative determination of organochlorines in fish tissue. *J. AOAC Int.* **2000**, *83*, 1334–1343.
(21) Youhnovski, N.; Schulz, D.; Schwarz, C.; Spiteller, G.; Schubert, K. Determination of hydroxyoctadecadienoic acids. *Z. Naturforsch.* **2002**, *58c*, 268–276.
(22) Moldovan, Z.; Jover, E.; Bayona, J. M. Gas chromatographic and mass spectrometric methods for the characterisation of long-chain fatty acids−application to wool wax extracts. *Anal. Chim. Acta* **2002**, *465*, 359–378.
(23) Jenske, R. Vetter, W. Highly selective and sensitive gas chromatography-electron-capture negative-ion mass spectrometry method for the indirect enantioselective identification of 2- and 3-hydroxy fatty acids in food and biological samples. *J. Chromatogr. A* **2007**, *1146*, 225–231.
(24) Wyatt, C. J.; Pereira, R. L.; Day, E. A. The lactone precursor in fresh milk fat: identification of the hydroxy fatty acids. *Lipids* **1966**, *2*, 208–211.
(25) Wyatt, C. J.; Pereira, R. L.; Day, E. A. Lactone precursor in fresh milk fat: Isolation and characterization of the precursor. *J. Dairy Sci.* **1967**, *50*, 1760–1763.
(26) Dimick, D. S.; Patton, S.; Kinsella, J. E.; Walker, N. J. The prevalence of aliphatic delta-lactones or their precursors in animal fats. *Lipids* **1966**, *1*, 387–390.

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