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Journal of Chromatography A, 793 (1998) 91–98

JOURNAL OF CHROMATOGRAPHY A

Gas–liquid chromatography–mass spectrometry of hydroxy fatty acids as their methyl esters *tert*.-butyldimethylsilyl ethers

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Received 18 June 1997; accepted 13 August 1997

Abstract

tert.-Butyldimethylsilyl ethers of secondary hydroxy fatty acid methyl esters (*t*BDMS-O-FAMEs) produce stable derivatives amenable to gas–liquid chromatography (GLC) and mass spectrometry (MS). Derivatives produce prominent molecular mass minus 57 $[M-57]^+$ fragment ions and unique marker fragment ions indicating the locations of the secondary hydroxyl groups along the aliphatic chain from the ω-2 carbon to carbon number 5 from the carboxylic terminus, in addition to yielding information regarding carbon chain length, and degree of unsaturation. The *t*BDMS-derivatives of C-2, C-3 hydroxy fatty acids and the unique GLC–MS data of γ- and δ-lactones are also presented. Though several *t*BDMS-O-FAMEs with centrally located hydroxyl groups were not chromatographically resolved, the combination of GLC retention times and monitoring of key diagnostic fragment ions of each *t*BDMS-derivative, when applied to mixtures containing all hydroxy isomers of palmitic through arachidic acid methyl esters, and to several monounsaturated, monohydroxylated fatty acid methyl esters, allowed for their unambiguous identification. Coupled with derivative stability, permitting their purification and concentration, this method was applied to the identification of trace lipids isolated from bovine skim milk which contained a complex mixture of hydroxy fatty acids of which 19 were newly identified. © 1998 Elsevier Science B.V.

Keywords: Fatty acids; Hydroxy fatty acids; Butyldimethylsilyl ethers; Lipids

1. Introduction

Hydroxy fatty acids are ubiquitous in nature and have been found as constituents of triacylglycerols, waxes, cerebrosides, and other lipids in plants, animals, insects and microorganisms [1–10]. Many of the methods employed for their detection and identification have been reviewed [11]. These include paper chromatography, column chromatography, thin-layer chromatography, high-performance liquid chromatography (HPLC) and gas–liquid chromatography (GLC) [11] and electrospray mass spectrometry and tandem mass spectrometry [12]. Because of their complexity and variety, hydroxy fatty acids are generally analyzed by GLC as their methyl esters with derivatization of their hydroxy function as their acetates, trifluoroacetates, trimethylsilyl ethers (TMS), and phenylurethanes [11]. More recently, the *tert*.-butyldimethylsilyl (*t*BDMS) moiety has been employed in the identification of several 2- and 3-hydroxy fatty acids (C-2 and C-3, respectively) as their *t*BDMS esters and ethers [13]. Because

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0021-9673/98/$19.00
PII S0021-9673(97)00876-5

of the *t*BDMS-ether's stability to hydrolysis [14], they have even been used as a means of separating and purifying monohydroxylated eicosanoids by HPLC prior to their subsequent analysis as their TMS-ether, methyl ester derivatives [15]. The use of the *t*BDMS derivative in determining the position of hydroxyl groups along the aliphatic chain of fatty acids, though, has been limited to the analysis by gas–liquid chromatography–mass spectrometry (GLC–MS) of several monohydroxyeicosatetraenoic acids as their *t*BDMS-ester and ether [16], and to a few reaction products resulting from lipid peroxidation in cancer cells [17] as their *t*BDMS-ether methyl esters.

Ongoing research in our laboratories has revealed the presence of very complex mixtures of hydroxy fatty acids in many biological materials and that most are present at low concentration ($<$0.1 mg/g fat). Since resolution by GLC of most of the isomeric forms of each hydroxy fatty acid chain length is not achievable [18] by any derivative noted above, thus making identification very difficult, the use of the *t*BDMS-ethers of the hydroxy fatty acid methyl esters (*t*BDMS-O-FAMEs) and their subsequent analysis by combined GLC–MS was selected to address this problem. Application of this technique to mixtures of synthetized secondary hydroxy fatty acid isomers with carbon chain lengths of 16 through 20 is described. With the exception of the spontaneous formation of γ- and δ-lactones of C4-OH and C5-OH fatty acids, which possess their own unique GLC and MS characteristics, the *t*BDMS-ethers of all hydroxy fatty acid methyl esters in a mixture were readily formed and identified by GLC–MS yielding information on chain length, location of the hydroxyl group and degree of unsaturation. Application of this method to a complex mixture of hydroxy fatty acids isolated from bovine skim milk illustrated the versatility of this method.

2. Experimental

2.1. Materials

Fatty acids, fatty acid methyl esters and ricinoleic acid methyl ester were purchased from Sigma (St. Louis, MO, USA). 2-Hydroxy- and 3-hydroxy fatty acid methyl esters were purchased from Matreya (Pleasant Gap, PA, USA). Reaction mixtures of monohydroxy monounsaturated fatty acid methyl esters, hydroxylated at ω-6, ω-7, ω-9, or ω-10, were synthesized from linoleic acid methyl ester via a partial oxymercuration–demercuration reaction [19]. Commercially unavailable monohydroxylated saturated fatty acid methyl esters were synthesized, as mixtures, from the respective saturated fatty acid via monohydroxylation of the aliphatic chain with 30% aqueous H_2O_2 in trifluoroacetic acid [20] followed by the formation of the methyl ester with 4% H_2SO_4 in dry methanol at 80°C for 60 min [21]. Employing the same reagents at 10°C, a mixture of ω-5, ω-6, ω-10, and ω-11 monohydroxy monounsaturated fatty acid methyl esters was synthesized from palmitoleic acid methyl ester and the mixture purified by normal-phase HPLC [15]. *N*-Methyl-*N*-*tert*.-butyldimethylsilyl-trifluoroacetamide (MTBSTFA) with 1% *tert*.-butyldimethylsilyl chloride (*t*BDMS-Cl) and *N*-*tert*.-butyldimethylsilylimidazole (TBSIM) were either synthesized in this laboratory [14,22] or were purchased from Regis (Morton Grove, IL, USA) and United Chemical Technologies (Bristol, PA, USA), respectively. All solvents were purchased from Aldrich (Milwaukee, WI, USA) and were redistilled prior to use. Dried bovine skim milk was purchased from Difco (Detroit, MI, USA).

2.2. Gas chromatography

Capillary GLC analysis was performed with a Varian GLC system, Model 3400CX (Varian Associates, Park Ridge, IL, USA) equipped with dual flame ionization detectors. The chromatographic column employed was a 30 m×0.25 mm I.D., fused-silica capillary column with bonded 0.25 μm film thickness of 100% methyl silicone (Quadrex, New Haven, CT, USA). The helium flow-rate was 5 ml/min, with injector and detector temperatures of 320 and 340°C, respectively. After an initial hold of 4 min at 80°C, the column was temperature programmed at 5°C/min to 340°C. EZChrom Chromatography Data System (San Ramon, CA, USA) was employed for data acquisition and compression.

2.3. Gas chromatography–mass spectrometry

Mass spectra were obtained on a Kratos MS 25 double-focusing mass spectrometer (Kratos, Urmston, Manchester, UK) interfaced with a Carlo Erba Model 4160 gas chromatograph equipped with a 100% methyl silicone capillary column and programmed as indicated above. Mass spectra were recorded at 70 eV with an ionization current of 100 μA, a source temperature of 280°C, and a transfer line temperature of 280°C. A Kratos Mach 3 Data System was employed for data acquisition, compression and manipulation.

2.4. Hydroxy fatty acid methyl ester standard solutions

Synthesized mixtures of isomeric hydroxy fatty acid methyl esters (HO-FAMEs) were prepared at a final concentration of approximately 1–2 mg each HO-FAME per ml of toluene. Solutions were kept in 25-ml serum vials sealed under an atmosphere of argon with a PTFE-faced silicone septum. For derivatization reactions, 50-μl aliquots of these stock solutions were employed. 12-Hydroxyoctadecanoic acid methyl ester, produced by the hydrogenation [17] of ricinoleic acid, was included as internal and reference standard in each standard mixture.

2.5. Isolation of hydroxy fatty acids from bovine skim milk

Bovine skim milk (2 g), dissolved in 5 ml of deionized water, was extracted with 4 volumes of chloroform–methanol (2:1, v/v). The chloroform layer was reduced to dryness in vacuo in a PTFE-faced screw-top test tube. The oily, waxy residue was then subjected to transesterification in 20 ml absolute methanol containing 4.0% sulfuric acid at 85°C for 2 h under argon atmosphere. After cooling the hydrolysis tube in an ice bath, 10 ml of ice cold water was then added and the contents cautiously mixed. The lipids were then extracted with 10 ml hexane. Aliquots of the hexane extract were then subjected to thin-layer chromatography employing 5×20 cm plates coated with a 250-μm layer of silica gel G (Analtech, Delaware, USA), and using hexane–diethyl ether (1:1, v/v) as the developing solvent. Hydroxy fatty acid methyl esters (R_F=0.7–0.8), visualized with iodine vapor, were extracted from the silica with diethyl ether–methanol (10:1, v/v) and dried in vacuo.

2.6. Derivatization of hydroxy fatty acid methyl esters

To the respective Reactivial, equipped with a small PTFE-coated stir bar and a PTFE-faced silicone septum, 50 μl each of toluene, DMF and pyridine were added to the sample to be derivatized and then mixed. Then 100 μl of TBSIM and 200 μl of MTBSTFA, containing 1% *t*BDMS-Cl, were sequentially added with stirring. The Reactivial was then heated at 85°C for 30 min and allowed to cool to room temperature. The resulting *t*BDMS-O-FAMEs were either directly analyzed or were washed prior to GLC–MS analysis by adding 1.0 ml hexane to the reaction mixture, which was then extracted three times each with 1.0 ml water to hydrolyze unreacted TBSIM and MTBSTFA and to remove imidazole, DMF and pyridine. The hexane solution could then be directly analyzed or reduced in volume via a stream of dry air.

3. Results and discussion

3.1. Derivatization

tert.-Butyldimethylsilylation of all standard hydroxy fatty acid methyl ester mixtures with TBSIM and MTBSTFA containing 1% *t*BDMS-Cl was complete within 30 min at 85°C. The resulting *t*BDMS-O-FAMEs were amenable to purification via aqueous extractions and concentration and, when stored in hexane, displayed no evidence of degradation after several months.

3.2. GLC–MS

The *t*BDMS-ethers of saturated, monohydroxy fatty acid methyl esters with carbon chain lengths of 16–20, possessing single secondary hydroxyl groups along the aliphatic chain, were analyzed by GLC–MS and the results are presented in Tables 1 and 2. As is characteristic of *t*BDMS derivatives [22–24]

Table 1
Comparison of the molecular mass-related fragment ions and their average relative intensities produced by each series of hydroxy fatty acid methyl esters[a]

Hydroxy fatty acid methyl ester	Fragment ion (average relative intensity)				
	$[M^{+\cdot}]$	$[M-15]^+$	$[M-57]^+$	$[M-57-32]^+$	
Palmitic	400 (3)	385 (7)	343 (72)	311 (100)	75(82)
Palmitoleic[b]	398 (2)	383 (6)	341 (61)	309 (100)	75(90)
Heptadecanoic	414 (3)	399 (6)	357 (65)	325 (100)	75(78)
Stearic	428 (2)	413 (7)	371 (60)	339 (100)	75(76)
Oleic[b]	426 (2)	411 (6)	369 (59)	337 (100)	75(78)
Nonadecanoic	442 (2)	427 (5)	385 (55)	353 (100)	75(73)
Arachidic	456 (2)	441 (3)	399 (52)	367 (100)	75(73)

[a]The relative intensities for each saturated hydroxy fatty acid methyl ester possessing a hydroxyl group at the ω-2-carbon through the respective δ-carbon (from the carboxylate terminus) were included in the series averaged.
[b]The monounsaturated hydroxy palmitoleic and oleic acid methyl esters, hydroxylated at ω-5, ω-6, or ω-10, and ω-6, ω-7, ω-9, or ω-10, respectively, were utilized.

and of the *t*BDMS ethers of fatty alcohols [25], the saturated and unsaturated *t*BDMS-O-FAMEs produced very weak molecular $[M^{+\cdot}]$ and $[M-15]^+$ fragment ions but prominent $[M-57]^+$ fragment ions at m/z 343, 357, 371, 385, and 399, and m/z 341, 355, 369, 383, and 397, respectively, at relative intensities greater than 50% (Table 1). Fragment ion $[M-57-32]^+$ typically served as the base peak. From the methylene unit data (Table 2), it is evident that the derivatives possessing the hydroxyl group closer to the ω-terminus were retained longer on the non-polar 100% methyl silicone column and that from the ω-8 carbon to the lactonizing carbons (carbon numbers 4 and 5 counting from the methyl ester terminus) very little difference in retention time was observed. This is appreciated more when ion chromatograms are plotted to display both mass and retention time for key fragment ions, as is shown Fig. 1 for the *t*BDMS-ethers of a mixture of hydroxy palmitic acid methyl ester isomers. A notable decrease in retention time was observed when the *t*BDMS-ether moiety is located on the ω-4 versus that at ω-3 carbon. This was observed for all hydroxy fatty acid methyl esters in this study. Cleavage between the carbon on the methyl ester side and the carbon bearing the *t*BDMS-ether moiety itself resulted in charge retention on the latter forming prominent marker ions (Table 2) which yield the precise location of the hydroxyl group. Fig. 1 graphically demonstrates the $[M-57]^+$ and $[M-57-32]^+$, i.e., m/z 343 and 311, respectively, and the prominent marker ions for the isomeric series of hydroxylations along palmitic acid methyl ester. The mass spectrum for palmitic acid methyl ester possessing a derivatized hydroxyl group on ω-5 carbon is shown in Fig. 2A, and it is characteristic of all saturated fatty acid methyl esters bearing a hydroxy group from the ω-2 carbon to the lactonizing carbons.

Though not shown in Table 2, monounsaturated *t*BDMS-O-FAMEs present with the same marker fragment ions up to the point where the degree of unsaturation occurs on the ω-side of the hydroxylation at which point the marker fragment ion is 2 a.m.u. less than the expected saturated fragment ion mass. This is clearly demonstrated in Fig. 2B in which a representative mass spectrum, employing palmitoleic acid methyl ester [16:1(Δ^9)] hydroxylated on the ω-10 carbon, is shown. Since the location of the double bond occurs on the ω-side of the hydroxyl group, the marker fragment ion produced is observed at m/z 269. In contrast, the *t*BDMS-ether of the hydroxy fatty acid ricinoleic acid [18:1(Δ^9)], for example, for which the hydroxyl group resides on the ω-7 carbon, the marker fragment ion is observed at m/z 229 (not shown) which is the expected value for a saturated ω-chain. For this latter case, it should be noted that the single degree of unsaturation for the *t*BDMS-ether of ricinoleic acid methyl ester is still reflected in its molecular

Table 2
Hydroxyl group location, associated marker fragment ion and methylene unit of hydroxy fatty acid methyl esters as their *tert*.-butyldimethylsilyl derivatives by GLC–MS analysis

	Location of hydroxyl group (ω carbon number)																	
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Marker fragment ion (m/z)[a]:	159	173	187	201	215	229	243	257	271	285	299	313	327	341	355	369		
Fatty acid methyl ester	Relative intensity *Methylene unit*																	
Palmitic	22	21	18	18	17	18	18	16	15	13	δ[b]	γ[b]	*C3*[b]	*C2*[b]				
	24.56	*24.45*	*23.99*	*23.87*	*23.50*	*23.48*	*23.45*	*23.43*	*23.41*	*23.41*	*19.88*	*19.96*	*23.36*	*23.33*				
Heptadecanoic	22	20	19	18	18	17	17	16	15	14	12	δ	γ	*C3*	*C2*			
	25.41	*25.32*	*25.10*	*24.72*	*24.66*	*24.61*	*24.57*	*24.55*	*24.54*	*24.55*	*24.55*	*20.89*	*20.98*	*24.47*	*24.43*			
Stearic	22	21	18	18	17	18	16	17	16	15	13	12	δ	γ	*C3*	*C2*		
	26.70	*26.45*	*25.94*	*25.82*	*25.75*	*25.69*	*25.41*	*25.39*	*25.37*	*25.38*	*25.38*	*25.42*	*21.87*	*21.96*	*25.35*	*25.31*		
Nonadecanoic	23	20	19	18	18	16	17	16	15	14	11	11	10	δ	γ	*C3*	*C2*	
	27.71	*27.34*	*27.11*	*26.71*	*26.64*	*26.58*	*26.54*	*26.53*	*26.51*	*26.51*	*26.52*	*26.54*	*26.55*	*22.85*	*22.94*	*26.35*	*26.29*	
Arachidic	23	20	16	15	15	13	14	13	13	11	10	10	9	7	δ	γ	*C3*	*C2*
	28.53	*28.55*	*27.95*	*27.84*	*27.87*	*27.41*	*27.38*	*27.36*	*27.34*	*27.34*	*27.34*	*27.34*	*27.36*	*27.39*	*23.81*	*23.89*	*27.34*	*27.28*

The *t*BDMS derivatives of hydroxy fatty acid methyl esters were analyzed by GLC–MS employing a 30 m×0.25 mm I.D. capillary column possessing a 0.25-μm film of bonded 100% methyl silicone phase. The helium flow-rate was 5 ml/min, with injector and detector temperatures of 320 and 340°C, respectively. After an initial hold of 4 min at 80°C the column was temperature programmed at a rate of 5°C/min to 340°C. Chromatographic retention times are expressed in methylene units (MU). Mass spectra were recorded at 70 eV with an ionization current of 100 μA, a source temperature of 250°C, and a transfer temperature 280°C.

[a]Marker fragment ion resulting from cleavage on the carboxyl side of the *t*BDMS-ether with charge retention on the alkyl ether moiety (i.e. m/z 187, $CH_3–(CH_2)_2–CH{=}O^+–Si(CH_3)_2C(CH_3)_3$).

[b]δ, γ, refer to the δ and γ lactones, and C-3 and C-2 refer to the C-3 and C-2 *t*BDMS-ethers (relative to the carboxyl carbon), respectively. Mass spectra of these products are discussed in the text.

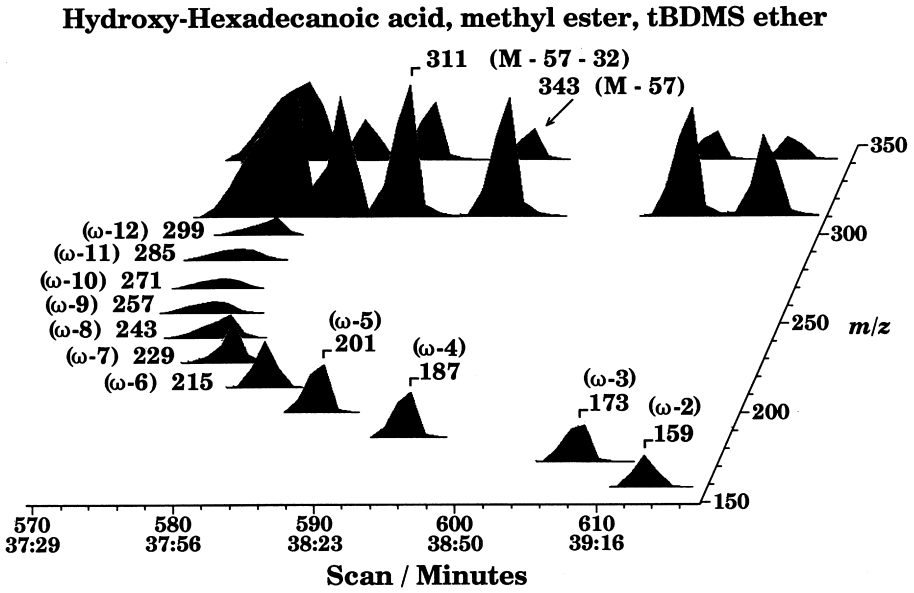

Fig. 1. Ion chromatograms (isometric display) for the *t*BDMS-ethers of the isomeric mixture of hydroxy palmitic acid methyl ester isomers. GLC–MS conditions were as described in Table 2.

mass-related fragment ions $[M-15]^+$, $[M-57]^+$ and $[M-57-32]^+$ at m/z 411, 369 and 337, respectively. It should be noted, though the location of the hydroxyl group and its relative location to the degree of unsaturation can be deduced, the location of the double bond itself may not be discernable.

As noted above, and in Table 2, lactones are observed when a hydroxyl group exists on carbon numbers 4 or 5 from a terminal carboxylic acid and are respectively designated as γ- and δ-lactones. Lactonization occurs spontaneously and is not avoided in the derivatization process presently reported. Approximately 15% of the 5-hydroxy fatty acid and less than 5% of the 4-hydroxy isomer form the *t*BDMS-ether methyl ester and present with the respective marker fragment ion indicated in Table 2. The γ- and δ-lactones, which predominate, elute chromatographically from the GLC well before the *t*BDMS-O-FAMEs of the same aliphatic chain length. The mass spectra of γ- and δ-lactones possess molecular ions (relative intensities ≃10%) and are dominated by the diagnostic cyclic ions at m/z 85 and 99, respectively, which serve as the base peaks and represent the loss of the alkyl groups from the cyclic lactone via α-cleavage. The typical mass spectrum of the γ-lactone resulting from 4-hydroxypalmitic acid is presented in Fig. 2C.

Finally, when the hydroxyl group resides on carbon number 2 (C-2) or 3 (C-3) relative to the carboxylic terminus (i.e., the α- or β-carbons, respectively) the resulting *t*BDMS-derivatives are readily distinguished from their counterparts possessing hydroxyl groups located nearer the ω-terminus via their unique retention times and characteristic fragmentation patterns. In contrast to the derivatized fatty acids which possessed more ω-oriented hydroxy groups, the mass spectra of the *t*BDMS-derivatives of C-2 and C-3 hydroxy fatty acid methyl esters are dominated by an intense m/z 89 ion, which usually serves the base peak (Fig. 2D), and a weak m/z 75 ion. Besides possessing unique retention times (Table 2), the *t*BDMS-ethers of C-2 and C-3 hydroxy fatty acid methyl esters are differentiated from each other by the characteristic and prominent $[M-57-28]^+$ and $[M-57-32]^+$ fragment ions, respectively.

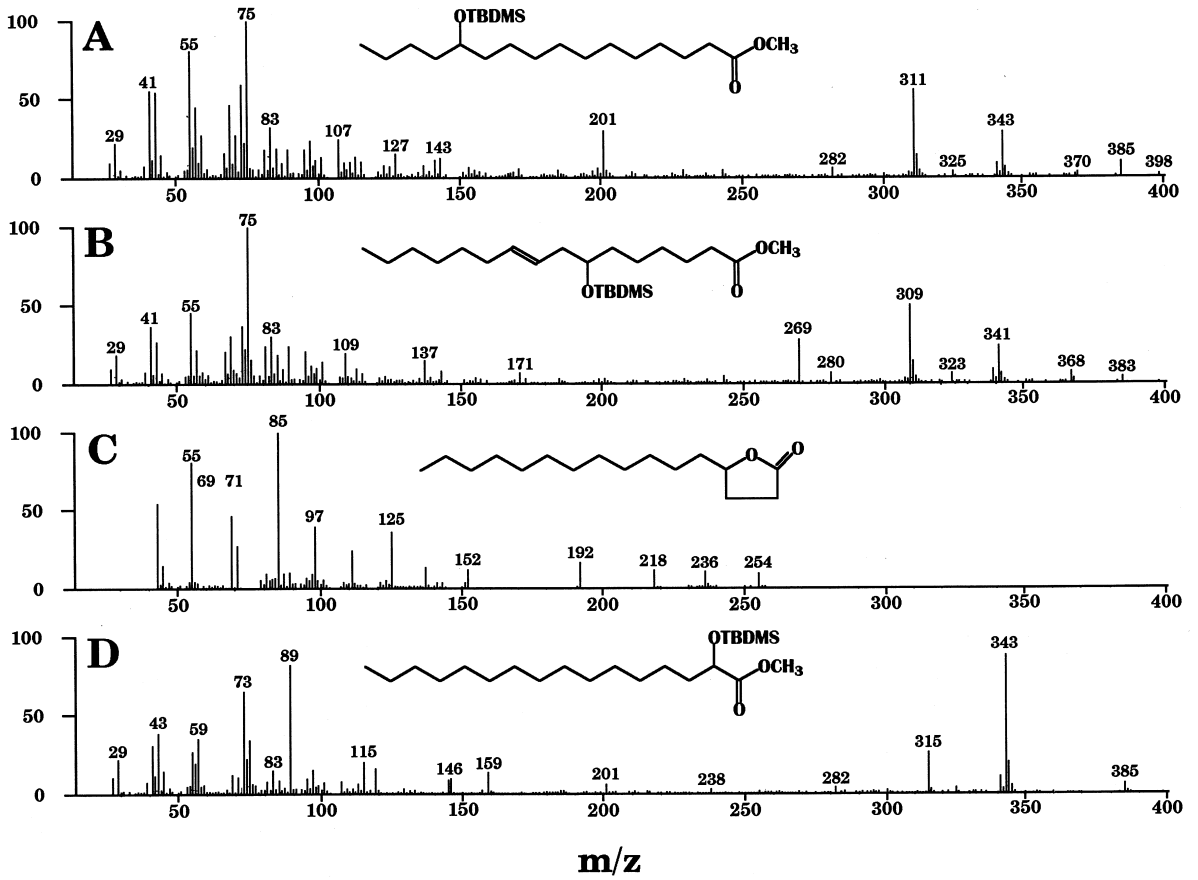

Fig. 2. Mass spectra of three C_{16} hydroxy fatty acid methyl esters and the γ-lactone. (A) *t*BDMS-ether of ω-5-hydroxy-palmitic acid methyl ester; (B) *t*BDMS-ether of ω-10-hydroxy-Δ^9-palmitoleic acid methyl ester; (C) γ-lactone originating from 4-hydroxy-palmitic acid; and (D) *t*BDMS-ether of 2-hydroxypalmitic acid methyl ester.

Table 3
Hydroxy fatty acids identified in bovine skim milk

Hydroxy fatty acid	Location of hydroxyl groups (peak number)[a]
Decanoic acid (capric)	C-2 (1) C3 (2)
Undecanoic acid	C-2 (3)
Dodecanoic acid (lauric)	C-2 (4) C-3 (5)
Tetradecanoic acid (myristic)	ω-6 (8)[b] ω-5 (9)[b] C-2 (6) C-3 (7)[b]
Pentadecanoic acid	C-2 (10) C-3 (11)
Hexadecanoic acid (palmitic)	ω-9 (13)[b] ω-8 (14)[b] ω-7 (16)[b] ω-6 (17)[b] ω-5 (18)[b] ω-4 (19)[b] ω-3 (20)[b] C-2 (12) C-3 (15)
Octadecanoic acid (stearic)	ω-10 (21)[b] ω-9 (22)[b] ω-8 (23)[b] ω-7 (24)[b] ω-6 (25)[b] ω-5 (26)[b] ω-4 (27)[b] ω-3 (28)[b] ω-2 (29)[b]
Arachidic	C-2 (30) C-3 (31)
Heneicosanoic	C-2 (32) C-3 (33)
Docosanoic	C-2 (34) C-3 (35)
Tricosanoic	C-2 (36) C-3 (37)
Tetracosanoic	C-2 (38)
Pentacosanoic	C-2 (39)

[a]Peak number referred to in Fig. 3.
[b]Not previously reported [24].

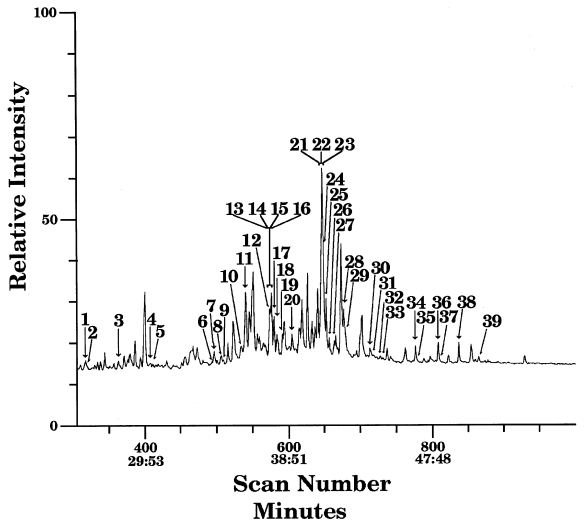

Fig. 3. Total ion chromatogram of the *t*BDMS-ethers of the hydroxy fatty acids isolated from bovine skim milk analyzed by GLC–MS, as described in Table 2. Peak labels refer to compound numbers presented in Table 3.

3.3. Identification of extracted hydroxy fatty acids from bovine skim milk

Since it has been reported that a complex mixture of hydroxy fatty acids could be found in bovine milk [26], the present method was applied to bovine skim milk for their identification. Fig. 3 presents the total ion chromatogram of the *t*BDMS derivatives of a bovine skim milk hydroxy fatty acid methyl ester profile. Based upon retention times, $[M-57]^+$ fragment ions and marker fragment ions, 39 saturated hydroxy fatty acids were identified (Table 3), 19 of which have not previously been reported.

In conclusion, a method is described in which the isomers of hydroxy fatty acid methyl esters are derivatized to their respective *t*BDMS-ether derivatives are identified by GLC–MS. The *t*BDMS-derivatives are readily prepared, have excellent chromatographic characteristics and, in complex mixtures, can yield the location of the hydroxyl group along the alkyl chain in addition to the chain length and degree of unsaturation.

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