Sphingopyxis italica, sp. nov., isolated from Roman catacombs

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The sequence of the 16S rRNA gene from strain SC13E-S71^T can be accessed at Genbank, accession number HE648058.

A Gram-negative, aerobic, motile, rod-shaped bacterium, strain SC13E-S71^T, was isolated from tuff, the volcanic rock where was excavated the Roman Catacombs of Saint Callixtus in Rome, Italy. Analysis of 16S rRNA gene sequences revealed that strain SC13E-S71^T belongs to the genus *Sphingopyxis*, and that it shows the greatest sequence similarity with *Sphingopyxis chilensis* DSMZ 14889^T (98.72%), *Sphingopyxis taejonensis* DSMZ 15583^T (98.65%), *Sphingopyxis ginsengisoli* LMG 23390^T (98.16%), *Sphingopyxis panaciterrae* KCTC12580^T (98.09%), *Sphingopyxis alaskensis* DSM 13593^T (98.09%), *Sphingopyxis witflariensis* DSM 14551^T (98.09%), *Sphingopyxis bauzanensis* DSM 22271^T (98.02%), *Sphingopyxis granuli* KCTC12209^T (97.73%), *Sphingopyxis macrogoltabida* KACC 10927^T (97.49%), *Sphingopyxis ummariensis* DSM 24316^T (97.37%) and *Sphingopyxis panaciterrulae* KCTC 22112^T (97.09%). The predominant fatty acids were $C_{18:1}\omega7c$, summed feature 3 (iso-$C_{15:0}$ 2OH and/or $C_{16:1}\omega7c$), $C_{14:0}$ 2OH and $C_{16:0}$. Predominant menaquinone was MK-10. Major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine and sphingoglycolipid. These chemotaxonomic data are common to members of the genus *Sphingopyxis*. However, a polyphasic approach using physiological tests, DNA base ratios, DNA-DNA hybridisation and 16S rRNA gene sequence comparisons showed that the isolate SC13E-S71^T belongs to a novel species within the genus *Sphingopyxis*, for which the name *Sphingopyxis italica* is proposed. The type strain is SC13E-S71^T (=DSM 25229^T =CECT 8016^T).

The genus *Sphingopyxis* was proposed by Takeuchi *et al.* (2001) to include the type species *Sphingopyxis macrogoltabida* and *Sphingopyxis terrae,* which Takeuchi *et al*. (1993) had previously described as *Sphingomonas macrogoltabidus* and *Sphingomonas terrae*. Currently, the genus *Sphingopyxis* comprises 16 species: *Sphingopyxis macrogoltabida* (Takeuchi *et al*., 1993, 2001), *Sphingopyxis terrae* (Takeuchi *et al.,* 1993, 2001), *Sphingopyxis alaskensis (*Vancanneyt *et al.,* 2001; Godoy *et al.,* 2003), *Sphingopyxis taejonensis* (Lee *et al*., 2001; Pal *et al.,* 2006), *Sphingopyxis witflariensis* (Kämpfer *et al*., 2002), *Sphingopyxis chilensis* (Godoy *et al*., 2003), *Sphingopyxis baekryungensis* (Yoon *et al*., 2005), *Sphingopyxis granuli* (Kim *et al*., 2005, 2011), *Sphingopyxis panaciterrae* (H. W. Lee *et al*., 2008, 2011), *Sphingopyxis ginsengisoli* (M. Lee *et al.,* 2008), *Sphingopyxis ummariensis* (Sharma *et al.,* 2010), *Sphingopyxis panaciterrulae* (Srinivasan *et al*., 2010), *Sphingopyxis bauzanensis* (Zhang *et al*., 2010), *Sphingopyxis soli* (Choi *et al*., 2010), *Sphingopyxis rigui* (Baik *et al*., 2012) and *Sphingopyxis wooponensis* (Baik *et al*., 2012). Members of this genus are Gram-negative, non-spore-forming, aerobic, chemo-organotrophic, catalase-positive, yellow or whitish-brown-pigmented bacteria with a DNA G+C content of 58.0-69.2 mol%. The type strains of these species have been isolated from sediment, soil, sludge and water; however, new species of *Sphingopyxis* have not yet been described either in subterranean environments or volcanic rock. In this study, we describe strain SC13E-S71^{T} retrieved from tuff, a volcanic rock from the Roman Catacombs of Saint Callixtus in Rome, Italy. A polyphasic approach showed that this isolate belongs to a novel species within the genus *Sphingopyxis*.

Strain SC13E-S71^T was isolated on tryptose soy agar (TSA; Oxoid) after two weeks at 28ºC. The methods used in this study have been described previously (Jurado *et al*., 2005a, b), unless indicated otherwise. Morphological, physiological and chemotaxonomic studies were carried out in triplicate on cultures on R2A agar (Difco) at 28ºC. Cell morphology, dimensions and motility were examined by phase contrast microscopy. Furthermore, motility was also checked on R2A broth containing 0.3% agar (Tambalo *et al.,* 2010). Oxidase activity was determined by monitoring the oxidation of dryslide oxidase (Becton Dickinson). Catalase production was indicated by the production of bubbles after mixing a cell suspension with a drop of 3% hydrogen peroxide solution on a slide. Acid production from a variety of substrates was tested using the API 50 CH system and API 50 CH B/E kit (bioMérieux), assimilation tests were carried out using the API 20NE kit (bioMérieux) and enzymatic activities were detected with API ZYM galleries (bioMérieux). API tests were performed following the manufacturer's instructions. Gram-reaction was determined by conventional Gram-staining and was confirmed by KOH-lysis test (Halebian *et al.,* 1981). Growth temperature was determined over the range 4-45ºC. Tolerance to NaCl was studied on R2A supplemented with 0-15% (w/v) NaCl. Growth at different pH values (4.0-11.0 at intervals of 0.5 pH unit) was assessed with R2A broth and R2A agar. Different media were tested for spore production: oatmeal agar (Difco), nutrient agar (Difco) and Bennett´s agar (Jones, 1949). Cellular fatty acids profiles were analysed in triplicate after 3 days on TSA at 28ºC according to the standard methodology described by Jurado *et al*. (2009). Polyamines were extracted and analysed by thin layer chromatography (TLC) according to Pedrol & Tiburcio (2001). Polar lipid profile, respiratory quinones and G+C

content of genomic DNA were determined by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). Genomic DNA extraction and amplification of 16S rRNA genes were performed as described by Laiz *et al*. (2009). The identification of phylogenetic neighbours was carried out by applying BLAST (Altschul *et al*., 1990) to the GenBank sequence database and the EzTaxon database (Chun *et al*., 2007). Pairwise 16S rRNA gene sequence similarities among the most closely related strains were determined using the global alignment algorithm on the EzTaxon server (http://www.Eztaxon.org) (Chun *et al*., 2007). For phylogenetic analyses, the nearly complete 16S rRNA gene sequence of strain SC13E-S71^T was aligned and compared with corresponding sequences of members of the genus *Sphingopyxis* and other representatives of taxa of the family *Sphingomonadaceae* using the multiple sequence alignment program CLUSTAL_X (Thompson et al., 1997). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura *et al*., 2011) and PHYLO_WIN (Galtier *et al*., 1996) with three treeing algorithms: the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) methods. Tree robustness was assessed by bootstrap resampling (1,000 replicates each). The degree of genomic relatedness among strain SC13E-S71^T and the most closely related species on the basis of 16S rRNA gene sequence similarity was determined by DNA–DNA hybridisation as described by Urdiain *et al.* (2008).

Cells of strain SC13E-S71^T were aerobic, Gram-negative, non-sporulating rods, catalase- and oxidase-positive. Strain SC13E-S71^T showed weak growth at concentrations of 2% (w/v) NaCl, although optimal growth occurred in the

absence of NaCl. Growth of strain SC13E-S71^T occurred in the temperature range of 10-30ºC, with an optimum at 25-30ºC. Table 1 shows other physiological characteristics of strain SC13E-S71^T, as well as numerous phenotypic differences from the phylogenetically closest species of the *Sphingopyxis* genus. Obvious differences were related with the presence or absence of enzymatic activities, assimilation of N-acetyl-glucosamine, adipic acid, arabinose, malate and mannose. Other differences included the production of acid from N-acetyl-glucosamine and aesculin. Further dissimilarities were noticed in fatty acid composition. The fatty acid profile of the strain SC13E-S71^T was similar to those of other type strains, but contained different amounts of fatty acids (Supplementary Table S1). The presence of $C_{18:1}\omega7c$ (30.0%), summed feature 4 (consisting of iso-$C_{15:0}$ 2OH and/or $C_{16:1}\omega7c$; 27.3%), $C_{14:0}$ 2OH (19.1%) and $C_{16:0}$ (7.2%) as the major fatty acids are a characteristic feature of the genus *Sphingopyxis* (Takeuchi *et al*., 2001). The characteristic difference between strain SC13E-S71^T and the other type strains of the genus *Sphingopyxis* was the absence of the fatty acid $C_{17:1}\omega6c$, which is generally found in members of this genus. Other differences were the presence of $C_{17:1}\omega7c$ (4.7%), which is absence in other type strains, and the high value of the fatty acid $C_{14:0}$ 2OH. Strain SC13E-S71^T contained ubiquinone Q-10 as the major respiratory quinone. Polar lipids analysis showed that strain SC13E-S71^T possessed diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, sphingoglycolipid and glycolipid (Supplementary Fig. S1). Strain SC13E-S71^T contained spermidine and spermine.

The 16S rRNA gene sequence of strain SC13E-S71^T indicated a phylogenetic relationship to the genus *Sphingopyxis,* as shown in the 16S rRNA gene phylogenetic tree (Fig. 1), where strain SC13E-S71^T formed a separate line of descent in the phylogenetic cluster of the genus *Sphingopyxis.* These results were supported by a high bootstrap value (96%). Most of the species included in this cluster shared 97% similarity in their 16S rRNA sequences. The closest taxa to strain SC13E-S71^T based on EzTaxon similarity searches were *S. chilensis* DSMZ 14889^T (GenBank accession number AF367204 with 98.72% similarity), *S. taejonensis* DSMZ 15583^T (AF131297, 98.65%), *S. ginsengisoli* LMG 23390^T (AB245343, 98.16%), *S. panaciterrae* KCTC12580^T (AB245353, 98.09%), *S. alaskensis* DSM 13593^T (CP000356, 98.09%), *S. witflariensis* DSM 14551^T (AJ416410, 98.09%), *S. bauzanensis* DSM 22271^T (GQ131578, 98.02%), *S. granuli* KCTC12209^T (AY563034, 97.73%), *S. macrogoltabida* KACC 10927^T (D13723, 97.49%), *S. ummariensis* DSM 24316^T (EF424391, 97.37%) and *S. panaciterrulae* KCTC 22112^T (EU075217, 97.09%). Strain SC13E-S71^T showed DNA-DNA relatedness of 35.0% with *S. chilensis* DSMZ 14889^T (reciprocal, 55.7%), 30.2 % with *S. taejonensis* DSMZ 15583^T (reciprocal, 39.2%), 48.9% with *S. ginsengisoli* LMG 23390^T (reciprocal, 59.0%), 42.9% with *S. panaciterrae* KCTC12580^T (reciprocal, 56.3%), 54.9% with *S. alaskensis* DSM 13593^T, 44.1% with *S. witflariensis* DSM 14551^T, and 53.4% with *S. bauzanensis* DSM 22271^T. These results indicate that strain SC13E-S71^T shows sufficient genomic coherence and hybridisation differences from its closest relatives to be considered a single species (Roselló-Mora & Amann, 2001; Stackebrandt *et al*., 2002). Furthermore, although the 16S rRNA nucleotide signature pattern is that of the genus *Sphingopyxis* described by

Takeuchi *et al*. (2001), strain SC13E-S71^T differs from all *Sphingopyxis* species in the nucleotides at position 1012 (C instead of T or A), 1013 (G instead of T, A or C); 1014 (T instead of G or C); 1021 (A instead of C or G) and 1022 (C instead of T, G or A) according to the *Escherichia coli* 16S rRNA gene sequence (GenBank accession number J01695).

The phenotypic and genotypic characteristics described above and in the species description below, together with the differences observed between strain SC13E-S71^T and previously described species of the genus *Sphingopyxis* reveal that strain SC13E-S71^T is a novel species within the genus *Sphingopyxis.* The name *Sphingopyxis italica* sp. nov. is proposed for this novel species.

Description of *Sphingopyxis italica* sp. nov.

Sphingopyxis italica (i.ta'li.ca. L. fem. adj. *italica* from Italy, the origin of the type strain).

Cells are aerobic, motile, Gram-negative, non-sporulating and rod-shaped (0.5-0.9 x 1.0-2.0 µm). Colonies are pale yellow, smooth, circular and 0.5 mm in diameter after 3 days at 28ºC on R2A agar. Catalase- and oxidase-positive. Does not reduce nitrate to nitrite. Growth occurs between 10 and 30ºC, optimum at 25-30ºC. Cells grow optimally in the absence of NaCl, with poor growth at 2% NaCl. The pH growth is between 4.5 and 8.5, with an optimum between pH 7.0 and 8.0. Does not produce indole. Produces acid from L-arabinose, D-galactose and aesculin but not from erythritol, D-glucose, D-

fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, N-acetyl-glucosamine, amygdalin, arbutin, sucrose, D-trehalose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, D-turanose, D-tagatose, D,L-arabitol, potassium gluconate, potassium 2-ketogluconate, L-fucose. Variable acid production from glycerol, D-arabinose, D-ribose, D,L-xylose, D-adonitol, methyl-β-D-xylopyranoside, salicin, cellobiose, D-maltose, D-lactose, D-melibiose, gentiobiose, D-lyxose, D-fucose and potassium 5-ketogluconate. Produces β-glucosidase, β-galactosidase, alkaline phosphatase, esterase (C1), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, β-glucuronidase, α,β-glucosidase, N-acetyl-β-glucosaminidase, valine arylamidase and trypsin but not arginine dihydrolase, urease, gelatinase, lipase (C14), cystine arylamidase, α-chymotrypsin, α-galactosidase, α-mannosidase or α-fucosidase. Assimilates glucose, arabinose, mannose, N-acetyl-glucosamine, malate and maltose, but not mannitol, potassium gluconate, capric acid, adipic acid, trisodium citrate or phenylacetic acid. Predominant fatty acids are $C_{18:1}\omega7c$, $C_{16:1}\omega7c$ or iso-$C_{15:0}$ 2OH, $C_{14:0}$ 2OH and $C_{16:0}$. Predominant respiratory lipoquinone is Q-10. Major polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine and sphingoglycolipid. The polyamines spermidine and spermine are present. The G+C content of the type strain is 65.7 mol%. The type strain, SC13E-S71^T (=DSMZ 25229^T = CECT 8016^T) was isolated from the tuff walls of the Roman catacombs of Saint Callixtus, Rome, Italy.

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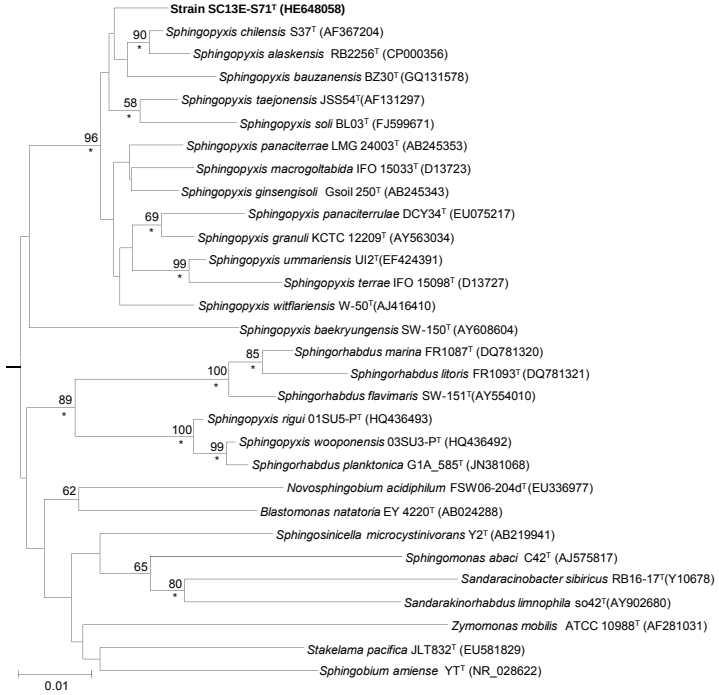
Table 1. Phenotypic characteristics of strain SC13E-S71^T and related species.

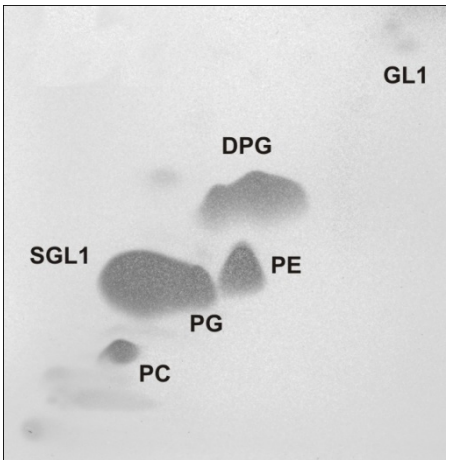
Strains: 1, SC13E-S71^T; 2, *S. chilensis* DSM 14889^T; 3, *S. taejonensis* DSM 15583^T; 4, *S. ginsengisoli* LMG 23390^T; 5, *S. alaskensis* DSM 13593^T; 6, *S. witflariensis* DSM 14551^T; 7, *S. panaciterrae* KCTC12580^T; 8, *S. bauzanensis* DSM 22271^T; 9, *S. granuli* KCTC12209^T; 10, *S. ummariensis* DSM 24316^T; 11, *S. macrogoltabida* KACC 10927^T; 12, *S. panaciterrulae* KCTC 22112^T. Data in columns 1-10 are from the present study. Data in columns 11 and 12 are from Srinivasan *et al*. (2010). Results from this study were obtained from cells grown under the same conditions.
+, positive; -, negative; (+) weakly positive; ND, not determined.

Characteristic	Strain											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Enzyme activities												
N-acetyl-β-glucosaminidase	+	-	-	-	+	-	-	-	-	-	+	+
Acid phosphatase	+	+	+	+	+	+	+	+	+	+	-	+
α-Chymotrypsin	-	-	+	-	(+)	-	+	-	-	-	+	-
Cystine arylamidase	-	-	-	(+)	-	-	+	(+)	+	+	-	+
β-Galactosidase	+	-	-	-	(+)	(+)	-	-	-	-	+	-
Lipase	-	-	-	-	-	-	-	-	-	-	(+)	-
Trypsin	(+)	+	+	(+)	(+)	(+)	+	+	+	+	-	+
Urease	-	-	-	-	+	-	-	-	-	-	-	-
Assimilation of												
N-acetyl-glucosamine	+	+	-	-	-	-	+	-	-	-	-	+
Adipic acid	-	+	+	(+)	(+)	+	+	+	+	+	-	-
Arabinose	+	-	-	-	-	-	-	-	-	-	+	-
Malate	+	+	+	(+)	(+)	-	+	+	-	+	-	+
Mannose	+	+	-	-	-	-	+	-	-	-	-	-
Acid produced from												
N-acetyl-glucosamine	-	+	-	-	-	-	-	-	-	-	-	+
Aesculin	+	+	+	+	+	+	+	+	+	+	-	+

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences, for selected set of taxa of the genus *Sphingopyxis* and other members of the family *Sphingomonadaceae*. The tree was constructed using the neighbour-joining method based on comparison of 1263 nt. Bootstrap values are expressed as percentages of 1,000 replicates; values <50% are not shown. Asterisks indicate that the corresponding branches were also recovered by the maximum-parsimony and maximum-likelihood treeing algorithms. Bar 0.01 nucleotide substitutions per site. *Erythrobacter aquimaris* SW-110^T (AY461441) was used as the outgroup.



Supplementary Fig. S1. Two-dimensional TLC of polar lipids of strain SC13E-S71^{T}. Plates have been staining with 5% molybdophosphoric acid to show all lipids. DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine, SGL1, sphingoglycolipid; GL1, glycolipid.



Supplementary Table S1. Major fatty acid composition of strain SC13E-S71^T and related type strains.

Strains: 1, SC13E-S71^T; 2, *S. chilensis* DSM 14889^T; 3, *S. taejonensis* DSM 15583^T; 4, *S. ginsengisoli* LMG 23390^T; 5, *S. alaskensis* DSM 13593^T; 6, *S. witflariensis* DSM 14551^T; 7, *S. panaciterrae* KCTC12580^T; 8, *S. bauzanensis* DSM 22271^T; 9, *S. granuli* KCTC12209^T; 10, *S. ummariensis* DSM 24316^T; 11, *S. macrogoltabida* KACC 10927^T; 12, *S. panaciterrulae* KCTC 22112^T. Data in columns 1-10 are from the present study. Data in columns 11 and 12 are from Srinivasan *et al*. (2010). Results from this study were obtained from cells grown under the same conditions. * ≤ 1%; ND, not detected; Summed feature 3 contains one or more of the following fatty acids: iso-$C_{15:0}$ 2OH and/or $C_{16:1}\omega7c$; Summed feature 4 contains: $C_{19:1}\omega6c$ and/or an unknown compound with an ECL of 18.846.

	Fatty acids	1	2	3	4	5	6	7	8	9	10	11	12
Saturated	$C_{14:0}$	*	1.2	*	2.9	*	*	*	*	*	*	*	1.5
	$C_{15:0}$	*	1.7	1.5	0.9	2.6	3.7	ND	*	*	*	*	2.1
	$C_{16:0}$	7.2	10.1	19.5	14.7	7.5	6.5	18.0	7.6	12.8	9.5	12.9	15.6
	$C_{17:0}$	ND	1.0	*	*	4.2	1.5	ND	*	*	*	*	2.3
	$C_{18:0}$	*	*	*	*	*	*	*	*	*	*	*	*
Unsaturated	$C_{16:1}\omega5c$	1.1	1.1	1.3	1.6	*	1.5	1.3	*	2.7	2.3	2.6	ND
	$C_{17:1}\omega6c$	ND	15.4	7.2	4.2	33.4	24.0	*	3.0	5.2	1.6	*	7.3
	$C_{17:1}\omega7c$	4.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	$C_{17:1}\omega8c$	ND	2.5	*	*	7.8	4.2	ND	ND	*	*	2.1	2.2
	$C_{18:1}\omega7c$	30.0	33.5	23.3	34.7	24.3	14.6	34.6	33.2	42.0	44.2	36.5	27.5
	$C_{18:1}\omega5c$	ND	*	*	1.2	*	*	*	*	1.6	*	*	1.5
	11-methyl $C_{18:1}\omega7c$	5.5	3.2	6.1	6.7	3.1	5.3	2.7	7.0	4.2	1.7	1.7	*
	$C_{19:0}$ cyclo $\omega8c$	ND	ND	ND	*	ND	ND	*	ND	*	ND	ND	ND
Hydroxy	$C_{14:0}$ 2OH	19.1	5.3	9.1	8.6	1.3	4.6	7.3	6.7	4.2	6.2	3.0	*
	$C_{15:0}$ 2OH	*	4.8	2.7	*	5.2	11.7	*	*	*	*	ND	3.2
	$C_{16:0}$ 2OH	2.3	2.4	2.4	1.4	1.2	3.3	1.2	3.5	2.0	2.4	2.3	ND
	$C_{16:1}$ 2OH	*	ND	ND	ND	ND	ND	ND	*	ND	ND	ND	ND
	iso-$C_{16:0}$ 3OH	1.6	*	ND	*	ND	ND	*	1.1	*	*	ND	ND
	iso-$C_{16:0}$ 2OH	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.3	ND
	iso- $C_{17:1}\omega9c$	ND	ND	*	ND	*	*	ND	*	*	*	ND	ND
Summed feature 3		27.3	15.8	23.5	20.1	6.1	17.4	32.3	34.4	21.7	28.5	35.0	18.6
Summed feature 4		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.8	15.4