Supplements

Lipidomic analysis of *Galdieria sulphuraria* cultivated at different pH

Milada Vítová [a], Franz Goecke [a], Karel Sigler [b], Tomáš Řezanka [b,*]

[a] *Institute of Microbiology, Czech Academy of Sciences, Laboratory of Cell Cycles of Algae, Centre Algatech, Opatovický mlýn 237, 379 81 Třeboň, Czech Republic*

[b] *Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague, Czech Republic*

2. Material and Methods

2.3. Isolation of lipids

The extraction procedure was based on the method of Bligh and Dyer [11], except that 2-propanol was substituted for methanol, since 2-propanol does not serve as a substrate for phospholipases [12]. The alcohol-water mixture was cooled, one part chloroform was added and the lipids from the lyophilized cells of different cultivations of *G. sulphuraria* were extracted for 30 min. Insoluble material was sedimented by centrifugation and the supernatant was separated into two phases. The aqueous phase was aspirated off and the chloroform phase was washed three times with two parts 1 M KCl each. The resulting chloroform phase was evaporated to dryness under reduced pressure.

Total lipid extracts were applied to Sep-Pak Vac Silica cartridge 35cc (Waters; with 10 grams of normal-phase silica), and TAGs were subsequently eluted from the cartridge with 40 mL of chloroform-methanol (9:1). Further, polar lipids were eluted from the cartridge by a mixture of 50 ml of methanol acidified with 0.1% of formic acid. The eluate of polar lipids was evaporated and the oil samples were dissolved in an acetonitrile-2-propanol-hexane mixture (1:1:1, v/v/v), which was injected for HPLC analysis.

2.4. FAMEs analysis

The lipids (both TAG and polar lipids) (~5 mg) were saponified overnight in 10% KOH-MeOH at room temperature. A fatty acid fraction obtained from saponification was partitioned between alkali solution (pH 9) and Et_2O to remove basic and neutral components. The aqueous phase, containing fatty acids, was acidified to pH 2 and extracted with hexane. The fatty acid fraction was methylated using CH_2N_2. GC-MS of fatty acid methyl ester (FAME) mixture was done on a Finnigan 1020 B in EI mode. Splitless injection was at 100

°C, and a fused silica capillary column (Supelcowax 10; 60 m x 0.25 mm i.d., 0.25 mm film thickness; Supelco, Prague) was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C/min to 180 °C and at 2 °C/min to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. All spectra were scanned within the range of *m/z* 50–500. The structures of FAMEs were confirmed by comparison of retention times and fragmentation patterns with those of the standard FAMEs (Supelco, Prague) [13,14].

2.5. HILIC-LC/MS-ESI

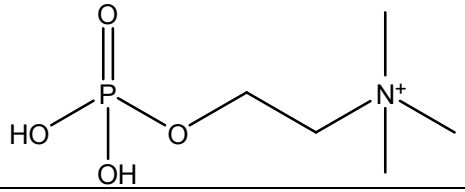
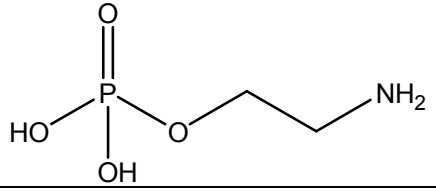
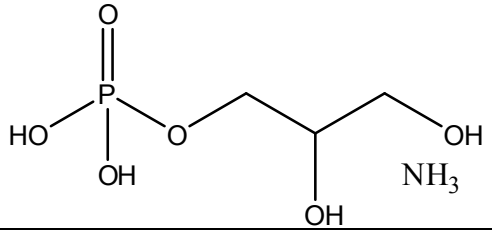
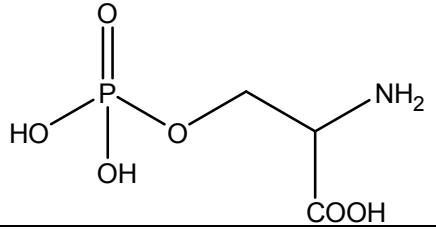
The HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Hewlett Packard, USA), and Ascentis® Express OH5, UHPLC HILIC column (2.7 μm particle size, L × I.D. 15 cm × 2.1 mm) (Supelco, Prague, Czech Republic) with an injection volume of 10 μL. UHPLC was performed at a flow rate of 0.33 mL/min for the polar lipids from all four cultivations (pH 1, 2, 3, and 4), with a linear gradient from mobile phase containing methanol/acetonitrile/aqueous 1 mM ammonium acetate (50:30:20, v/v/v) to methanol/acetonitrile/aqueous 1 mM ammonium acetate (10:70:20, v/v/v) for 60 min or with a linear gradient from mobile phase containing hexane/2-propanol/formic acid/24% aqueous ammonium (80:19:0.5:0.5, v/v/v/v) to 2-propanol/water/formic acid/24% aqueous ammonium 90:9:0.5:0.5 (for separation of DGTA and DGTS, respectively) for 50 min. Column temperature was 35 °C and a re-equilibration period was 30 min between runs. The whole UHPLC flow was introduced into the ESI source without any splitting.

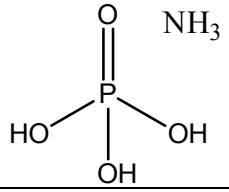
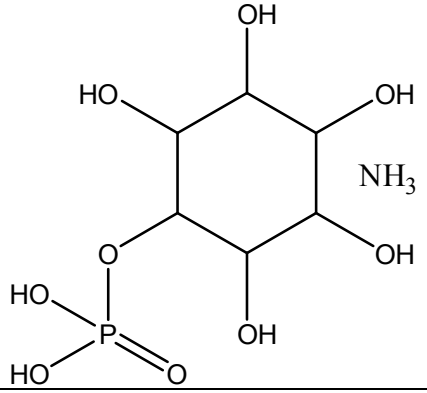
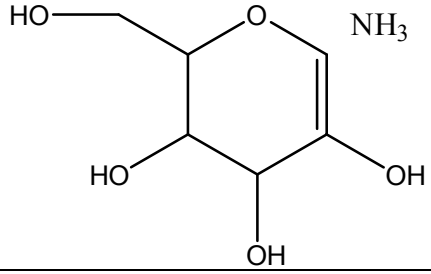
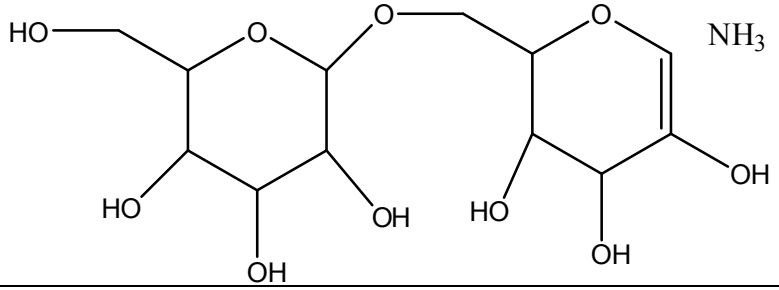
LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany), a high resolution hybrid mass spectrometer equipped with a heated electrospray interface (HESI) was used. ESI–MS analysis was performed in the FT positive ion mode. MS spectra were acquired with target mass resolution of R = 105,000 at *m/z* 800. The ion spray voltage was set at 3.5 kV and the scan range of the instruments was set at *m/z* 200-1500. Nitrogen was used as a nebulizer gas - set at 18 arbitrary units (sheath gas) and 7 arbitrary units (auxiliary gas). Helium was used as a collision gas for CID experiments. The CID normalization energy of 35% was used for the fragmentation of parent ions. The MS/MS product ions were detected by the high resolution FT mode. Further source conditions in the positive mode: vaporizer temperature, 250°C; capillary temperature, 230°C; capillary voltage, 50 V; tube lens voltage, 170 V.

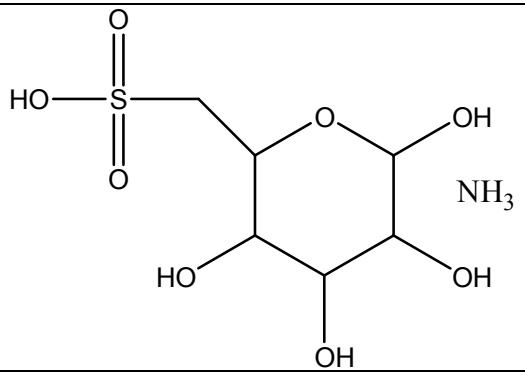
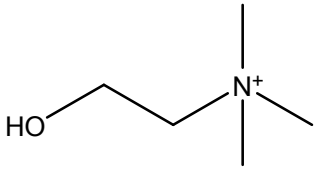
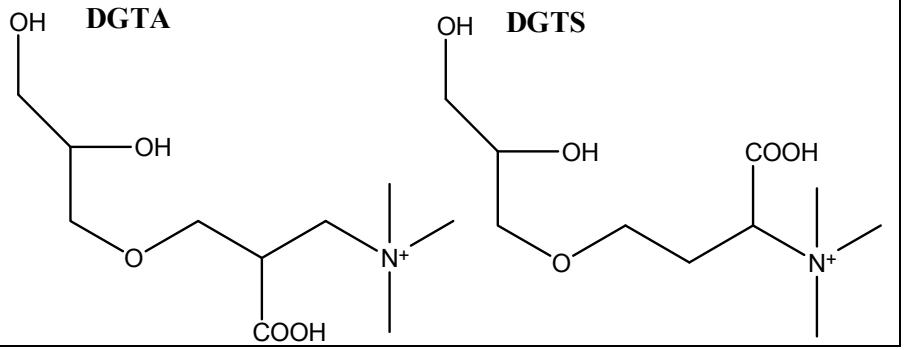
The same apparatus was used for negative ion mode. MS spectra were acquired with target mass resolution of R = 30,000 at *m/z* 400. The ion spray voltage was set at -2500V and the scan range of the instruments was set at m/z 200-2000. Nitrogen was used as a nebulizer gas - set at 18 arbitrary units (sheath gas) and 7 arbitrary units (aux gas). Helium was used as

68 a collision gas for CID experiments. The CID normalization energy of 35% was used for the
69 parent ions fragmentation. The MS/MS product ions were detected by the high resolution FT
70 mode. The H-ESI temperature was set to 250°C.
71
72

Table 1S. Mass spectrometry in positive ion mode for molecular-ion analysis of 12 classes of polar lipids using a HILIC(diol)-ESI-MS/MS.

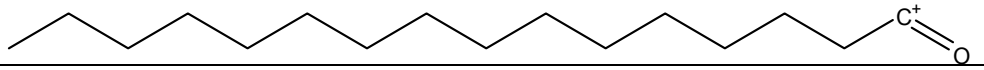
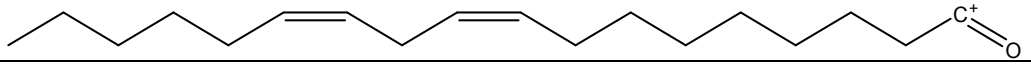
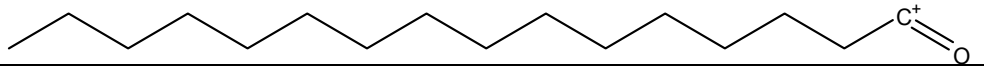
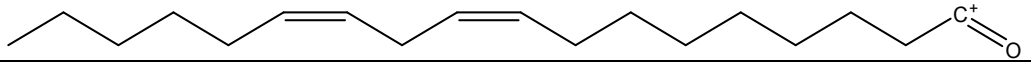
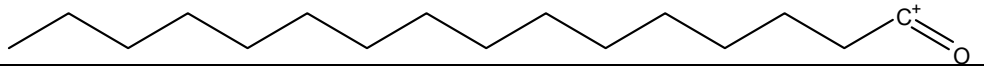
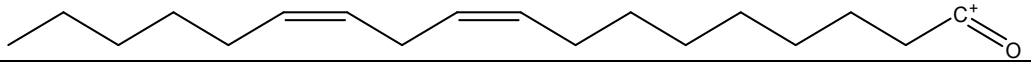
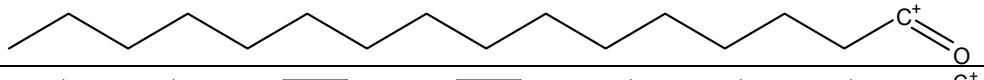
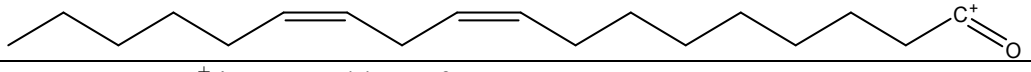
Polar lipids	**Lipid class**	**Molecular ion**	**Scan type**	**Neutral loss or product ion mass (Da)**	**Molecular formula of fragment neutral loss or product ion**	**Structures**
Phospholipids	PC	$[M + H]^+$	PIS	184	$[C_5H_{15}NO_4P]^+$	
	PE	$[M + H]^+$	NL	141	$[C_2H_8NO_4P]$	
	PG	$[M + NH_4]^+$	NL	189	$[C_3H_{12}NO_6P]$	
	PS	$[M + H]^+$	NL	185	$[C_3H_8NO_6P]$	

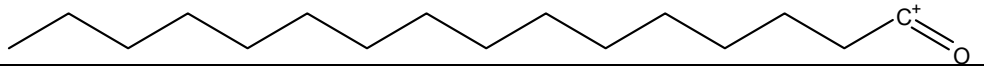
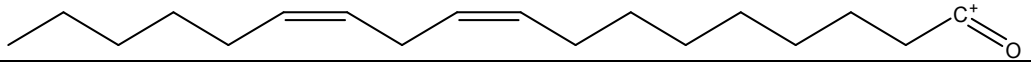
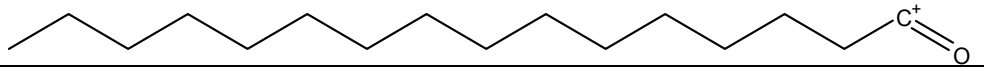
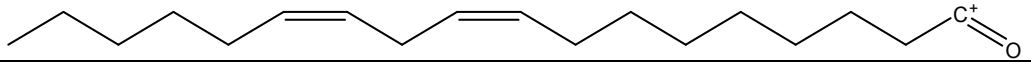
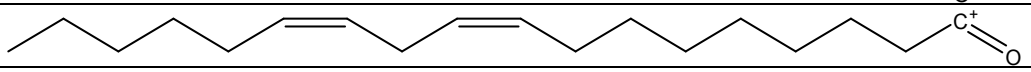
	PA	$[M + NH_4]^+$	NL	115	$[H_6NO_4P]$	
	PI	$[M + NH_4]^+$	NL	277	$[C_6H_{16}NO_9P]$	
Glycolipids	MGDG	$[M + NH_4]^+$	NL	179	$[C_6H_{13}NO_5]$	
	DGDG	$[M + NH_4]^+$	NL	341	$[C_{12}H_{23}NO_{10}]$	

	SQDG	$[M + NH_4]^+$	NL	261	$[C_6H_{15}NO_8S]$	
Betaine lipids	DGCC	$[M + H]^+$	PIS	104	$[C_5H_{14}NO]^+$	
	DGTA and/or DGTS	$[M + H]^+$	PIS	236	$[C_{10}H_{22}NO_5]^+$	

PC $[M+H]^+$ ions in positive ion mode with Precursor of 184.1 (Pre 184.1);
PE $[M+H]^+$ ions in positive ion mode with Neutral Loss of 141.0 (NL 141.0);
PG $[M+NH_4]^+$ in positive ion mode with NL 189.0 for PG;
PI $[M+NH_4]^+$ in positive ion mode with NL 277.0;
PS $[M+NH_4]^+$ in positive ion mode with NL 185.0;
PA $[M+NH_4]^+$ in positive ion mode with NL 115.0;
MGDG $[M+NH_4]^+$ in positive ion mode with NL179.1;
DGDG $[M+NH_4]^+$ in positive ion mode with NL 341.1.

Table 2S.

m/z	**DGDG**
238.1291	$[C_9H_{16}O_6+NH_4]^+$ i.e. Gal+part of glycerol
239.2375	
263.2375	
400.1819	$[C_{15}H_{26}O_{11}+NH_4]^+$ i.e. 2xGal+part of glycerol
516.3536	$[M+NH_4\text{-Gal-}R_1COOH]^+$
492.3536	$[M+NH_4\text{-Gal-}R_2COOH]^+$
654.4065	$[M+NH_4\text{-FA (18:2)}]^+$
678.4065	$[M+NH_4\text{-FA (16:0)}]^+$
934.6467	$[M+NH_4]^+$
	DGTA
236.1492	$[C_{10}H_{22}NO_5+H]^+$ i.e. head group
239.2375	
263.2375	
456.3684	$[\text{lyso-DGTA(16:0)-}H_2O+H]^+$
474.3789	$[\text{lyso-DGTA(16:0)+}H]^+$
480.3684	$[\text{lyso-DGTA(18:2)-}H_2O+H]^+$
498.3789	$[\text{lyso-DGTA(18:2)+}H]^+$
677.5356	$[M+H\text{-}59]^+$ i.e. $[M+H\text{-}N(CH_3)_3]^+$
753.6357	$[M+NH_4]^+$
	DGTS
236.1492	$[C_{10}H_{22}NO_5+H]^+$ i.e. head group
239.2375	
263.2375	
456.3684	$[\text{lyso-DGTS(16:0)-}H_2O+H]^+$
474.3789	$[\text{lyso-DGTS(16:0)+}H]^+$
480.3684	$[\text{lyso-DGTS(18:2)-}H_2O+H]^+$
498.3789	$[\text{lyso-DGTS(18:2)+}H]^+$
649.5049	$[M\text{-}87]^+$ i.e. $[M\text{-}CH_2\text{-}CH_2\text{-}N+(CH_3)_3]^+$
677.5356	$[M+H\text{-}59]^+$ i.e. $[M+H\text{-}N(CH_3)_3]^+$
691.6109	$[M\text{-}NH_4\text{-}CO_2]^+$
753.6357	$[M+NH_4]^+$
	MGDG
238.1291	$[C_9H_{16}O_6+NH_4]^+$ i.e. Gal+part of glycerol
239.2375	
263.2375	
492.3536	$[M+H\text{-sn2 FA}]^+$ i.e. neutral loss of sn2 RCOOH group

516.3536	$[M+H\text{-sn1 FA}]^+$ i.e. neutral loss of sn1 RCOOH group
609.5332	$[M+NH_4\text{-Gal}]^+$
772.5939	$[M+NH_4]^+$
	PA
239.2375	
263.2375	
393.2401	$[M+H\text{-sn2 FA}]^+$ i.e. neutral loss of sn2 RCOOH group
411.2506	$[M+H\text{-sn2 RCH=C=O}]^+$ i.e. loss of sn1 acyl chain as ketene (RCH=C=O)
417.2403	$[M+H\text{-sn1 FA}]^+$ i.e. neutral loss of sn1 RCOOH group
435.2506	$[M+H\text{-sn1 RCH=C=O}]^+$ i.e. loss of sn1 acyl chain as ketene (RCH=C=O)
690.5074	$[M+NH_4]^+$
	PC
478.3292	$[M+H\text{-59-sn2 FA}]^+$ i.e. neutral loss of sn2 RCOOH group -59
496.3397	$[M+H\text{-59-sn2 RCH=C=O}]^+$ i.e. loss of sn1 acyl chain as ketene (RCH=C=O) -59
502.3294	$[M+H\text{-59-sn1 FA}]^+$ i.e. neutral loss of sn1 RCOOH group -59
520.3395	$[M+H\text{-59-sn1 RCH=C=O}]^+$ i.e. loss of sn1 acyl chain as ketene (RCH=C=O) -59
575.5039	$[M+H\text{-}183]^+$ i.e. $[M+H\text{-phosphocholine}]^+$
699.4964	$[M+H\text{-}59]^+$ i.e. $[M+H\text{-N(CH3)3}]^+$
758.5693	$[M+H]^+$
	PE
239.2375	
263.2375	
410.2672	$[M+NH_4\text{-}43\text{-}C_{17}H_{31}COOH]^+$
428.2777	$[M+NH_4\text{-}43\text{-}C_{16}H_{29}CH{=}C{=}O]^+$
434.2672	$[M+NH_4\text{-}43\text{-}C_{15}H_{31}COOH]^+$
452.2777	$[M+NH_4\text{-}43\text{-}C_{15}H_{30}{=}C{=}O]^+$
575.5039	$[M+NH_4\text{-}147]^+$ $((NH_4O)(HO)P(O)(OCH_2CH_2NH_2))$
592.5305	$[M+NH_4\text{-}141]^+$ $((HO)_2P(O)(OCH_2CH_2NH_2))$
690.5074	$[M+NH_4\text{-}43]^+$ (aziridine)
733.5496	$[M+NH_4]^+$
	PG
239.2375	
263.2375	
410.2671	$[690\text{-}R_2COOH]^+$
434.2672	$[690\text{-}R_1COOH]^+$
484.3039	$[M\text{-}R_2COOH]^+$
508.3042	$[M\text{-}R_1COOH]^+$
575.5037	$[M+NH_4\text{-polar part}]^+$ i.e. $[M+NH_4\text{-}(HO)_2P(O)OCH_2CH(OH)CH_2OH]^+$

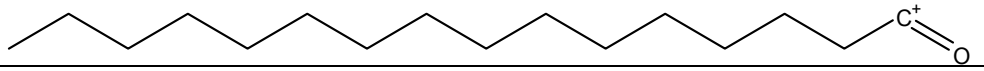
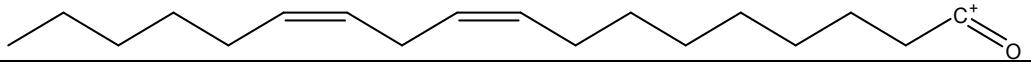
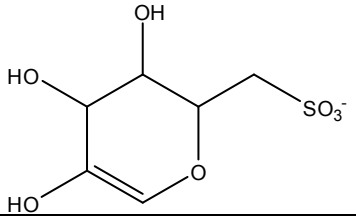
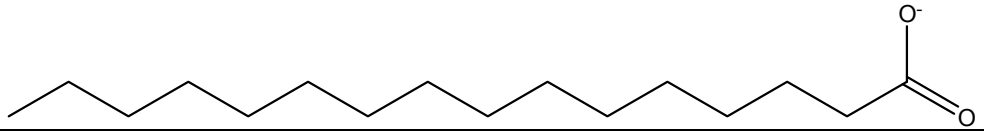
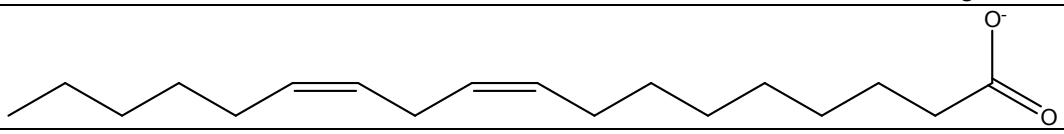
690.5074	$[M+NH_4\text{-glycerol}]^+$ i.e. $[M+NH_4\text{-}HOCH_2CH(OH)CH_2OH]^+$
764.5442	$[M+NH_4]^+$
	PSC
239.2375	
263.2375	
464.2719	$[\text{lyso-PSC}(16:0)\text{-}H_2O+H]^+$
482.2825	$[\text{lyso-PSC}(16:0)+H]^+$
488.2719	$[\text{lyso-PSC}(18:2)\text{-}H_2O+H]^+$
506.2825	$[\text{lyso-PSC}(18:2)+H]^+$
700.5043	$[M+H\text{-}61]^+$ i.e $[M+H\text{-}CH_2SCH_3]^+$
761.5155	$[M+H]^+$
	SQDG
225.0075	
255.2330	
279.2330	
537.2739	$[M\text{-sn2 FA } (18:2)]^-$
561.2739	$[M\text{-sn1 FA } (16:0)]^-$
817.5141	$[M-H]^-$

Fig. 1S. Natural 16:0/18:2-PC.

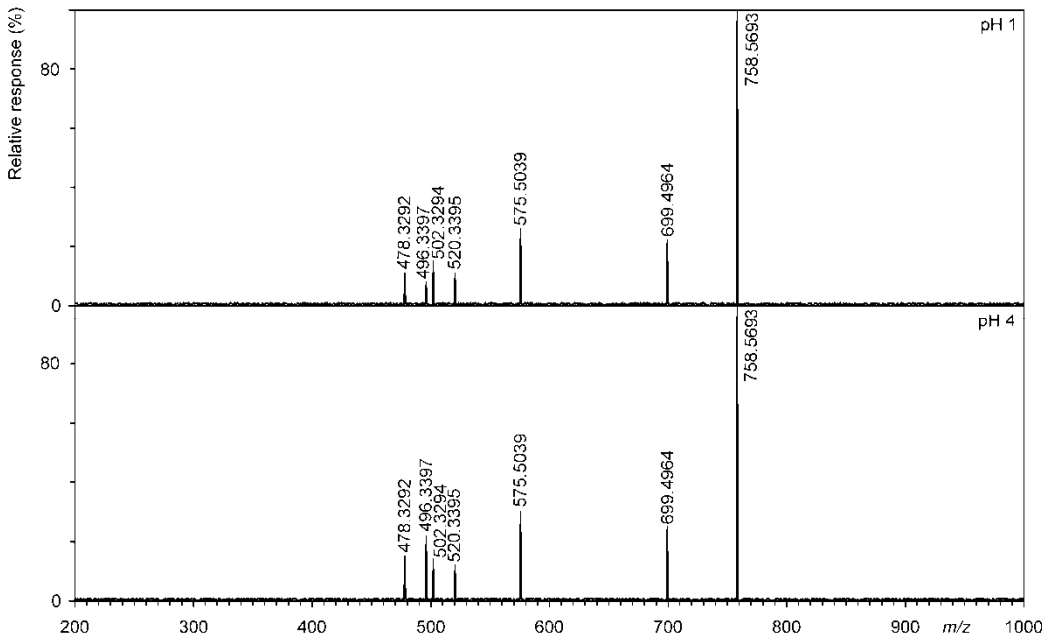


Fig. 2S. Standard of 16:0/18:2-PC

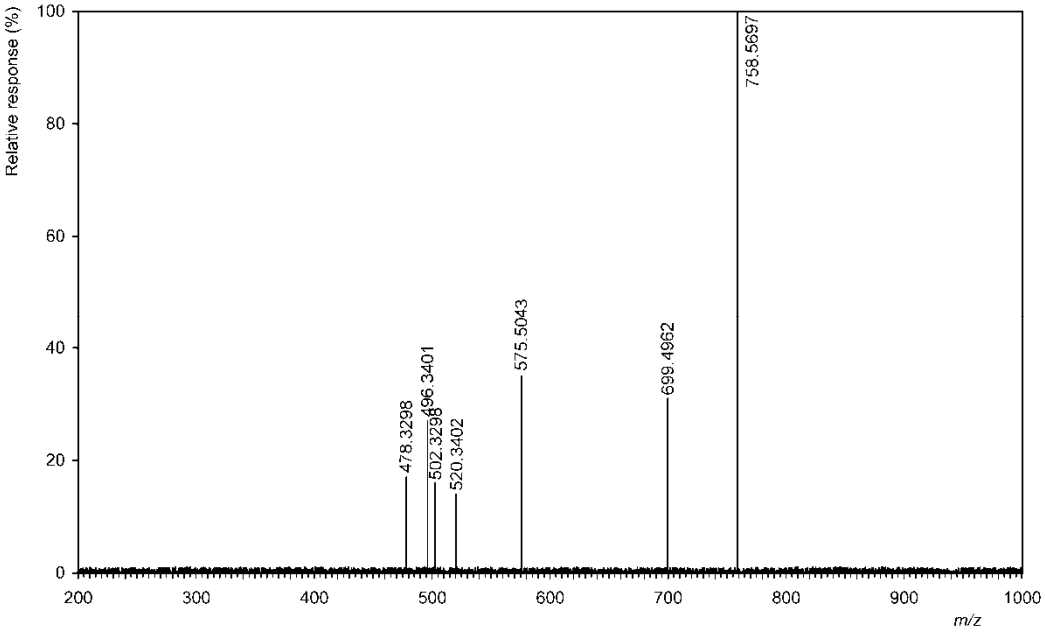


Fig. 3S. Standard 18:3/16:0 SQDG

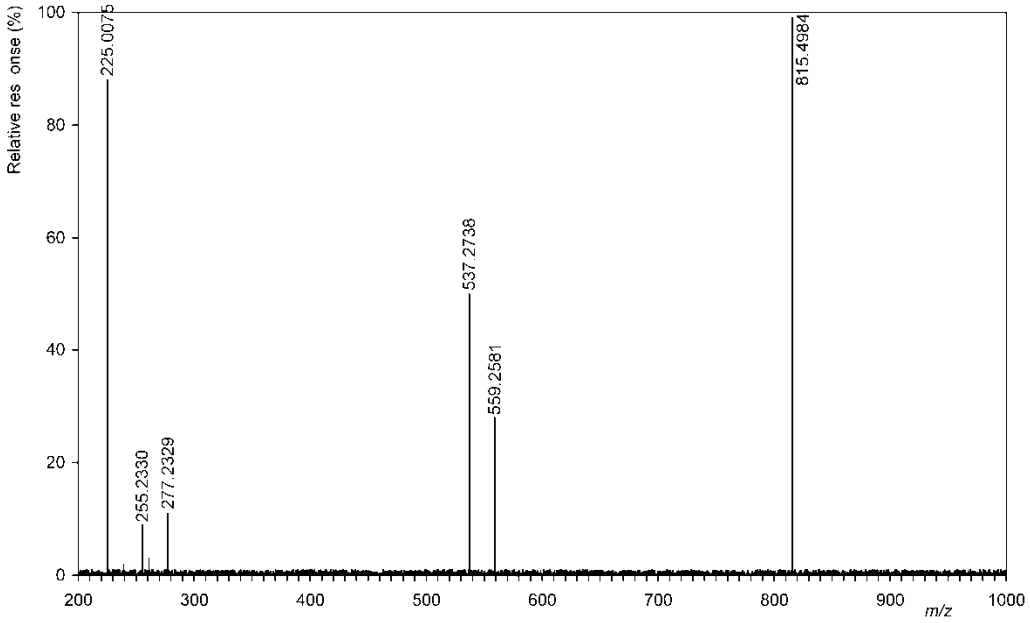


Fig. 4S. Natural 16:0/18:2-DGTS

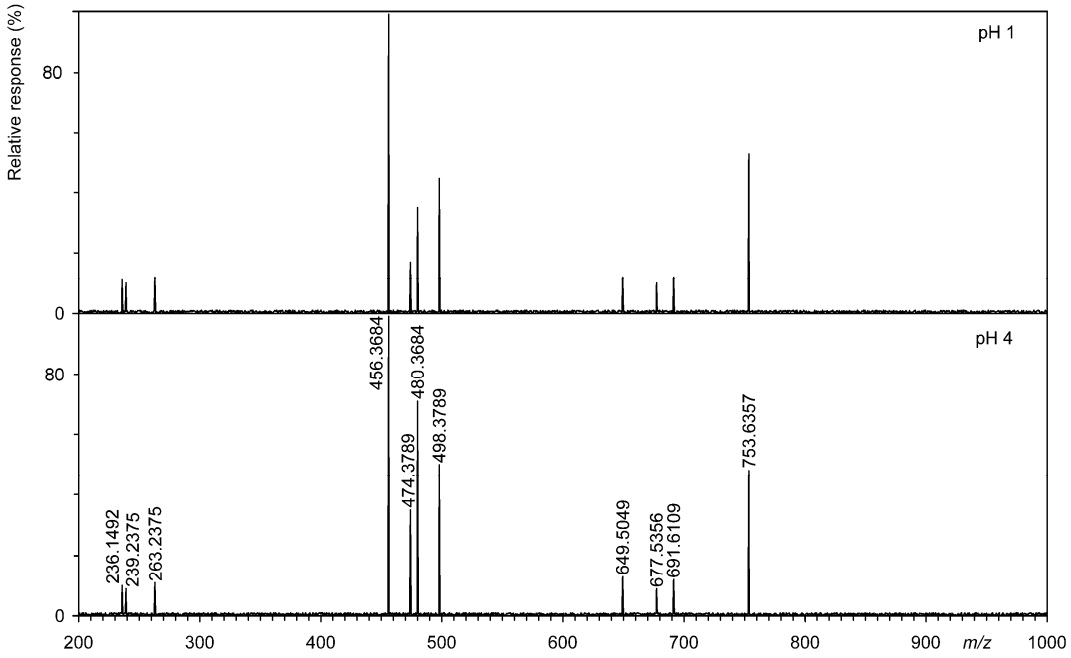


Fig. 5S. Natural 16:0/18:2-PA

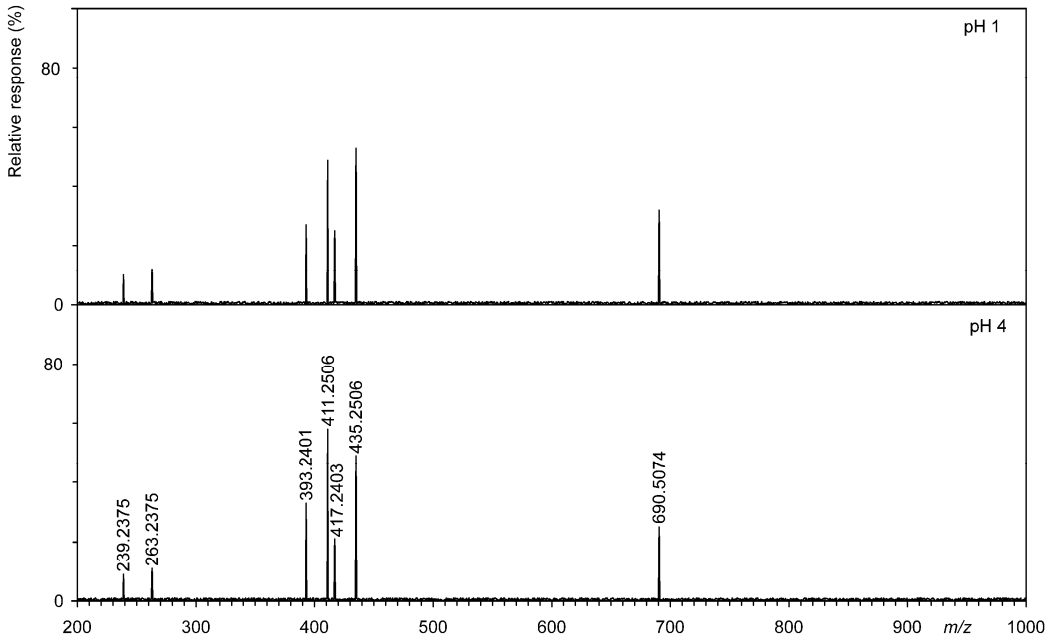


Fig. 6S. Natural 16:0/18:2-PE

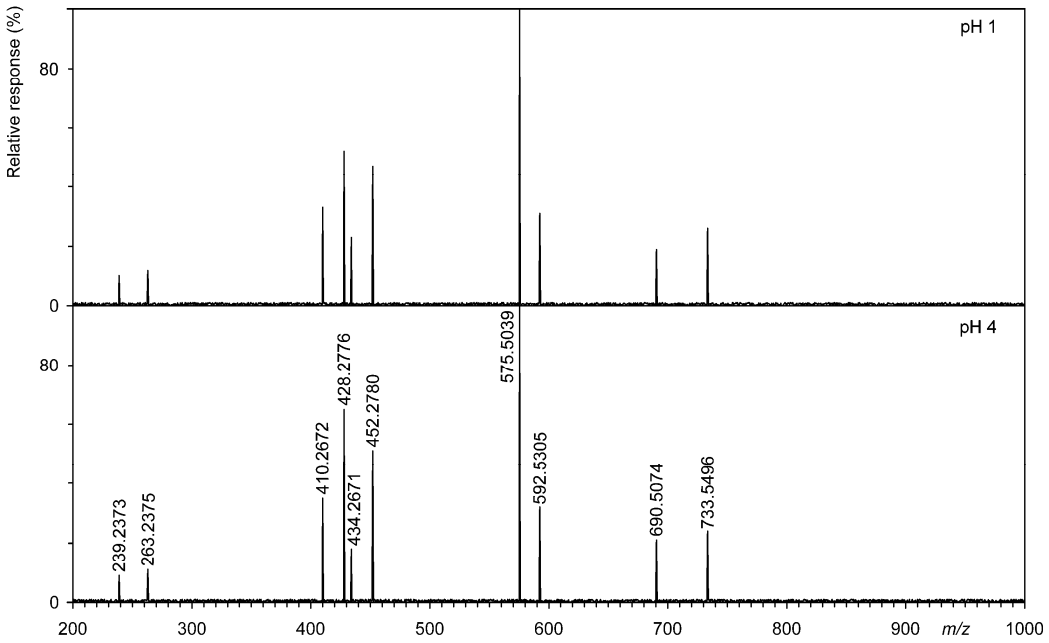


Fig. 7S. Natural 16:0/18:2-PG

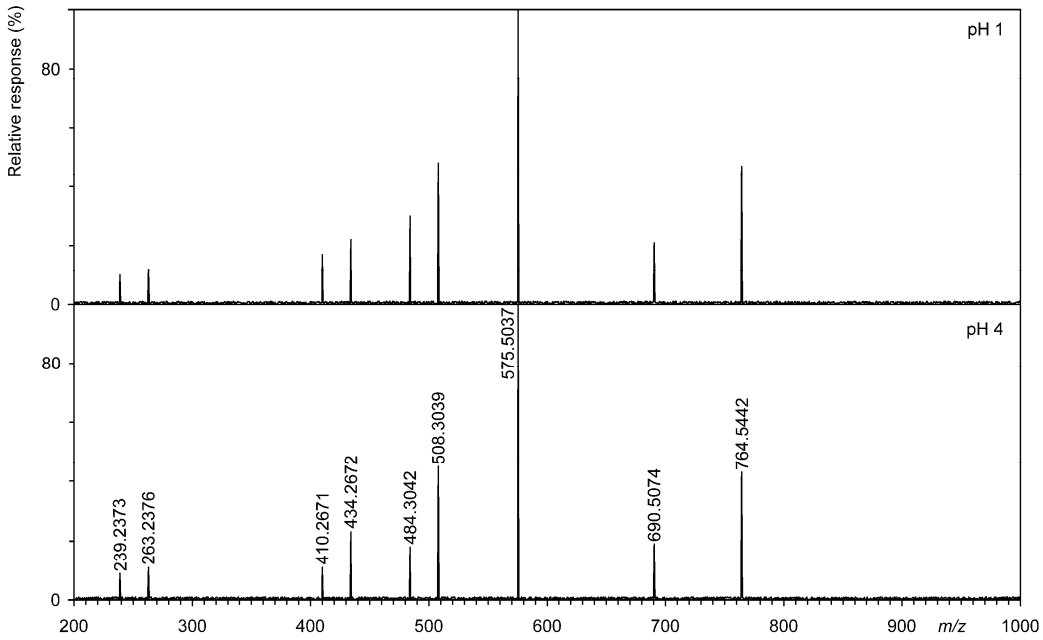


Fig. 8S. Natural 16:0/18:2-PSC