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Detailed structural characterization of cardiolipins from various biological sources using a complex analytical strategy comprising fractionation, hydrolysis and chiral chromatography

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ABSTRACT

Cardiolipins (1,3-bis(*sn*-3′-phosphatidyl)-*sn*-glycerol) (CLs) are widespread in many organisms, from bacteria to higher green plants and mammals. CLs were observed in Gram-positive bacterium of the genus *Kocuria*, brewer's yeast *Saccharomyces*, the green alga *Chlamydomonas*, spinach and beef heart. A mixture of molecular species of CLs was obtained from total lipids by hydrophilic interaction liquid chromatography (HILIC), and these were further separated and identified by reversed phase LC/MS with negative tandem electrospray ionization. The majority of CLs molecular species from each organism were cleaved using phospholipase C from *Bacillus cereus*. This phospholipase cleaves CLs into 1,2-diglycerols and phosphatidylglycerol 3-phosphates, which were then separated. After CLs cleavage, diacylglycerols such as *sn*-1,2-diacyl-3-acetyl-glycerols (i.e., triacylglycerols) were separated and identified by chiral chromatography/MS-positive tandem ESI. Significant differences in the composition of the molecular species between the 3-(3-*sn*-phosphatidyl) and 1-(3-*sn*-phosphatidyl) moieties of CLs were found in all organisms tested. Molecular species of CLs that contained four different fatty acids were identified in all five samples, and CLs containing very long chain fatty acids were identified in yeast. In addition, CLs containing both enantiomers (at the *sn*-2 carbon) were present in the bacterium tested. These findings were further supported by data already published in GenBank where, in the same family - Micrococcaceae - both enzymes responsible for chirality in the *sn*-2 position, glycerol-3-phosphate and glycerol-1-phosphate dehydrogenases, were present.

1. Introduction

Cardiolipins (CLs) is the trivial name for lipids with the structure 1,3-bis(*sn*-3′-phosphatidyl)-*sn*-glycerol. The trivial name "cardiolipin" is derived from the fact that this group of phospholipids was first found in the hearts of animals. These phospholipids have four acyl groups, three chiral centers and can carry up to two negative charges. This unique structure makes it one of the most difficult to identify, because, for example, with four different fatty acids (FAs), there can theoretically exist up to 256 (4^4) molecular species, including, regioisomers and enantiomers. Cardiolipins are present in both prokaryotic and eukaryotic cells. In bacteria, they are primarily components of membranes, and in eukaryotic organisms, they are located in the mitochondria. Schlame et al. [1] hypothesized that the dominant species of CLs contained only one or two types of fatty acids, which would result in a sharp decline in the number of possible molecular species. But with the 14 major fatty acids, including polyunsaturated forms, in eukaryotes can produce 38,416 distinct CLs species [2]. However, the development of chromatography and mass spectrometry has shown that this is not the case. For example, in the paper of Lee et al. [3], CLs with four different FAs were commonly present. From 121 CLs molecular species, a total of 17 CLs were identified, containing four different fatty acids, i.e. 14% of total CLs.

Cardiolipin, together with phosphatidylglycerol (PG), is the major phospholipid in bacteria, whether Gram-negative or Gram-positive. An example is the presence of CLs in *Escherichia coli* [4], where 53 CLs (with an odd or even number of carbons in acyl groups) from 60:2-CL to 72:0-CL (for nomenclature, see Ex-

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perimental methods), were identified by mass spectrometry (MS). Further, CLs in *Bacillus licheniformis* were analyzed by hydrophilic interaction liquid chromatography - electrospray ionization mass spectrometry (HILIC-ESI-MS) and several molecular species were identified [5]. For example, the 62:0-CL contained three molecular species, i.e. 15:0/15:0/15:0/17:0, 15:0/16:0/15:0/16:0, and 14:0/17:0/16:0/15:0. Bacteria of the genus *Kocuria* (formerly *Micrococcus*, [6]) contain CLs as one of the major phospholipids (see for example, dozens of articles published in the International Journal of Systematic and Evolutionary Microbiology). Analysis of *Rhodobacter sphaeroides* by reversed phase-high performance liquid chromatography (RP-HPLC) coupled with tandem MS (MS^2) with electrospray ionization (ESI) showed that, e.g., 64:1-CL is a mixture of three molecular species, with four different FAs present in each [7].

Molecular species of CLs have been identified in algae as well as in higher plants [8]. Another studied organism was the green alga *Chlamydomonas reinhardtii*. The gene encoding cardiolipin synthase [9] and fatty acids of CLs [10] have been identified in this alga. In addition to the classical plant model (*Arabidopsis thaliana*), barley leaves, mung bean hypocotyls, and spinach leaves were also analyzed by liquid chromatography-tandem MS. Molecular species having the same molecular weight, such as 18:3/18:2/18:2/18:1-CL versus 18:2/18:2/18:2/18:2-CL, were separated and subsequently identified by RP-HPLC/tandem MS [8].

Twenty-four CLs with different FAs were identified in a wild strain of *Saccharomyces cerevisiae* by LC-MS, in particular from 57:1-CL to 72:4-CL [11]. Unlike engineered yeast strains, in wild strains, no molecular species containing an unsaturation number of five have been identified. In the wild type *S. cerevisiae* BY4741, CLs were proven to contain up to four different FAs in one molecular species. Altogether, nineteen molecular species were identified by negative ESI tandem MS, five of which contained four different FAs [12].

Considerable attention has been paid to the analysis of CLs in mammals. Dozens of articles have been published, primarily describing the analysis of CLs by LC-MS [13–15]. One of the first attempts to analyze molecular species of CLs was made more than 40 years ago [16]. Total CLs were separated by RP-HPLC, individual peaks were collected and FAs were determined by gradient elution RP-HPLC. Twenty peaks were identified, and with few exceptions (18:3_18:3_18:2_18:0), each peak was a mixture of several molecular species of CLs. For several decades, it was believed [17] that only a few molecular species were present in mammalian hearts, and this phenomenon was called "molecular symmetry in cardiolipins". In recent years, mainly due to constantly evolving LC/MS techniques, dozens of molecular species have been identified in bovine heart CLs, both highly asymmetric (four different FAs in one molecule), as well as odd and even FAs [18]. Some CLs obtained from bovine heart and northern red snapper (*Lutjanus campechanus*) show the presence of four different FAs [19]. For example, CL 78:13-CL at *m/z* 1521.9801 is a mixture of twenty molecular species, nine of which contain four different FAs. It is clear from the above papers that the most commonly used modern analytical method is LC-MS with various modifications. Ideally, CLs are separated from other lipids, most often by HILIC, and further molecular species of CLs are separated by RP-HPLC. Identification is usually performed by ESI tandem MS, usually in negative mode.

Based on these papers and our previous articles describing the identification of regioisomers and enantiomers of different lipids, we analyzed CLs from five different organisms. The Gram-positive bacterium *K. rhizophila*, the green alga *C. reinhardtii*, the bottom-fermenting brewing yeast *S. pastorianus*, spinach and bovine heart were used to cover as wide a range of organisms as possible. Using several analytical techniques, i.e. hydrophilic interaction liquid chromatography (HILIC), reversed phase-high performance liquid chromatography/tandem high-resolution electrospray ionization mass spectrometry (RP-HPLC/HR-ESI-MS/MS) in negative ionization mode and enzymatic cleavage by phospholipase C, we were able to prove that in all five sources were present molecular species containing four different fatty acids.

2. Experimental methods

2.1. Chemicals and standards

Phospholipase C from *Bacillus cereus* (≥200 units/mg protein), preparative TLC Glass Plates L × W 20 cm × 20 cm, silica gel 60 matrix, polymeric binder, fluorescent indicator, 1′,3′-bis[1,2-dioleoyl-*sn*-glycero-3-phospho]-glycerol, 1′,3′-bis[1,2-dipalmitoleoyl-*sn*-glycero-3-phospho]-glycerol, 1′,3′-bis[1,2-dimyristoleoyl-*sn*-glycero-3-phospho]-glycerol, 1′,3′-bis[1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho]-glycerol (sodium salts), commercial bacterial FAMEs standard solution (BAME), PUFA No.1 Marine source, PUFA No.2 (Animal Source), PUFA No.3 from Menhaden Oil, analytical standards and all other reagents used were of analytical grade were purchased from Merck (previously Sigma-Aldrich and/or Supelco, Prague, Czech Republic). All other reagents used were of analytical grade and purchased from Merck (Prague, Czech Republic).

TAG enantiomers were prepared according to previously published papers [20–22]. Briefly, 13.2 mg (0.1 mmol) of 1,2- or 2,3-isopropylidene-*sn*-glycerol, 0.1 mmol of acetic acid, 13.4 mg (0.11 mmol) of 4-dimethylaminopyridine and 22.6 mg (0.11 mmol) of *N,N´*-dicyclohexylcarbodiimide in 2 mL of dichloromethane were stirred at 20°C for 2 h (Supporting Information Fig. S1). The acetonide was deprotected by the reaction with 0.3 mL of TFA at −20°C for 30 min and the mixture was then neutralized by a cold solution of 2 mol/L sodium hydroxide. The resulting monoacylglycerols were extracted by a mixture of chloroform:methanol (4:1, *v/v*, 3 mL) and the chloroform layer was evaporated. The monoacylglycerols were stirred with 2.2 mmol of palmitic acid, 0.22 mg of 4-dimethylaminopyridine, and 0.24 mmol of *N,N*≈dicyclohexylcarbodiimide in 2 mL of dichloromethane for 1.5 h at 20°C. The enantiomers were extracted from the reaction mixture using hexane, evaporated, and injected in hexane solution to the chiral LC–MS.

2.2. Sample preparation of organisms

Cultivation of the Gram-positive bacterium *Kocuria rhizophila* CCM 552 has been described previously, [19]. The cultivation of the green alga *Chlamydomonas reinhardtii* is described in Supplements. Bottom-fermenting brewer's yeast *Saccharomyces pastorianus* was obtained from a large brewery in the Czech Republic (yearly beer output of 130.000.000 L) [23]. Beef heart and spinach were purchased from a local butcher and gardener, respectively. Cells of all microorganisms were separated by centrifugation and lyophilized.

2.3. Isolation and fractionation of lipids

The extraction procedure was based on the described method [24] with modifications as previously described [25]. Briefly, the lyophilized cells were suspended in a dichloromethane:methanol mixture (2:1, *v/v*) for 30 minutes with stirring, after which dichloromethane and water were added and the dichloromethane phase was evaporated to dryness under reduced pressure. Total lipids were dissolved in the mobile phase (acetonitrile:2-propanol (99:1, *v/v*)) for further analysis by LC-MS.

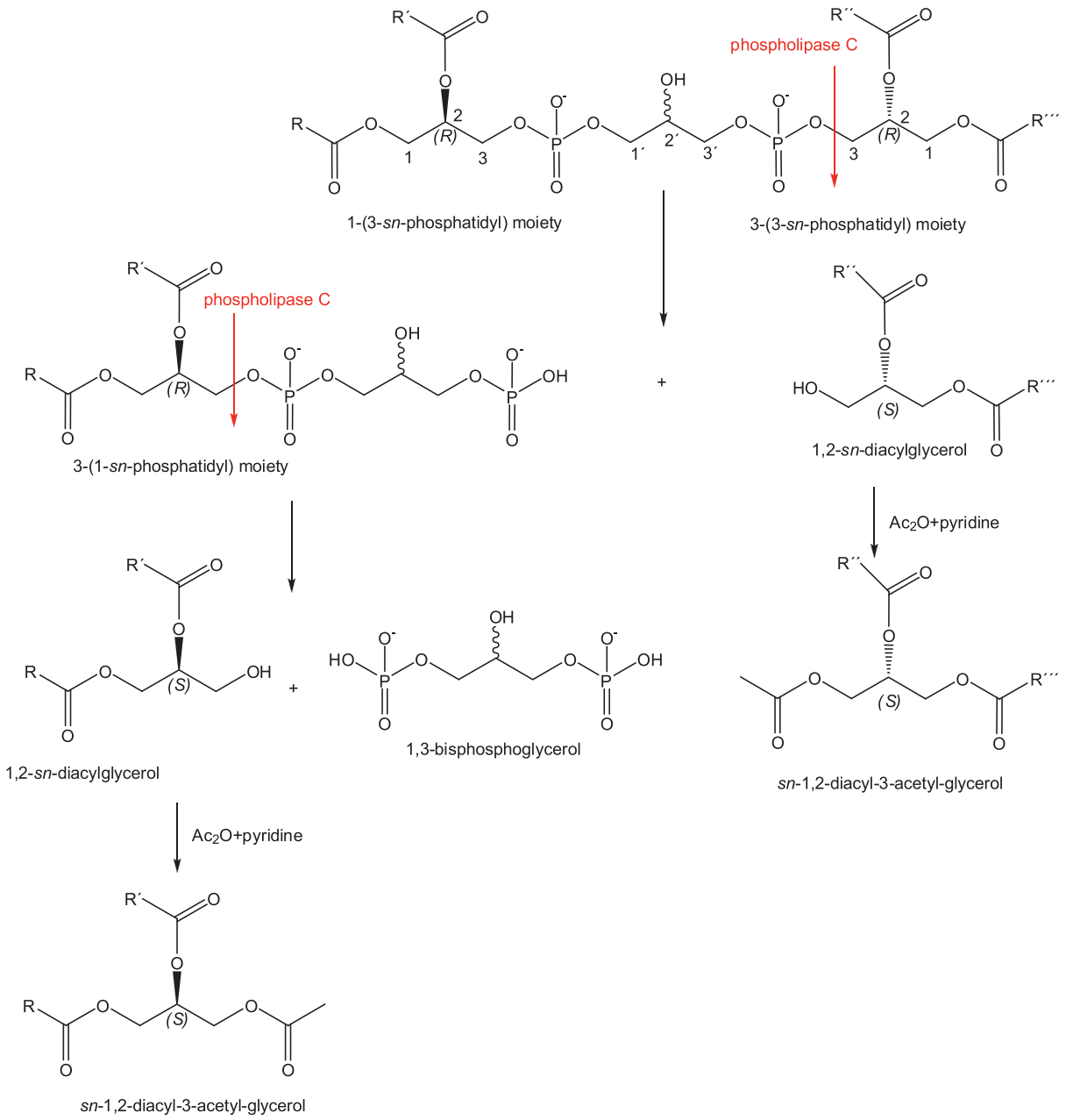

Fig. 1. Hydrolysis of cardiolipin by commercially obtained phospholipase C. The hydrolysis has two reaction steps. In the first, from the 3-(3-*sn*-phosphatidyl) group of CLs, 1,2-diglycerol is produced and at the same time phosphatidylglycerol 3-phosphate is formed from the 1-(3-*sn*-phosphatidyl) group of CLs. This is further hydrolyzed by excess phospholipase C and, with a longer incubation period, to 1,2-diglycerol, see [44].

2.4. Preparation and analysis of fatty acid methyl esters

Methyl esters were prepared from CLs using standard procedures. A detailed description of the preparation and the GC-MS analysis is provided in the Supplementary Information. The mass spectra in the electron ionization (EI) mode were analyzed as described previously [26,27].

2.5. Shotgun lipidomics

An LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA), a high-resolution hybrid mass spectrometer equipped with a heated electrospray interface (H-ESI), was operated in positive and negative ionization modes. Flow Injection Analysis (FIA) was used for sample introduction into the H-ESI ion source. Acetonitrile:water (50:50, *v/v*) was used at a flow rate of 150 μL/min. The MS scan range was performed in the Fourier transform mode and recorded within a window between 200–2000 *m/z*. Mass spectra were acquired with target mass resolution of R = 110,000 at *m/z* 800 and the ion spray voltage was set at 3.5 kV (in the positive ionization mode) and -2.5 kV (in the negative ionization mode). Both ionization modes used the following parameters: sheath gas flow, 18 arbitrary units (AU); auxiliary gas flow, 7 AU; ion source temperature, 250°C; capillary temperature, 230°C; capillary voltage, 50 V; and tube lens voltage, 170 V. Helium was used as a collision gas for collision-induced dissociation (CID) experiments. The CID normalization energy of 35% was used for the fragmentation of parental ions. The MS/MS product ions were detected in high resolution FT mode. The calibration of the MS spectrometer was conducted using a Pierce LTQ Orbitrap

Table 1
Identification of cardiolipin molecular species by shotgun analysis.

bacterium			yeast			alga			spinach			heart		
mol spec[a]	$[M-H]^-$	abun (%)	mol spec[b]	$[M-H]^-$	abun (%)	mol spec[c]	$[M-H]^-$	abun (%)	mol spec[d]	$[M-H]^-$	abun (%)	mol spec[e]	$[M-H]^-$	abun (%)
58:0	1267.8711	12.2	58:3	1261.8249	4.0	**64:10**	**1331.8085**	**14.6**	66:3	1373.9493	1.7	68:7	1393.9179	7.6
59:0	1281.8867	47.6	58:2	1263.8401	2.6	**64:6**	**1339.8704**	**79.3**	66:2	1375.9649	2.2	**68:6**	**1395.9336**	**5.3**
60:0	1295.9024	89.3	**58:1**	**1265.8593**	**1.5**	66:7	1365.8856	39.9	**68:6**	**1395.9336**	**4.2**	68:5	1397.9490	9.4
61:1[a]	**1307.9024**	**25.4**	60:3	1289.8574	7.2	66:6	1367.9009	15.7	68:5	1397.9490	5.3	68:4	1399.9650	3.1
61:0	1309.9180	100.0	**60:2**	**1291.8707**	**3.4**	66:5	1369.9165	21.2	68:4	1399.9650	3.0	**68:2**	**1403.9988**	**4.7**
62:1	1321.9182	7.2	60:1	1293.8904	1.8	**66:4**	**1371.9380**	**48.5**	70:10	1415.9024	2.4	69:8	1405.9181	1.1
62:0	**1323.9338**	**11.7**	62:3	1317.8840	16.4	**68:8**	**1391.9010**	**16.2**	70:9	1417.9180	2.9	68:1	1406.0119	3.3
63:1	1335.9336	6.4	62:2	1319.9010	7.1	**68:7**	**1393.9179**	**41.9**	70:8	1419.9335	7.6	69:7	1407.9337	1.0
63:0	1337.9493	15.9	62:1	1321.9182	2.6	**68:6**	**1395.9336**	**29.8**	70:7	1421.9492	9.3	69:6	1409.9493	1.2
64:1	**1349.9491**	**8.3**	64:4	1343.9041	14.2	68:5	1397.9490	27.8	**70:6**	**1423.9650**	**13.7**	70:10	1415.9024	13.4
64:0	1351.9650	11.4	64:3	1345.9184	9.6	**68:4**	**1399.9650**	**100.0**	70:5	1425.9806	5.6	70:9	1417.9180	8.6
65:1	**1363.9649**	**6.3**	64:2	1347.9318	5.8	70:8	1419.9335	16.2	70:4	1427.9960	3.9	70:8	1419.9335	11.6
65:0	1365.9806	9.5	**64:1**	**1349.9491**	**1.4**	**70:6**	**1423.9650**	**14.1**	72:12	1439.9023	15.8	70:7	1421.9492	14.4
66:0	1379.9962	5.2	66:4	1371.9380	60.2	70:5	1425.9806	58.6	72:11	1441.9178	27.2	**70:6**	**1423.9650**	**19.7**
67:0	1394.0119	3.1	66:3	1373.9463	28.9	72:12	1439.9023	25.3	72:10	1443.9337	87.1	70:5	1425.9806	25.9
			66:2	1375.9669	4.5	72:11	1441.9178	21.7	72:9	1445.9493	100.0	70:4	1427.9960	16.5
			66:1	**1377.9709**	**2.7**	72:10	1443.9337	17.7	72:8	1447.9650	42.8	71:8	1433.9493	1.1
			68:4	1399.9650	100.0	72:9	1445.9493	30.8	72:7	1449.9806	16.4	71:7	1435.9651	1.0
			68:3	1401.9769	31.8	72:8	1447.9650	16.2	**72:6**	**1451.9963**	**11.2**	72:12	1439.9023	26.3
			68:2	**1403.9988**	**36.8**	72:7	1449.9806	35.9	72:4	1456.0276	2.5	72:11	1441.9178	23.2
			70:4	1427.9960	78.2	**72:6**	**1451.9963**	**80.3**				72:10	1443.9337	18.4
			70:3	1430.0070	25.7	72:5	1454.0082	13.1				72:9	1445.9493	47.7
			70:2	1432.0336	4.4	72:4	1456.0276	7.1				72:8	1447.9650	100.0
			72:4	1456.0276	14.7							72:7	1449.9806	88.3
			72:3	1458.0362	3.8							**72:6**	**1451.9963**	**54.9**
												73:8	1461.9806	1.3
												74:10	1471.9648	31.6
												74:9	**1473.9806**	**8.7**
												74:8	**1475.9963**	**46.2**
												74:6	**1480.0276**	**24.6**
												76:11	1497.9806	65.3

[a] Bold are CLs that have been found by MS^2 to contain four FAs, for example molecular species having $[M-H]^-$ at *m/z* 1395.9336, see Fig. 2S (Supplements).

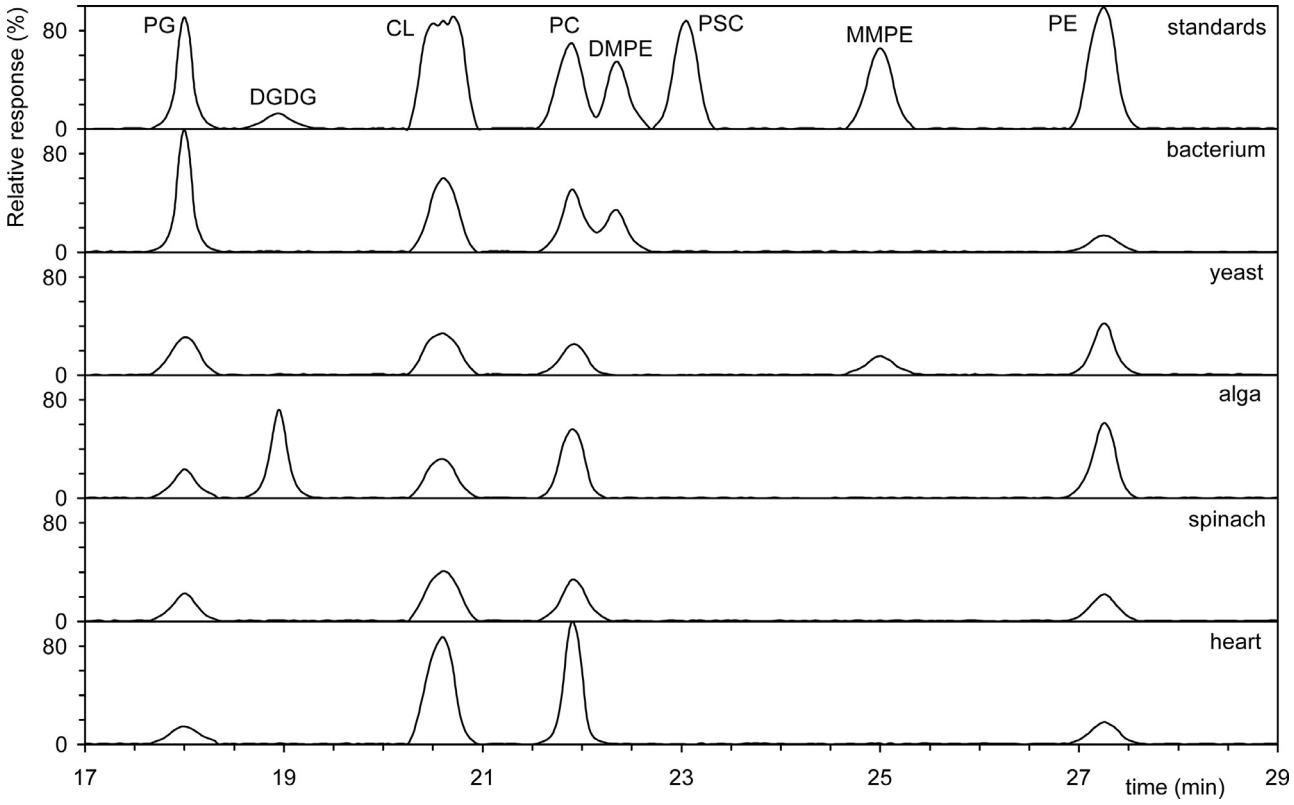

Fig. 2. Hydrophilic interaction liquid chromatography - HILIC/ESI-MS separation of major lipid classes of the five different biological sources (Gram positive bacterium *Kocuria rhizophila*, green alga *Chlamydomonas reinhardtii,* bottom brewer's yeast *Saccharomyces pastorianus*, spinach, and bovine heart). The TIC (total ion current) was filtered and exhibits ions in the interval from 200 to 1900 Da. Abbreviations: phosphatidylglycerol (PG), digalactosyldiacylglycerol (DGDG), cardiolipin, i.e. diphosphatidylglycerol (CL), phosphatidylcholine (PC), dimethyl PE (DMPE), phosphatidylsulfocholine (PSC), monomethyl PE (MMPE), phosphatidylethanolamine (PE).

Table 2
Cardiolipins containing very long chain fatty acids from yeast.

$[M-H]^-$	Molecular species of cardiolipins	Abundance[a]
1736.3406	22:1_24:1_24:1_22:1	1.00
1740.3719	22:0_24:0_24:1_22:1	0.73
1744.4032	22:0_24:0_24:0_22:0	0.53
1796.4345	24:0_24:0_24:1_24:1 + 22:0_26:0_24:1_24:1	1.05
1800.4658	22:0_26:0_26:0_22:0	0.41
1828.4971	22:0_24:0_26:0_26:0	0.36
1852.4971	26:0_26:0_24:1_24:1	0.39

[a] abundance of total CLsDue to the natural representation of the isotopes ^{13}C and ^{2}H, i.e. D, the ion at *m/z* 1741.3752 with the formula $C_{100}{}^{13}C_1H_{193}O_{17}P_2{}^-$ has a higher abundance than the ion $[M-H]^-$ at *m/z* 1740.3718 with the formula $C_{101}H_{193}O_{17}P_2{}^-$. The ion abundance ratio is 100:91.5.

positive and/or negative ion calibration solution (Thermo Fisher Scientific, San Jose, CA, USA). The internal lock mass was used in mass spectra acquisition, i.e., 255.2330 *m/z* $[M-H]^-$ palmitic acid in the negative ESI. The mass accuracy was better than 0.9 ppm. The chemical structures of compounds were confirmed with the help of the spectral database LIPID MAPS® Lipidomics Gateway (http://www.lipidmaps.org/).

2.6. HILIC-ESI-MS

The HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Hewlett Packard, USA), and Ascentis® Express HILIC, 2.7 μm particle size, HPLC Column, L × I.D. 150 mm × 4.6 mm Merck (formerly Supelco, Prague, Czech Republic) with an injection volume of 30 µL. HPLC was performed at a flow rate of 1.03 mL/min 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. The column temperature was 35°C and the re-equilibration period between runs was 30 min. The 10% HPLC flow was introduced into the ESI source and 90% of the flow containing fractions of lipid classes were collected manually. In order to have sufficient material, the fractionation was repeated up to five times and the corresponding fractions were pooled, evaporated to dryness and re-dissolved in an appropriate volume of mobile phase and used for RP-HPLC. The system backpressure reached 1000 bars during the gradient analysis. The LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany) high-resolution hybrid mass spectrometer was used for the ESI-MS analysis, under conditions as described above.

2.7. Separation and identification of CLs by RP-HPLC/MS^2

The LC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Agilent, Santa Clara, CA, USA) and three Luna Omega 1.6 µm, C18, 100 Å, LC Columns L × I.D. 150 × 2.1 mm, connected in series. Although the efficiency stated by the manufacturer was not achieved, i.e. 350,000 theoretical plates/meter (see manufacturer's literature), the efficiency achieved in our case was ~311,000 theoretical plates/meter. 1′,3′-bis[1,2-dimyristoleoyl-*sn*-glycero-3-phospho]-glycerol with tR 63.9 min was used as an internal standard, with a flow rate of 0.95 mL/min, an injection volume of 10 μL, and column temperature of 25°C. The five pooled corresponding fractions of CLs from HILIC were separated using a solvent gradient program with the solvent A:B mixture from 50% B to 100% B in 80 min (flow rate 1.0 mL/min). Solvent A contained acetonitrile-2-propanol (1:1, *v/v*) and solvent B contained acetonitrile-2-propanol (2:3, *v/v*), both con-

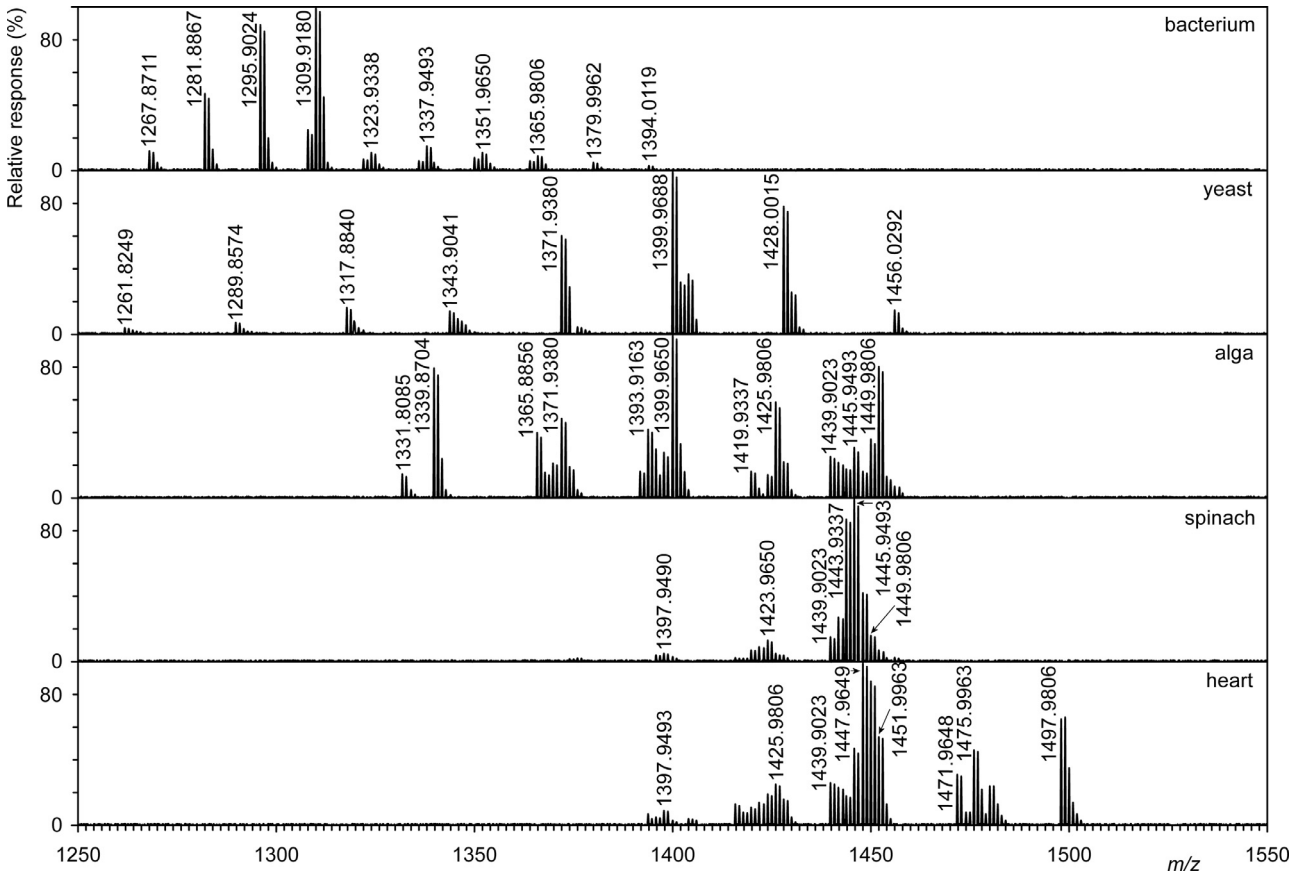

Fig. 3. The high resolution MS spectra of CLs (in ESI negative ionization mode) acquired by the shotgun lipidomic analysis of five different biological materials. Numbers correspond to the accurate mass values; the details to all detected mass peaks are provided in Table 1.

taining 2.5 mmol/L of ammonium acetate from 5 min to 105 min, and a hold for 10 min. The composition was returned to the initial conditions over 10 min. The 10% HPLC flow was introduced into the ESI source and 90% of the flow containing molecular species of CLs were collected manually. For ESI-MS analysis, the LTQ Orbitrap Velos was used, under conditions as described above.

2.8. Phospholipase C hydrolysis

Corresponding peaks (nine runs) eluted at different intervals were pooled, evaporated to dryness and hydrolyzed by phospholipase C. Approximately 1 mg of CLs and commercially available phospholipase C (2 units) from *Bacillus cereus* were dispersed in 1 mL Tris buffer (17.5 mM; pH 7.3) with 1% calcium chloride (0.1 mL) and diethyl ether (0.3 mL) [28]. The mixture was shaken vigorously at 37°C for approximately 30 min. The degree of hydrolysis was determined by TLC (silica gel with fluorescent indicator on 20 cm × 20 cm TLC plates; Merck, Prague, Czech Republic), mobile phase dichloromethane:methanol:acetic acid (65:25:8, vol/vol/vol)). 1,2-Diacylglycerols from the 3-(3-*sn*-phosphatidyl) group of cardiolipin and phosphatidylglycerol 3-phosphate from the 1-(3-*sn*-phosphatidyl) group of cardiolipin were isolated, see Fig. 1. Complete polar head cleavage of phosphatidylglycerol 3-phosphate was performed by repeated hydrolysis using phospholipase C. The 1,2-diacylglycerols formed by both cleavages were esterified as previously described [20]. Briefly, the resulting DAGs were acetylated [29] by standing overnight in a mixture of acetic anhydride:pyridine (3:2, *v/v*) at 20°C to 1,2-diacyl-3-acetyl-*sn*-glycerols i.e. AcTAGs. These AcTAGs were further separated and identified by chiral-HPLC-ESI-MS^2.

2.9. AcTAG analysis using chiral-HPLC-ESI-MS^2

The LC system used for separation in the chiral mode was the same as the system used in the reversed-phase mode. AcTAGs (~1 mg/mL in mobile phase) were chromatographically separated on two Astec cyclobond TM I 2000 DMP (3,5-dimethyl-phenyl- carbamate modified β-cyclodextrin) chiral LC columns (5 μm, 25 cm × 4.6 mm) (Sigma-Aldrich) connected in series. The mobile phase was a gradient from 95% A and 5% B at 0 min to 45% A and 55% B over 180 min, and then isocratically maintained for 30 min, where A was hexane and B was a hexane-2-propanol (97:3, *v/v*) mixture [30,31]. The flow rate, column temperature, and injection volume were 0.45 mL/min, 24°C, and 10 μL, respectively. Other conditions were the same as those used for RP-HPLC/MS^2, as described above. The elution order of enantiomers from the chiral column was determined on the tR of both synthetically prepared isomers, see subchapter 2.1 (Chemicals and standards).

2.10. Limits detection and quantitation

The limit of detection (LOD), and the limit of quantitation (LOQ) of LC/ESI-MS method were determined based on the analysis by (14:0)-CL with the final concentration (10 fM, 100 fM, 1 pM, 10 pM, 100 pM, and 1 nM, 1 nM). LOD and LOQ were determined from signal-to-noise ratios 3 and 10, respectively. The reproducibility of the peak area was determined from six consecutive (14:0)-CL measurements for low (100 fM) and high (1 nM) concentrations. The LOD of (14:0)4-CL) was 147 fmol (186 pg (picogram, i.e. 10^{-12})) which is an order of magnitude identical to the LOD reported by Lee et al. [3], who published LOD = 105.2 (fM) for $(14:0)_4$-CL. The LOQ we measured was 490 fM.

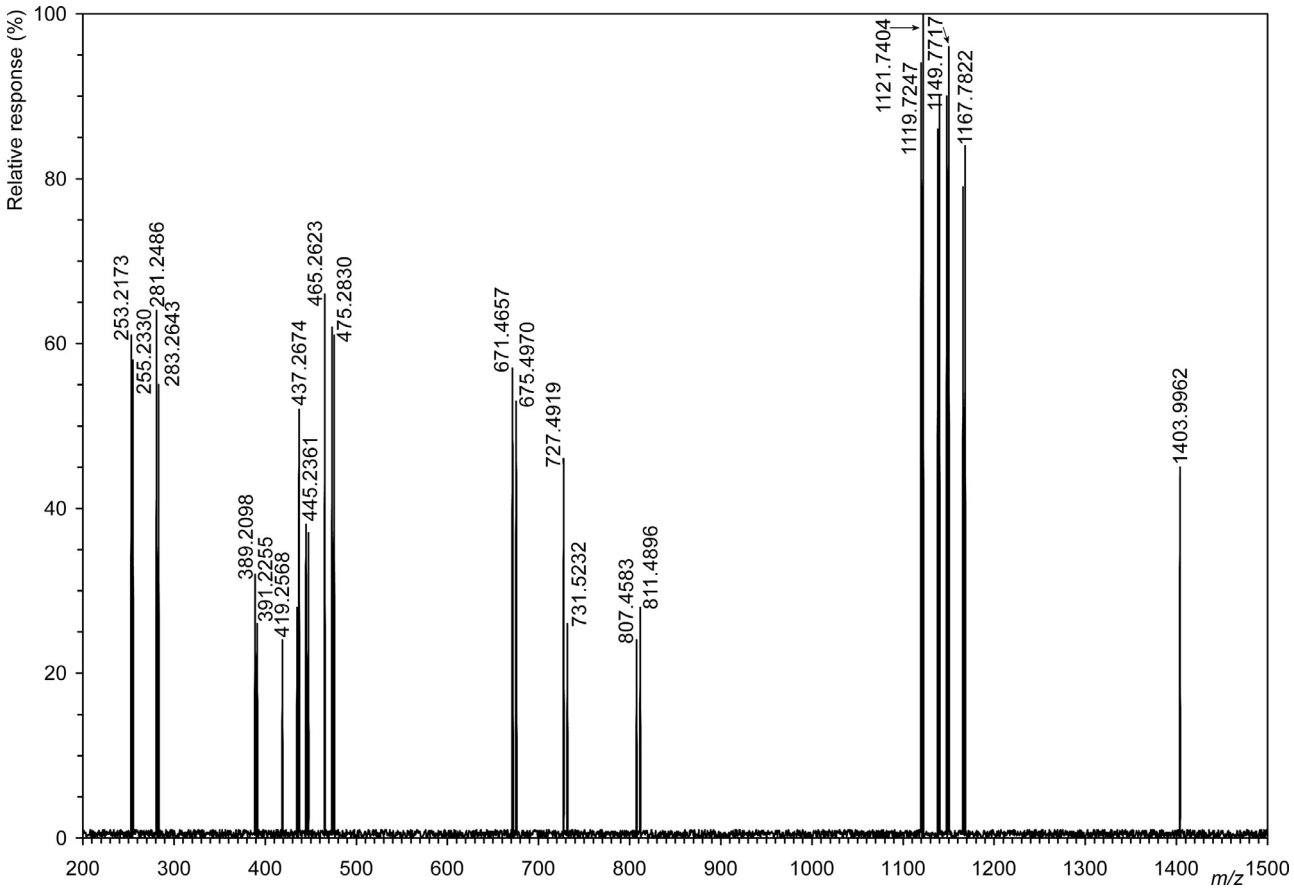

Fig. 4. Tandem MS of 68:2-CL (yeast) 16:0_18:0_16:1_18:1-CL. For experimental details, see Experimental methods, in all figures, including mass spectra, see Results.

2.11. Statistical analysis

The statistical analysis was performed using the IBM SPSS Statistics (Statistical Package for the Social Sciences; IBM Corp, 2013) Statistics software, version 26 (IBM® Corporation, Armonk, NY, USA).

2.12. Lipid nomenclature

Lipid nomenclature was applied according to Liebisch et al. [32], see Supplementary Information.

3. Results and discussion

3.1. Analysis of fatty acids and HILIC

Individual samples from all five organisms were lyophilized and extracted according to Bligh-Dyer [24] for simultaneous inactivating phospholipases [25]. Fatty acids of total cardiolipins were analyzed and identified by GC-MS (Table 1S Supplements). The analysis of FAMEs confirmed the presence of such fatty acids as are described in the literature (bacteria [20], yeast [23], alga [9] spinach [8], heart [16]).

Total lipids were separated by HILIC chromatography. The chromatogram depicted in Fig. 2 shows the separation of total lipids over the interval from 17 to 29 min and demonstrated a base-line separation of all major lipid classes. Despite the fact that HILIC separation is a commonly used method for separating total lipids into individual classes and was previously utilized for separation of CLs from bacterium *K. rhizophila* [20], yeast *S. pastorianus* [23], green algae [33], spinach, i.e. a dicotyledonous plant [8], and mammalian heart [13], the arrangement of HILIC chromatography used in our study was not sufficiently effective for separation of the standards ($(14:1)_4$-, $(16:1)_4$-, and $(18:1)_4$-CLs).

3.2. Shotgun lipidomic analysis

After HILIC separation, CLs were first analyzed by shotgun lipidomics (Fig. 3). Dozens of molecular species were identified and their descriptions and abundance are given in Table 1. It is clear that the prokaryotic organism *K. rhizophila*, had a completely different CLs composition from the other four eukaryotic organisms. *S. pastorianus* differed from other eukaryotic organisms in its content of fatty acids (see also below). In the eukaryotes algae, dicotyledonous plants and mammals, the CLs contained polyunsaturated fatty acids from 16:4 (algae) to 20:5 (heart), (Table 1).

Fragmentation of CLs [34] was used to determine the structure of individual molecular species. As an example, one species had a molecular mass value of 1403.9962 Da for the $[M\text{-}H]^-$ ion. The tandem mass spectrum is shown in Fig. 4. The probable structure and *m/z* values of appropriate ions are given in Table 2S. Diagnostic ions confirming the structure 16:0_18:0_16:1_18:1-CL (68:2-CL, named according to the nomenclature, see Supplements). This structure was supported by six ions of type **a,** **a**+56, **a**+136, **b,** **b**+56, and **b**+136 (according to Hsu et al. [35]). This procedure was also performed for other molecular species. The mixture of CLs at *m/z* 1395.9336 ($[M\text{-}H]^-$) comprised a total of seven $RCOO^-$ type ions that could be identified by tandem MS (ions of the type $RCOO^-$ of appropriate fatty acids - 16:4, 16:1, 16:0, 18:3, 18:2, 18:1, and 18:0), (Fig. 2S). Similarly, CLs that contained four different fatty acids were identified. In Table 1, the relevant molecular species are indicated in bold. Separation of CLs molecular species having the

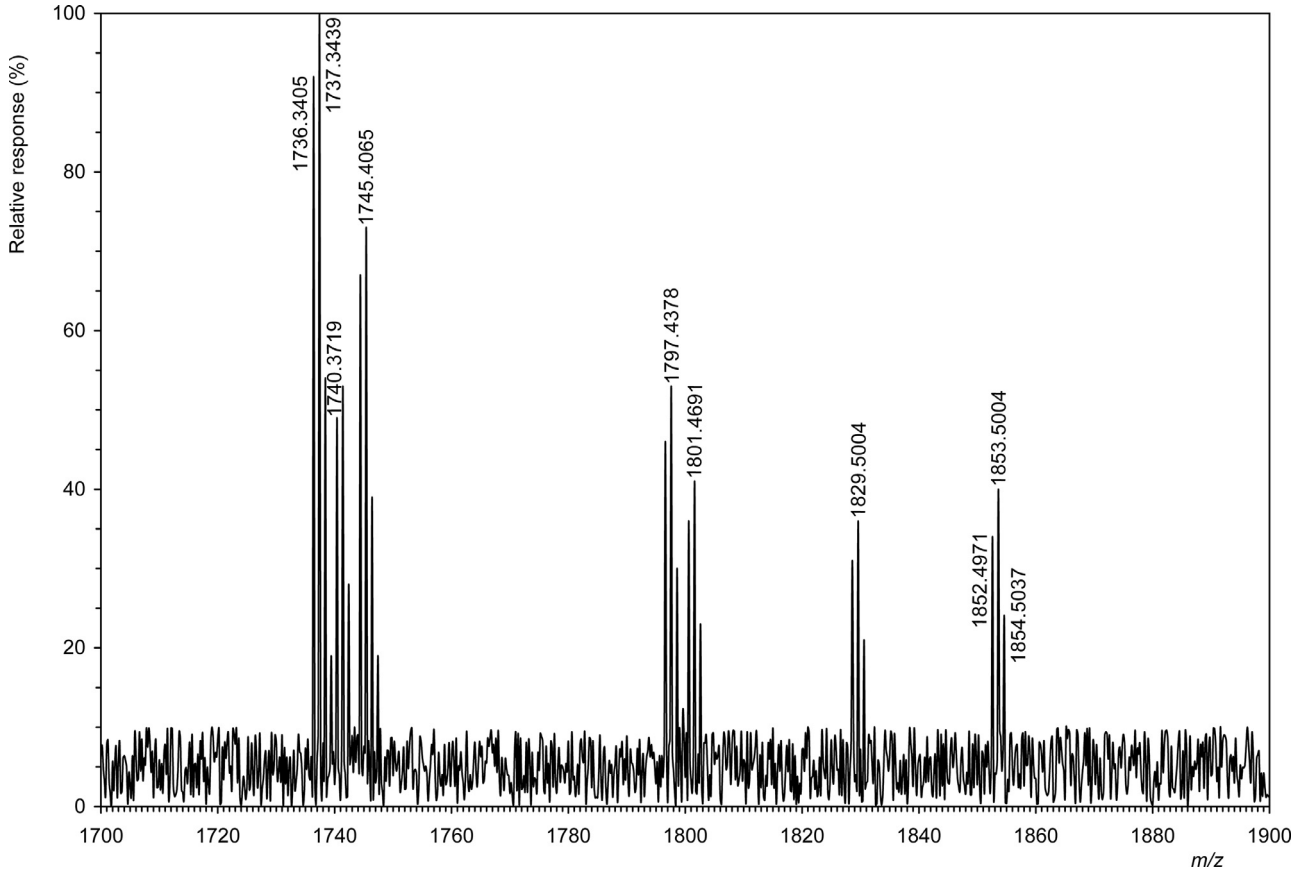

Fig. 5. Shotgun lipidomic analysis of CLs from yeast. Only the interval of cardiolipinś [M-H]$^-$ containing very long chain fatty acids is shown.

same summary formula and differing only in FAs is very difficult. We identified only one paper [8] where two or even three molecular species having the same summary formula were separated from each other; for example, a mixture of CLs 72:9, in which fatty acids 18:3_18:2_18:2_18:2 (tR = 16.0538 min) and 18:3_18:3_18:2_18:1 (tR = 16.3526 min) were identified (see their Table 1).

Yeast CLs have a diversity of fatty acid chains. For this reason, we measured ions up to 2000 Da. Based on the extension of this window, several ions in the region of 1700 to 1900 Da were identified (Fig. 5). After further analysis, i.e. tandem MS, see e.g. ion at *m/z* 1740.3719 ([M-H]$^-$), it was possible to prove that molecular species containing very long chain fatty acids (VLCFAs) were present. These molecular species are listed in Table 2. The following acid moieties (acyls) at *m/z* 339.3269 (docosanoic acid, 22:0 FA), 367.3582 (tetracosanoic acid, 24:0 FA), 365.3425 (tetracosenoic acid, 24:1 FA), and 337.3112 (docosenoic acid, 22:1 FA) were identified. The MS^2 spectrum was dominated by the [M-H]$^-$ ion at *m/z* 1740.3719; the ion corresponding to the double charged [M-2H]$^{2-}$ ion at *m/z* 870.1764 was minor under the conditions used. The spectrum (Fig. 6) was further dominated by six ions, designated by Hsu et al. [35] as **a, b, a**+56, **b**+56, **a**+136 and **b**+136 having *m/z* values 843.6848, 839.6535, 899.7111, 895.6797, 979.6774, and 975.6461 Da (Fig. 3S). Based on the *m/z* values of these ions, two phosphatidyl moieties, C46:0 and C46:2, were identified in the CLs molecules. Furthermore, other ions, the structures of which are shown in Table 2S, were found. These ions were formed by further cleavage of phosphatidic acid-like or phosphatidylethanolamine-like fragmentation, as described by Hsu et al. [35]. Similarly, the structure of the ions **a**(**b**)+56 and **a**(**b**)+136, and their fragmentation products, have been proven. The final step was to identify the connection between the two diacylglycerol moieties and the central glycerol, and this was achieved based on the tandem MS of [M-H]$^-$ finding that the ion **a** at *m/z* 843.6848 had a higher abundance than the ion **b** at *m/z* 839.6535, as previously described [35]. Based on this, the structure 22:0_24:0_24:1_22:1-CL was assigned to CLs containing four different FAs. Their presence in yeast phospholipids is not so surprising, since the presence of VLCFAs has already been described in phosphatidylethanolamines and/or phosphatidylcholine [23].

This analytical method makes it possible to determine up to hundreds of molecular species in a very short time, as has been described previously [36]. Based on shotgun lipidomics and tandem MS, it was confirmed, (Table 1), that CLs molecular species containing four different FAs existed in all five samples, and twenty-eight CLs containing four fatty acids were identified by MS^2.

3.3. Separation and identification of CLs by RP-HPLC/MS_2

The separation of mixed CLs from all five sources is shown in Fig. 7. Relevant peaks after RP-HPLC (marked in bold, Fig. 7) were collected and hydrolyzed by phospholipase C. Relevant peaks were those for which the appropriate molecular species was shown to contain four different fatty acids, determined by shotgun analysis MS^2. For peaks that were not separated on the base line, only a part of the peak was collected. The phospholipase C selectively hydrolyzes molecular species of CLs, as was previously described [28] and is also shown in Fig. 1. The difference in hydrolysis rate, i.e. the preferential cleavage of 3-(3-phosphatidyl moiety of CLs) was used to determine the CLs regioisomers. The resulting diacylglycerols (DAGs) were separated by TLC from phosphatidyl glycerol

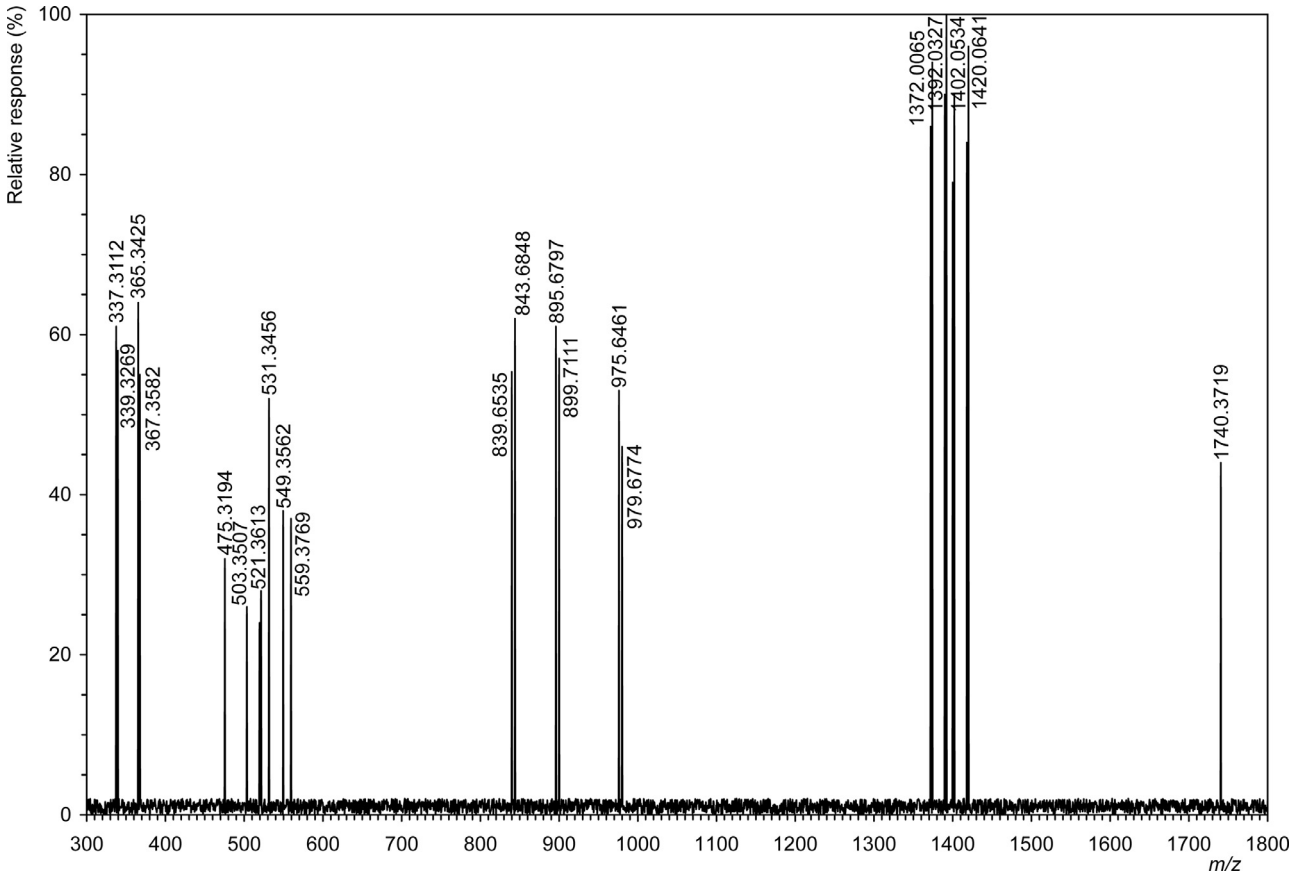

Fig. 6. Tandem MS of ion at *m/z* 1740.3719 ([M-H]$^-$). Based on the m/z values for the four acyls, i.e. at m/z 339.3269 (docosanoic acid), 367.3582 (tetracosanoic acid), 365.3425 (tetracosenoic acid), and 337.3112 (docosenoic acid) and six dominant ions a, b, a+56, b+56, a+136 and b+136, the structure of cardiolipin was determined to be 22:0_24:0_24:1_22:1-CL.

3-phosphates, which were further cleaved by excess of phospholipase C to form DAGs. The resulting DAGs were acetylated to give *sn*-1,2-diacyl-3-acetyl triacylglycerols (AcTAGs) and they were identified by chiral-HPLC-tandem MS as described previously [21].

3.4. AcTAG analysis using chiral-HPLC-ESI-MS^2

In this work, we furthered the use of chiral-HPLC analysis. The individual AcTAGs obtained by selective hydrolysis of the respective molecular species of CLs by phospholipase C (see Experimental methods and Fig. 1), and subsequent derivatization, were separated as AcTAGs. The analysis of AcTAGs was not a problem and thus proved the structure of native CLs (Table 3), e.g. 64:10-CL. Two AcTAGs were identified by chiral-HPLC-tandem MS, i.e. *sn*-16:2/16:3/2:0 from the 3-(3-phosphatidyl moiety of CLs) and *sn*-16:1/16:4/2:0 from the 3-(1-phosphatidyl moiety of CLs). The key ions confirmed the above structures were four neutral loss ions of RCOOH + NH_3 from $[M+NH_4]^+$ having values of 351.2530, 349.2373, 353.2686, and 347.2217 Da. The resulting structure of native 64:10-CL was *sn*-16:2/16:3/16:1/16:4-CL. The procedure was similar for 64:6-CL, where four major ions at m/z 353.2686, 351.2535, 355.2848, and 349.2379 were again identified by tandem MS. Other CLs were similarly analyzed in this way and are given in Table 3. In some cases, the CLs identified in two organisms (alga and yeast) using a shotgun lipidomic approach contained the same number of carbon atoms and the same number of double bonds (e.g. 68:4-CL). However, the resulting analysis using AcTAGs gave completely different results. In alga, *sn*-16:1/18:1/18:2/16:0-CL was present (key ions neutral loss of RCOOH + NH_3 from $[M+NH_4]^+$ at m/z 381.2999, 379.2843, 355.2843, and 353.2686), whereas in yeast, highly symmetrical 16:1/18:1/18:1/16:1-CL was obtained. In the second molecular species, key ions of the neutral loss type RCOOH + NH_3 from $[M+NH_4]^+$, were obtained, having values of 381.2999 and 353.2686 Da, (Tables 3Sa-3Sc for the structure of AcTAGs ions and Fig. 4S for the mass spectra).

If the peak from RP-HPLC/MS^2 of CLs contained two or more molecular species, analysis of the structure of the appropriate CLs was more difficult. An example is 61:1-CL from *K. rhizophila*. Following isolation of the mixture of DAGs after hydrolysis by phospholipase C and separation by chiral-HPLC, four AcTAGs, i.e. 15:0/15:0/2:0, 15:0/16:1/2:0, 14:0/15:0/2:0 and 16:0/16:1/2:0 were identified. Based on these results, it was possible to determine that the native 61:1-CL contained two molecular species, namely *sn*-15:0/15:0/15:0/16:1-CL and *sn*-14:0/15:0/16:0/16:1-CL. In other cases, the procedure was similar. However, it is important to mention that, for example, in the case of 72:6-CL, the analysis of a molecular species containing four different FAs, i.e. stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) acids was unsuccessful. We believe that further progress can, in principle, only be achieved with the successful separation of molecular species of native CLs. The theoretically possible number of molecular species of CLs containing four different fatty acids is 256 (4^4), including the regioisomers and enantiomers.

Another example where FAs in CLs differed diametrically was 70:6-CL, where 18:2/18:2/18:2/16:0-CL was dominant in the heart, whereas for the alga and spinach, it was 18:3/18:2/18:1/16:0-CL. Based on this analysis, we determined that the molecular species of CL in mammalian tissue contained only two FAs, whereas in

Table 3
RP-HPLC of CLs from five sources.

tR (min)	heart	spinach	alga	yeast	bacterium	ACN:DB[a]	mol spec_1	mol spec_2	mol spec_3
38.08	0.09	0.24	**2.01**	0.00	0.24	64:10	16:1_16:2_16:3_16:4		
47.21	0.05	0.16	0.06	0.88	0.00	58:3			
48.82	3.96	4.08	3.49	0.13	0.24	72:12			
53.38	0.08	0.22	0.07	0.57	0.08	58:2			
54.23	3.50	7.34	2.99	0.13	0.03	72:11			
55.40	0.02	0.22	0.01	1.58	0.03	60:3			
58.79	2.74	23.64	2.44	0.13	0.24	72:10			
59.04	0.11	0.08	**10.94**	0.15	0.03	64:6	16:0_16:1_16:2_16:3		
59.38	0.06	0.08	0.03	**0.75**	0.19	60:2	14:1_14:0_16:0_16:1		
59.63	0.08	0.03	0.11	**0.33**	0.16	58:1	12:0_14:0_16:0_16:1		
60.88	0.12	0.11	**2.23**	0.18	0.03	68:8	16:1_18:1_18:2_16:4	16:1_18:1_16:1_18:3	
62.73	0.15	0.16	0.10	3.59	0.05	62:3			
63.62	7.16	27.17	4.25	0.09	0.00	72:9			
64.73	0.02	0.08	0.11	0.07	3.20	58:0			
64.81	4.72	0.08	0.10	0.04	0.05	74:10			
64.93	1.68	1.90	2.23	0.09	0.05	70:8			
64.98	0.05	0.00	2.17	0.04	0.11	66:6			
66.02	0.06	0.14	0.07	1.56	0.21	62:2			
66.23	1.07	0.11	**5.78**	0.15	0.16	68:7	16:1_18:1_18:2_16:3		
66.82	9.90	0.19	0.11	0.09	0.19	76:11			
67.13	0.03	0.14	0.01	0.22	**6.67**	61:1	15:0_15:0_15:0_16:1	14:0_15:0_16:0_16:1	
67.52	0.11	0.08	0.04	0.04	12.54	59:0			
68.39	0.12	0.24	0.06	3.11	0.08	64:4			
69.63	15.23	11.41	2.23	0.02	0.08	72:8			
69.98	**1.22**	0.14	0.12	0.20	0.13	74:9	18:2_18:2_18:1_20:4	18:1_18:2_18:2_20:4	18:0_18:2_18:3_20:4
70.18	2.13	2.45	0.08	0.07	0.08	70:7			
70.47	0.02	0.03	2.92	0.13	0.16	66:5			
70.95	**0.76**[b]	**1.09**[b]	**4.11**[b]	0.11	0.08	68:6			
71.18	0.12	0.16	0.10	0.13	23.75	60:0			
71.42	0.08	0.03	0.01	2.10	0.19	64:3			
70.95	0.14	0.19	0.12	0.57	1.87	62:1			
73.70	0.11	0.14	**6.69**	13.20	0.03	66:4	16:1_18:1_18:0_16:2		
74.54	0.00	0.11	0.03	1.27	0.03	64:2			
74.77	0.03	0.08	0.04	0.11	26.68	61:0			
75.14	13.40	4.35	4.95	0.07	0.16	72:7			
76.03	2.89	**3.53**	1.94	0.09	0.27	70:6	18:2_18:2_18:2_16:0	18:3_18:2_18:1_16:0	
76.51	**7.00**	0.24	0.08	0.13	0.21	74:8	18:0_18:1_18:3_20:4	18:2_18:2_18:0_20:4	18:1_18:2_18:1_20:4
76.93	0.08	0.22	0.03	0.11	1.60	63:1			
78.35	0.06	0.46	0.06	6.33	0.16	66:3			
79.13	0.46	0.82	13.79	**21.90**	0.00	68:4	16:1_18:1_18:0_16:2	16:1_18:1_18:1_16:1	
80.26	0.09	0.03	0.07	0.15	**2.93**	62:0	15:0_15:0_16:0_16:0	14:0_15:0_16:0_17:0	
80.63	3.81	1.36	8.08	0.15	0.16	70:5			
80.94	**8.22**[c]	**1.90**[c]	**11.08**[c]	0.13	0.08	72:6			
81.42	0.00	0.27	0.08	**0.31**	**2.13**	64:1	15:0_16:0_16:0_17:1	15:0_16:0_16:1_17:0	14:0_16:0_16:1_18:0
82.63	0.14	0.27	0.10	0.09	0.13	74:7			
82.41	0.05	0.60	0.12	0.99	0.24	66:2			
84.20	2.44	1.06	0.03	17.14	0.03	70:4			
84.43	0.02	0.22	1.81	0.22	0.21	72:5			
85.22	0.11	0.19	0.10	0.13	4.00	63:0			
85.63	**3.65**	0.22	0.06	0.20	0.19	74:6	18:0_18:1_18:2_20:3		
87.51	0.11	0.05	0.12	0.13	**1.60**	65:1	16:0_16:0_16:0_17:1	15:0_16:1_17:0_17:0	15:0_16:0_17:0_17:1
88.44	0.11	0.68	0.98	3.22	0.03	72:4			
90.63	0.12	0.11	0.01	0.22	2.93	64:0			
91.82	0.02	0.14	0.04	5.63	0.08	70:3			
92.33	0.61	0.27	0.03	**8.07**	0.05	68:2	16:0_18:0_16:1_18:1		
95.06	0.12	0.16	0.12	**0.59**	0.24	66:1	18:1_18:0_16:0_14:0		
95.86	0.14	0.03	0.06	0.02	2.40	65:0			
98.82	0.02	0.08	0.11	0.83	0.05	72:3			
101.85	0.09	0.19	0.03	0.96	0.27	70:2			
102.42	0.00	0.00	0.07	0.09	1.33	66:0			
107.11	0.46	0.19	0.14	0.09	0.05	68:1			
109.58	0.14	0.05	0.04	0.18	0.80	67:0			

[a] The retention in RP-HPLC increases with increasing equivalent carbon number (ECN) defined as the total carbon number (ACN) in all acyl chains minus two times the number of DBs, i.e. ECN=ACN−2DB.

[b] five molecular species of CLs, see 18:3_18:2_16:1_16:0-CL, 16:1_16:1_18:2_18:2-CL, 16:0_18:1_18:1_16:4-CL, 16:0_18:0_18:2_16:4-CL, and 18:3_18:3_16:0_16:0-CL, were identified, but analysis by AcTAGs was not successful.

[c] four molecular species of CLs, see 18:2_18:2_18:1_18:1-CL, 18:0_18:1_18:2_18:3-CL, 18:2_18:2_18:2_18:0-CL, and 18:3_18:2_18:1_18:0-CL, were identified, but analysis by AcTAGs was not successful.

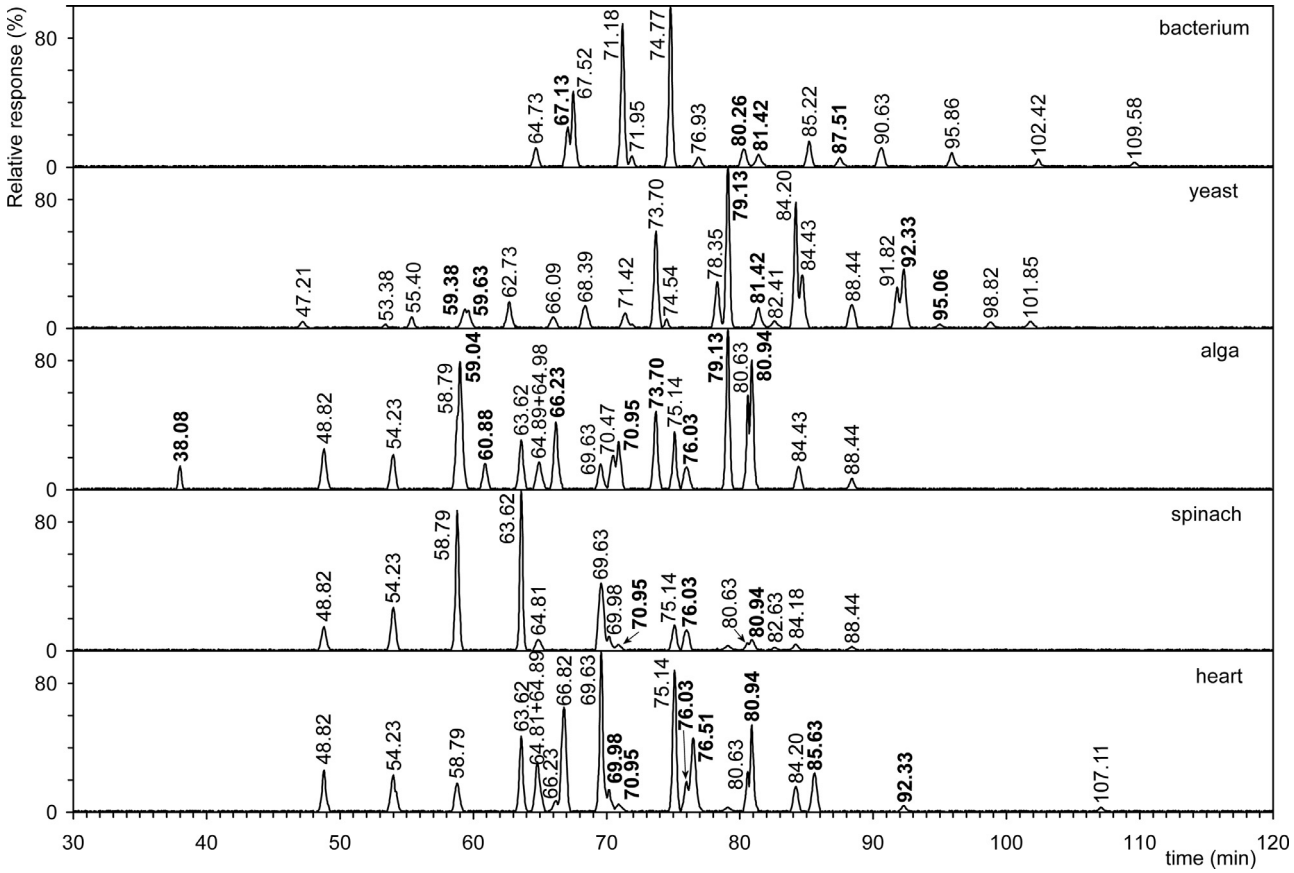

Fig. 7. Separation of cardiolipins on three reversed phase columns connected in series. The peaks marked in bold were collected and further hydrolyzed by commercially obtained phospholipase C. The TIC was filtered and indicates ions in the interval from 900 to 2000 Da.

photosynthetic organisms (alga or spinach) four FAs were present. In particular, both by shotgun analysis and by a combination of RP-HPLC of CLs, hydrolysis by phospholipase C and subsequent chiral-HPLC combined with identification using tandem MS, it was possible to prove that in all five organisms, there are molecular species of CLs that contain four different fatty acids. Furthermore, significant differences in molecular species of CLs were found between prokaryotic *K. rhizophila* and four eukaryotic organisms (Table 3). In *K. rhizophila*, CLs containing both even and odd acyls and with a maximum of one double bond were identified. In *S. pastorianus*, molecular species of CLs were identified that contained acyls from C14 to C26 and fatty acids had a maximum of one double bond. The other three organisms contained molecular species of CLs with fatty acids from C16 up to C20, but fatty acids had up to five double bonds. Molecular species of CLs containing VLCFAs have also been identified in yeast by shotgun analysis. This result complements previously published articles on the presence of these acids in phospholipids and triacylglycerols [23, 37].

Chiral analysis of the resulting AcTAGs, (Fig. 8), showed that AcTAGs from *K. rhizophila* were mainly mixtures of R and S enantiomers. Furthermore, some AcTAGs were analyzed with respect to their chirality and configuration of the *sn*-2 carbon. Two standards were synthesized (see Experimental methods and Fig. 1S). Based on the chiral-HPLC of these standards (Fig. 8 and Table 4), it was possible to determine that the S enantiomer of AcTAGs always predominated, meaning that the major R enantiomer was present in the phospholipid (Fig. 1). In the case of all four eukaryotic organisms, the proportion of R enantiomers was several times lower.

Twenty-two molecular species of TAGs isolated from the seed oil of *Santalum album* were separated by RP-HPLC and chiral HPLC methods and identified by positive electrospray ionization tandem MS detection (ESI^+-MS) [20]. Itabashi and Kuksis [38] published an extensive article describing the chirality of phosphatidylglycerols (PGs) (precursors of CLs biosynthesis) from plants, eggs and a bacterium. For all PGs, it was determined using chiral-HPLC derivatives (dinitrophenylurethanes of PGs) that natural PGs had the R configuration on the *sn*-2 carbon.

Analysis of human prostate cancer DU145 cells showed that the methylation by labeled CD_3I significantly increased sensitivity [39]. Cardiolipins having four different FAs, e.g., ((16:1,18:2),(18:2,20:4) or (14:0,16:0),(16:1,18:1) were also found in human cells. The use of isotope-labeled methylation based quantitation with reversed phase nanoflow ultrahigh performance liquid chromatography-electrospray ionization-tandem mass spectrometry (nUPLC-ESI-MS/MS) resulted in the determination of *sn*-glycero-3-phosphate moieties and fatty acids in each diacylglycerol were performed.

Based on the experimental results, we believe that the following hypothesis can be formulated. The key starter unit in phosphatidic acid (PA) and also all phospholipids, including CLs, is glycerol phosphate. This compound can exist either as (*S*)-2,3-dihydroxypropyl dihydrogen phosphate and (*R*)-2,3-dihydroxypropyl dihydrogen phosphate (Fig. 5S), sometimes also called *sn*-glycerol-1-phosphate, or as *sn*-glycerol-3-phosphate. The *sn*-glycerol-1-phosphate is a phosphoric ester of glycerol, which is a component of Archaea lipids [40], whereas *sn*-glycerol-3-phosphate is present in bacteria [41] and in all eukaryotic organisms, e.g. in yeast [42]. The *sn*-glycerol-3-phosphate is a biosyn-

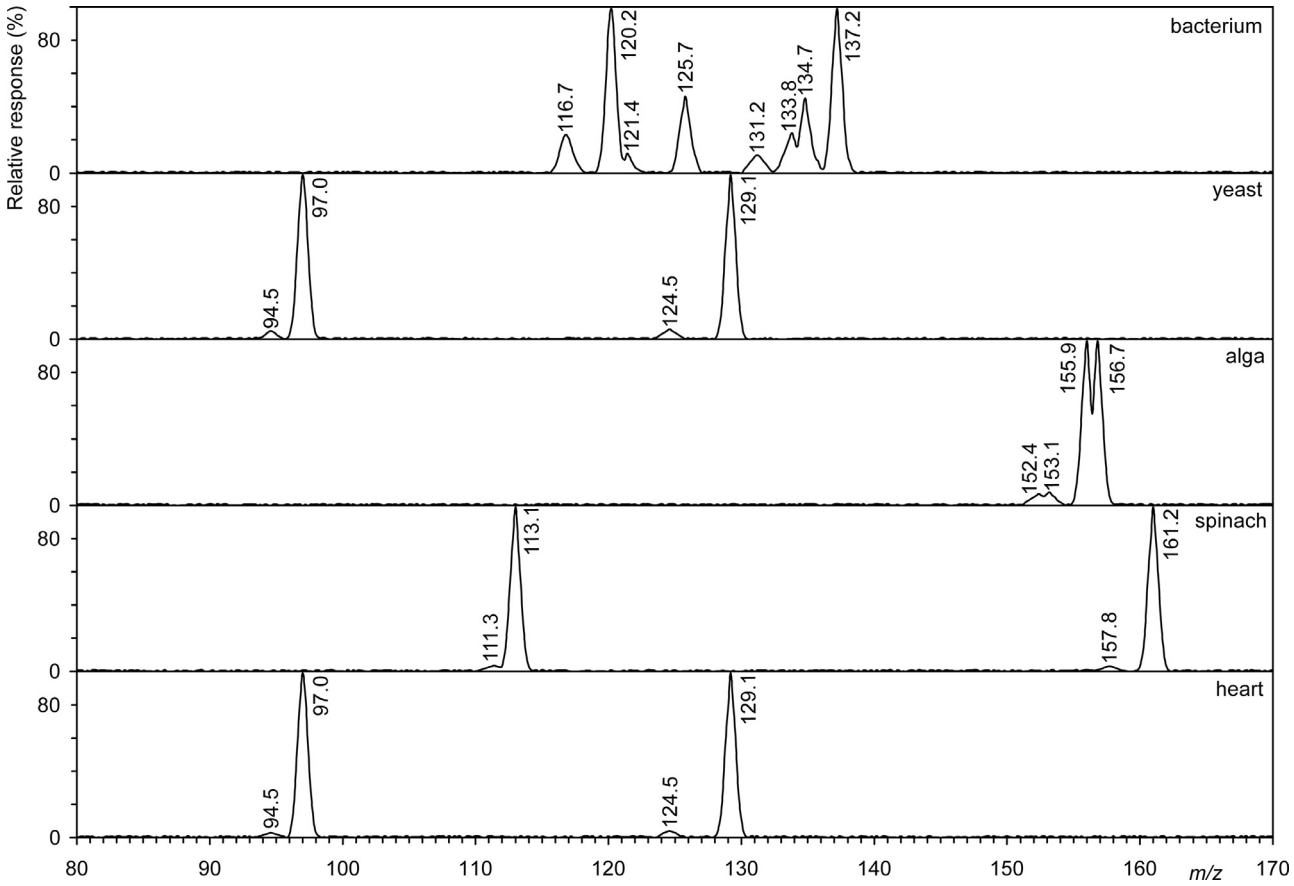

Fig. 8. Chiral separation of natural AcTAG, i.e. enantiomers and a regioisomers, see Table 4. TIC was filtered and indicates the type of ions $[DAG]^+$, i.e. ions resulting from loss of neutral RCOOH + NH_3 from $[M+NH_4]^+$ in the interval from 450 to 650 Da. The $[DAG]^+$ type ion was always the base peak.

Table 4
Molecular species of AcTAGs identified by chiral-HPLC/ESI-MS^2.

tR[a]	Relative Abundance %)	mol spec	ACN[b]	DB[b]	ECN[b]
94.5	5,0	16:0/18:0/2:0	36	0	36
94.5	3,0	16:0/18:0/2:0	36	0	36
97.0[c]	**100,0**	**16:0/18:0/2:0**	**36**	**0**	**36**
97.0	**100,0**	**16:0/18:0/2:0**	**36**	**0**	**36**
111.3	3,0	18:1/16:0/2:0	36	1	34
113.0	**100,0**	**18:1/16:0/2:0**	**36**	**1**	**34**
116.7	23,0	15:0/15:0/2:0	32	0	32
120.2	**100,0**	**15:0/15:0/2:0**	**32**	**0**	**32**
121.4	12,0	16:0/16:1/2:0	34	1	32
124.5	6,0	16:1/18:1/2:0	36	2	32
124.5	4,0	16:1/18:1/2:0	36	2	32
125.7	**46,0**	**16:0/16:1/2:0**	**34**	**1**	**32**
129.1	**100,0**	**16:1/18:1/2:0**	**36**	**2**	**32**
129.1	**100,0**	**16:1/18:1/2:0**	**36**	**2**	**32**
131.2	11,0	14:0/15:0/2:0	31	0	31
133.8	24,0	15:0/16:1/2:0	33	1	31
134.7	**45,0**	**14:0/15:0/2:0**	**31**	**0**	**31**
137.2	**100,0**	**15:0/16:1/2:0**	**33**	**1**	**31**
152.4	7,0	16:1/16:2/2:0	34	3	28
153.1	8,0	16:0/16:3/2:0	34	3	28
155.9	**100,0**	**16:1/16:2/2:0**	**34**	**3**	**28**
156.7	**100,0**	**16:0/16:3/2:0**	**34**	**3**	**28**
157.8	3,0	18:3/18:2/2:0	38	5	28
161.0	**100,0**	**18:3/18:2/2:0**	**38**	**5**	**28**

[a] Blue font - molecular species Gram positive bacterium *Kocuria rhizophila,* brown font - molecular species from bottom brewer's yeast *Saccharomyces pastorianus*, green font - molecular species from green alga *Chlamydomonas reinhardtii*, black font - molecular species from spinach, red font - molecular species from heart.

[b] The retention in NARP-HPLC increases with decreasing equivalent carbon number (ECN) defined as the total carbon number (ACN) in all acyl chains minus two times the number of DBs, i.e. ECN=ACN−2DB.

[c] Bold – *S* enantiomers.

thetic precursor if PA is esterified with a coenzyme A ester. The reaction is catalyzed by glycerol-3-phosphate acyltransferase at the *sn*-1 position, to form lysophosphatidic acid. This compound is further acylated by acylglycerophosphate acyltransferase at the *sn*-2 position to form a key intermediate in the biosynthesis of phospholipids, i.e. phosphatidic acid (Fig. 5S).

The hypothesis that a bacterium of the genus *Kocuria* uses two pathways, i.e. two starter units ((*S*)-2,3-dihydroxypropyl dihydrogen phosphate and (*R*)-2,3-dihydroxypropyl dihydrogen phosphate) is likely true because both glycerol-3-phosphate dehydrogenase (GenBank KUM38942.1) and glycerol-1-phosphate dehydrogenase (GenBank SDP79711.1) are present in the genus *Arthrobacter*, which means that both glycerol phosphates (*sn*-glycerol-1-phosphate and its enantiomer *sn*-glycerol-3-phosphate) can be biosynthesized. Since the genus *Arthrobacter* and the genus *Kocuria* belong to the same family - Micrococcaceae, we assume that both enantiomers can also be synthesized in the genus *Kocuria*. A recent paper [43] reports that bacteria of the phylum *Candidatus* Cloacimonetes of the Fibrobacteres – Chlorobi – Bacteroidetes group may contain mixed lipids based on glycerol-3-phosphate and glycerol-1-phosphate.

The relevant enzyme (glycerol-1-phosphate dehydrogenase) has also been found in flowering plants such as the golden kiwifruit (*Actinidia chinensis*) (GenBank: PSR98690.1) or *Gossypium arboreum*, commonly called tree cotton (GenBank: KHG18172.1).

4. Conclusion

Cardiolipins were successfully separated by RP-HPLC where molecular species of CLs differed by at least one of two values, either the number of carbon atoms or the number of double bonds. Unfortunately, even though a combination of three columns in series were used, with an efficiency of 325,000 theoretical plates/meter (determined by the $(14:0)_4$-CL standard), the regioisomers of CLs could not be separated. Therefore, a combination of chromatographic methods and enzymatic hydrolysis was used, which, due mainly to removal of the polar head, allowed the separation of hydrolytic products, i.e. *sn*-1,2-diacyl-3-acetyl TAGs. It was found by chiral-HPLC that AcTAGs, especially in bacteria, are a mixture of two enantiomers. It has therefore been hypothesized that either *sn*-glycerol-1-phosphate or *sn*-glycerol-3-phosphate is a precursor of PA and thus CLs biosynthesis. This finding strongly suggests that the bacterium supports a biosynthetic route described so far only in Archaea. Further analysis shows that some CLs contained four different FAs and thus lacked symmetry. In addition, CLs from prokaryotic and eukaryotic organisms differed significantly. A difference was also found between non-photosynthetic and photosynthetic organisms, i.e. mammals versus dicotyledonous plants and alga. In conclusion, this combination of methods, although very time consuming, facilitates the identification of molecular species of CLs in any organism, as shown from five different sources. With a substantial increase in the efficiency of reversed phase chromatography, further separation of molecular species may be expected in the future.

CRediT author statement

Milada Vítová: Cultivation of the green alga, Data curation, Methodology, Writing. Milena Stránská: Cultivation of the Gram-positive bacterium, Data curation, Methodology, Writing. Andrea Palyzová: Data curation, Methodology, Writing- Original draft preparation. Tomáš Řezanka: Conceptualization, Software, Writing-Reviewing and Editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Supplementary materials

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