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Separation and identification of diacylglycerols containing branched chain fatty acids by liquid chromatography-mass spectrometry

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ABSTRACT

A combination of two chromatographic and two enzymatic methods was used for the analysis of molecular species of lipids from Gram-positive bacteria of the genus *Kocuria*. Gram-positive bacteria contain a majority of branched fatty acids (FAs), especially iso- and/or anteiso-FAs. Two strains *K. rhizophila* were cultivated at three different temperatures (20, 28, and 37°C) and the majority phospholipid, i.e., the mixture of molecular species of phosphatidylglycerols (PGs) was separated by means of hydrophilic interaction liquid chromatography (HILIC). After enzymatic hydrolysis of PGs by phospholipase C and derivatization of the free OH group, the *sn*-1,2-diacyl-3-acetyl triacylglycerols (AcTAGs) were separated by reversed phase HPLC. Molecular species such as i-15:0/i-15:0/2:0, ai-15:0/ai-15:0/2:0, and 15:0/15:0/2:0 (straight chains) were identified by liquid chromatography-positive electrospray ionization mass spectrometry. The tandem mass spectra of both standards and natural compounds containing iso, anteiso and straight chain FAs with the same carbons were identical. Therefore, for identification of the ratio of two regioisomers, i.e. i-15:0/ai-15:0/2:0 vs. ai-15:0/i-15:0/2:0, they were cleavage by pancreatic lipase. The mixture of free fatty acids (FFAs) and 2-monoacylglycerols (2-MAGs) was obtained. After their separation by TLC and esterification and/or transesterification, the fatty acid methyl esters were quantified by GC-MS and thus the ratio of regioisomers was determined.

It has been shown that the ratio of PG (containing as majority i-15: 0 / i-15: 0, i-15: 0 / ai-15: 0 and / or ai-15: 0 / i-15: 0 and ai-15: 0 / ai-15: 0 molecular species) significantly affected the membrane flow of bacterial cells cultured at different temperatures.

1. Introduction

The major components of cell membranes are phospholipids, which form a semipermeable barrier that maintains cellular homeostasis. Bacteria do not form static structures, but constantly modify the lipid composition of their membranes in response to changes in the physiological environment, i.e., external adverse conditions such as temperature, osmolarity, salinity or pH [1].

Gram-positive bacteria such as firmicutes, e.g., the genus *Kocuria* and many others, are characterized by a cytoplasmic membrane and a thick murein cell wall [2]. Membranes consist mostly of glycerophospholipids composed of two fatty acids, glycerol backbone, phosphate group and a variable head group. Membrane lipids of gram-positive species, e.g., genus *Kocuria*, contain a high proportion of C15 chain branched fatty acids (br-FA) and a substantial amount of C17 br-FA in membrane lipids [3,4,5,6,7,8,9]. The major polar lipids of the genus *Kocuria* are phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG), among many others [3,4,9].

Since Gram-positive bacteria do not usually contain polyunsaturated FA in the membranes, they use a different mechanism to preserve membrane fluidity at low temperatures. One basic possibility for cell membranes to be fluidized, i.e., they have the correct phase transition temperature, is a change of the polar head. It has been reported [10] that a change of e.g., choline for ethanolamine to dipalmitoyl phospholipid leads to an increase in the phase transition temperature of 22 °C (16:0/16:0-PC (41 °C) versus 16:0/16:0-PE (63 °C)). The second option is to modify the acyl chains. For instance, in the series 15:0/15:0-PC, i-15:0/i-15:0-PC, and ai-15:0/ai-15:0-PC the phase transition temperature decreases from 35.0 °C to 6.5 °C to -13.9 °C [11]. Third possibility for cells to influence the phase transition temperature is to rearrange acyl chains on the glycerol backbone of the phospholipid. It was found, for example, that 15:0/17:0-PG has a phase transition temperature of 41.2 °C, whereas its regioisomer 17:0/15:0-PC has only 36.2 °C [12]. Of course, all three possibilities can be combined.

The analysis of phospholipids and TAGs are often described in the literature, including e.g., spectral database LIPID MAPS® Lipidomics Gateway (http://www.lipidmaps.org/). However, no one has yet comprehensively described the behavior of phospholipids

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and TAGs, where the acyls in these lipids are not only straight chains, but are branched. Only vague mentions, either describing that the compounds cannot be separated [13,14] or lipids with branched acyls can be partially separated have been published. [15].

Separation of *sn*-positional isomers (regioisomers) of intact phospholipids can be performed by RP-HPLC only provided the acyls have sufficiently different chain lengths and degrees of unsaturation. The regioisomer with the unsaturated chain attached to the *sn*-1 position elutes first, while the regioisomer with the unsaturated chain attached to the *sn*-2 position has higher retention time [16]. In the case of two synthetic regioisomers (16:0/18:1-PC and 18:1/16:0-PC), i.e., those having chains of similar length while the number of double bond(s) varies by one, the separation was sometimes successful. One possibility is combining collision with ozone-induced dissociation (CID/OzID). Based on the extracted ion chromatogram for CID/OzID products at *m/z* 405 (indicative of 18:1/16:0-PC), it was possible to quantify the two regioisomers. Two regioisomers of PC, i.e., 18:0/16:1-PC and 16:1/18:0-PC, were identified by tandem MS based on $RCOO^-$ (palmitoyl and/or oleoyl) ion intensities, but were not separated by HPLC [17]. Similar results for two regioisomers (18:1/18:0-PC and 18:0/18:1-PC) were published by Batarseh et al. [18]. The extracted-ion chromatograms showed one peak, whereas the diagnostic product ions corresponding to 18:1 at *sn*-1, i.e., ion at *m/z* 405 and ion at *m/z* 407 (18:0 at *sn*-1) show partial separation of the above-mentioned molecular species. Using differential mobility spectrometry method, it was possible to determine 18:0/16:1-PC and/or 16:1/18:0-PC as cationized ions with Ag^+, Li^+, Na^+, and K^+ [19]. Unfortunately, fully saturated acyls such as the pair of regioisomers 16:0/18:0-PC and 18:0/16:0-PC have not yet been separated.

As shown above, intact phospholipids are difficult to separate by HPLC. One of the possible reasons is probably their high polarity. Conversely, after hydrolysis with phospholipase C, the resulting diacylglycerols (DAGs) can be separated by RP-HPLC after suitable derivatization. Two papers [20,21] deal with the separation of diacylglycerols in the form of tris(3,5-dimethylphenylurethanes). There is no problem to separate such pairs of regioisomers (16:0/18:1-PC - 18:1/16:0 PC, 18:1/18:0-PC - 18:0/18:1-PC, or 14:0/16:0-PC - 16:0/14:0-PC) that were not previously separated.

Unfortunately, none of the above papers is concerned with the analysis of regioisomers of phospholipids containing branched FAs. In addition, branched FAs, as mentioned above, have a majority representation in phospholipids Gram-positive bacteria [22].

Based on our previous experience with the separation of triacylglycerols containing branched (iso and/or anteiso) and unbranched FAs [23], we have used a combination of two chromatographic and two enzymatic techniques in this article. In particular, total lipids were separated by hydrophilic interaction liquid chromatography (HILIC), with PG identified as the major phospholipid. Furthermore, free 1,2-diacylglycerols (DAGs) were obtained from phospholipase C, which were derivatized to *sn*-1,2-diacyl-3-acetyl-triacylglycerols (AcTAGs). Separation of these compounds into molecular species was performed by RP-HPLC. All peaks were identified by high-resolution electrospray ionization (ESI) MS. An exception is mixture of regioisomers (i15:0/ai15:0/2:0 versus ai15:0/i15:0/2:0), which has not been separated. After enzymatic hydrolysis of this mixture by pancreatic lipase, free fatty acids (FFAs) and 2-monoacylglycerols (2-MAGs) were analyzed as methyl esters by GC-MS.

2. Experimental methods

2.1. Chemicals and standards

Acetonitrile, 2-propanol, hexane, dichloromethane, ergosterol (Es), Bacterial Acid Methyl Ester (BAME) Mix, 1-palmitoyl-2-myristoyl-sn-glycero-3-phosphocholine (16:0-14:0 PC), 1,2-dipentadecanoyl-sn-glycero-3-phosphocholine (15:0-15:0 PC), 1-palmitoyl-(13-methylmyristoyl)-sn-glycero-3-phosphocholine (16:0-i15:0 PC), 1-palmitoyl-(12S-methylmyristoyl)-sn-glycero-3-phosphocholine (16:0-ai15:0 PC), 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine (18:1-16:0 PC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (16:0-18:1 PC), phospholipase C from *Clostridium perfringens* (*C. welchii*) Type XIV, lyophilized powder, 150 units/mg protein were purchased from Merck (previously Sigma-Aldrich, Prague, Czech Republic).

Cholesteryl oleate (SE), triolein (TAG), diolein (DAG), oleic acid (FFA), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (16:0-18:1-PC), respective PO-PC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PO-PE), 1,2-dioleoyl-*sn*-phosphatidylethanolamine (PE), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (PS), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidylcholine (LPC), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidic acid, Na-salt (LPA), and 1,2-dipentadecanoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) were from Larodan (Malmö, Sweden). 1,2-Dioleoyl-*sn*-glycero-3-phospho-(1′-myo-inositol) (ammonium salt) (PI) were purchased from Avanti (Avanti Polar Lipids, Inc., Alabama, USA).

2.2. Cultivation of microbial cultures

K. rhizophila CCM 552 and CCM 4950 were obtained from the Czech Collection of Microorganisms (CCM), Brno, Czech Republic. The cultures of bacteria were grown in 100 mL of a mineral medium (in g/L; NaCl 0.5, $Na_2HPO_4.12\ H_2O$ 14.62, KH_2PO_4 3.0, and NH_4Cl 1) supplemented with yeast extract (10 g/L) and trace elements (in mg/L; $MgSO_4.7H_2O$ 200, $CaCl_2.2H_2O$ 10, $FeSO_4.7H_2O$ 1.5). The cultures were incubated at 20, 28, and 37°C on an orbital shaker (200 rpm) for 48 h. Supernatants and pellet cultures were separated by centrifugation (12,000 g, 10 min, 4°C) and the cell pellets were subsequently lyophilized.

2.3. Isolation of lipids

Isolation and pretreatment of lipids was carried out according to Bligh and Dyer [24], with modifications as described previously [20]. For a full description of the experiments, see the Supplements.

2.4. Analysis of fatty acids as FAMEs and 3-pyridylcarbonyl esters

Analysis of fatty acids in the form of methyl esters (FAME) was described previously [25,26]. A complete description of all conditions of FAME and 3-pyridylcarbonyl esters analysis by GC/MS is in the Supplements.

2.5. HILIC-LC/MS-ESI

The total lipids were identified and quantified by LC/MS. A detailed description of negative ESI is again described in the Supplements.

2.6. Phospholipase C hydrolysis

PGs (~ 3 mg) and commercially available phospholipase C (5 units) from *Clostridium perfringens* 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) [27]. The mixture was shaken vigorously at 37°C for approximately 45 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,

Table 1
Identified fatty acids (relative percentage) during cultivations of two strains *Kocuria rhizophila* CCM 552 and CCM 4950 at three different temperatures (20°C, 28°C, and 37°C).

FAME	CCM 552 20°C	CCM 552 28°C	CCM 552 37°C	CCM 4950 20°C	CCM 4950 28°C	CCM 4950 37°C
iso-C13:0	0.2 ± 0.1[a]	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.2	0.4 ± 0.2	0.5 ± 0.2
anteiso-C13:0	0.3 ± 0.2	0.2 ± 0.2	0.1 ± 0.1	0.5 ± 0.2	0.3 ± 0.2	0.2 ± 0.2
iso-C14:0	2.1 ± 0.5	2.8 ± 0.5	3.2 ± 0.8	1.4 ± 0.8	1.9 ± 0.5	2.7 ± 0.6
C14:0	1.7 ± 0.4	1.3 ± 0.5	0.6 ± 0.3	1.6 ± 0.4	1.1 ± 0.4	0.6 ± 0.3
iso-C15:0	5.7 ± 0.9	9.2 ± 0.9	20.4 ± 2.1	6.3 ± 0.9	12.3 ± 2.6	22.4 ± 2.1
anteiso-C15:0	71.2 ± 2.8	69.4 ± 2.9	61.7 ± 2.8	73.3 ± 2.6	68.5 ± 3.1	59.9 ± 3.0
C15:1	2.0 ± 0.6	1.3 ± 0.4	1.0 ± 0.2	2.1 ± 0.5	1.4 ± 0.4	1.0 ± 0.3
C15:0	1.2 ± 0.4	0.8 ± 0.3	0.4 ± 0.2	1.3 ± 0.3	1.0 ± 0.2	0.6 ± 0.2
iso-C16:0	1.8 ± 0.7	2.4 ± 0.9	2.7 ± 0.6	1.2 ± 0.4	1.8 ± 0.3	2.4 ± 0.8
C16:1ω-7	3.8 ± 0.5	2.9 ± 0.8	1.7 ± 0.4	2.5 ± 0.8	1.8 ± 0.5	1.0 ± 0.4
C16:0	3.2 ± 0.9	2.6 ± 0.5	1.6 ± 0.4	2.9 ± 0.7	2.3 ± 0.4	1.5 ± 0.6
anteiso-C17:1ω-9	1.5 ± 0.4	2.2 ± 0.7	1.7 ± 0.8	1.5 ± 0.6	2.3 ± 0.6	1.8 ± 0.7
iso-C17:0	0.7 ± 0.2	1.0 ± 0.2	1.3 ± 0.7	0.9 ± 0.4	1.3 ± 0.2	2.0 ± 0.5
anteiso-C17:0	2.1 ± 0.3	1.6 ± 0.3	1.1 ± 0.6	1.9 ± 0.7	1.5 ± 0.5	0.8 ± 0.3
iso-C18:0	0.5 ± 0.2	0.9 ± 0.4	1.2 ± 0.8	0.6 ± 0.2	1.0 ± 0.4	1.6 ± 0.5
C18:1ω-9	1.2 ± 0.4	0.7 ± 0.2	0.4 ± 0.2	1.1 ± 0.3	0.6 ± 0.2	0.5 ± 0.2
C18:0	0.9 ± 0.3	0.5 ± 0.1	0.4 ± 0.3	1.0 ± 0.2	0.6 ± 0.3	0.4 ± 0.3

a Mean ± S.D. from three measurements.

vol/vol/vol)). The resulting DAGs were acetylated [28] by standing overnight in a mixture of acetic anhydride/pyridine (3:2, v/v) at 20°C to monoacetyldiacylglycerols i.e. AcTAGs. These AcTAGs were further separated and identified by LC-MS. Similarly, AcTAGs have been previously separated [29,30,31].

2.7. RP-HPLC/ESI-MS^2

AcTAGs were analyzed by an LC-MS system containing three reversed phase columns connected in series, see Supplements. The mass spectrometer LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany), a high-resolution hybrid mass spectrometer equipped with a liquid chromatograph was used, see also Supplements. This instrument was used for tandem mass spectra, multiple precursor ion scans (PIS), and selected ion monitoring (SIM).

2.8. Pancreatic lipase hydrolysis, i.e., determination of fatty acids at the sn-2 position

The collected peak containing the mixture of i-15:0/ai-15:0/2:0 and/or ai-15:0/i-15:0/2:0 that eluted at an interval of 623-633 min, pancreatic lipase (Merck, Prague, Czech Republic), 150 µL Tris buffer, 25 µL sodium cholate (1 g/L), and 10 µL calcium chloride (2.2%) were mixed in a vial, heated at 40°C for 1 minute, and stirred for 2 minutes in a Vortex mixer. After cooling to 20°C, 50 µL of 6 M HCl and 0.2 mL of diethyl ether were added. The reaction mixture was extracted with 0.2 ml diethyl ether three times. The combined ether extracts were separated by TLC (silica gel with fluorescent indicator on 20 cm × 20 cm TLC plates) using a mobile phase hexane/diethyl ether/acetic acid (60:39:1, v/v/v). FFAs and *sn*-2 MAGs were scraped off and were extracted from silica with diethyl ether and evaporated to dryness. *sn*-2 MAGs were transesterified to FAMEs as described previously [32]. Briefly, FAMEs were prepared from FFAs by means of methanolic BF_3 [33], see Supplements.

2.9. Statistics

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), to explore the metabolic relationships between different temperatures at 20°C, 28°C, and 37°C. Three replicates (n = 3) of each analysis were used. Data were presented as means ± SD.

3. Results and discussion

3.1. Fatty acids analysis

The data in Table 1 were obtained by analyzing both fatty acid methyl esters (FAMEs) and 3-pyridylcarbinol esters (formerly called picolinyl esters). Saturated FAs from C14 up to C18, odd and even numbered with a straight- and branched (iso and/or anteiso) chains were identified and quantified. The order of elution of FAMEs from the capillary column is iso- anteiso-, normal- (straight) chains. The differences in mass spectra of FAMEs are very small, so it is preferable to use 3-pyridylcarbinol esters to identify them.

3-Pyridylcarbinol esters of FAs, which show different mass spectra, were used to identify branched chains, see Figs 1S and 2S (Supplements). The mass spectra of iso and/or anteiso 3-pyridylcarbinol esters resemble mass spectra of saturated straight chain 3-pyridylcarbinols, except for the very obvious gap of 28 Da between ions $[M-15]^+$ and $[M-43]^+$, respectively, and between ions $[M-29]^+$ and $[M-57]^+$. These gaps represent the loss of the penultimate and/or ante-penultimate carbon atom and the methyl groups attached thereto.

3.2. Hydrophilic interaction liquid chromatography - HILIC

Total lipids of both strains of *K. rhizophila* CCM 552 and CCM 4950 were analyzed by HILIC, see Fig. 1. All polar lipids were separated on the base line, unfortunately some highly polar lipids (U_1 - U_6) were not identified. Phosphatidylglycerols have been identified as major phospholipids and were manually collected and analyzed further by direct inlet high-resolution negative ESI. It is apparent from Table 2 that the mixture of PGs contains more than ten of the molecular species, with 15:0/15:0-PG being the majority. Precursor ion $[M-H]^-$ at *m/z* 693.4712 was identified in tandem MS (Fig. 2) and two pairs of ions at *m/z* 469.2572 loss of *sn*-1 acyl chains as ketenes (RĆH=C=O) from $[M-H]^-$ and *m/z* 451.2466 neutral loss of RCOOH groups from $[M-H]^-$ versus *m/z* 395.2204 loss of acyl chains as ketenes (RĆH=C=O) and glycerol from $[M-H]^-$ and *m/z* 377.2099 neutral loss of RCOOH groups and glycerol from $[M-H]^-$ were identified. Other ions, e.g., ions at *m/z* 619.4344 (loss of glycerol from precursor ion), glycerophosphoglycerol ion at *m/z* 245.0432, acyl ion at *m/z* 241.2173 and ion at *m/z* 152.9958, loss arising of H_2O from glycerol-3-phosphate ion. These ions fully confirm the structure of 15:0/15:0-PG. Furthermore, the tandem MS of natural PG having $[M-H]^-$ at *m/z* 764.5447 and the commercial

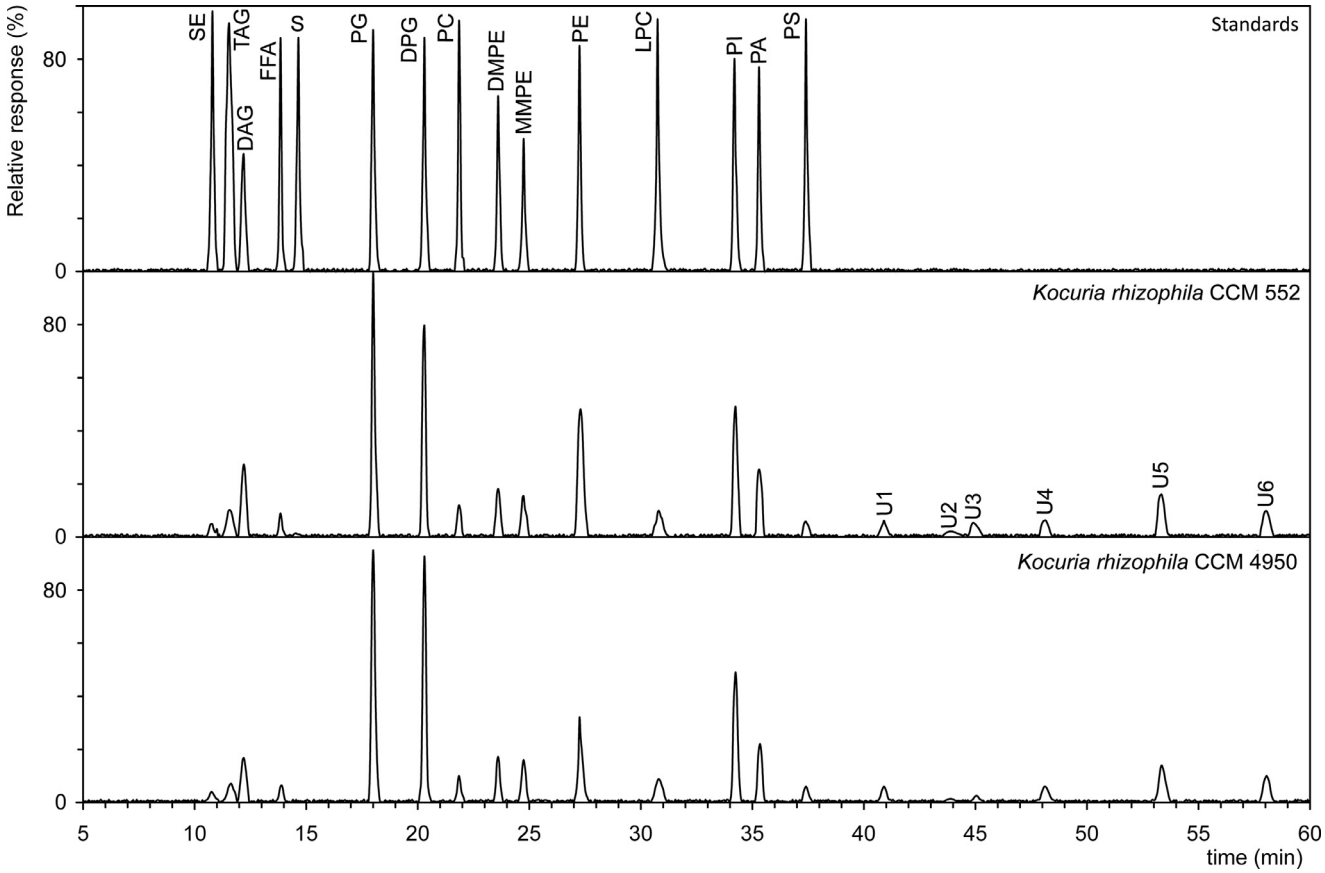

Fig. 1. Hydrophilic interaction liquid chromatography - HILIC/ESI-MS separation of standards (top) and major lipid classes of the two bacteria (*Kocuria rhizophila* CCM 552 and *K. rhizophila* CCM 4950). TIC was filtered and indicates the ions in the interval from 200 to 1900 Da. Abbreviations: SE (sterol ester), TAG (triacylglycerol), DAG (diacylglycerol), FFA (free fatty acid), S (sterol), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylcholine (PC), monomethyl PE (MMPE), dimethyl PE (DMPE), phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC), phosphatidylinositol (PI), phosphatidic acid (PA) and phosphatidylserine (PS), U1-U6 unknown.

Table 2
Selected data from cultivation of strain CCM 4950 at 20°C. The peak of PGs (Fig. 2) was analyzed by high resolution negative ESI.

	[M-H]$^-$	molecular species (acyls)	abundance	molecular species (acyls)		Abundance
665.4401	15[a]	13	0.97	14	14	0.51
677.4399	14	15	0.29			
679.4556	15	14	**2.15**[b]			
691.4558	14	16	**2.95**	15	15	0.58
693.4712	15	15	**61.98**	16	14	0.30
703.4556	16	15	0.34			
705.4710	15	16	**3.42**	14	17	0.38
707.4869	16	15	**7.46**	17	14	0.35
717.4711	16	16	0.64			
719.4871	15	17	0.68	16	16	0.35
721.5025	17	15	**8.20**			
731.4870	17	16	0.42			
733.5025	15	18	0.21	17	16	0.38
735.5182	18	15	**7.44**			

a acyl carbon number.
a bold value, these molecular species were also identified by LC-MS as AcTAGs, see Fig. 4.

sample are exactly the same, see Fig. 2. Later, it was found, that the natural sample contained six molecular species. At this resolution, the two ions (at *m/z* 227.2017 and at *m/z* 255.2330, respectively) corresponding to the 14:0 and 16:0 acids are not sufficiently distinct to be seen in Fig. 2.

Unfortunately, no differences were found between branched and unbranched molecular species. As shown in Fig. 3, two commercially obtained standards, i.e., 16:0/i15:0-PC, respectively, 16:0/ai15:0-PC, have identical mass spectra. Regiosomeric PCs can be distinguished by ESI-MS based on abundances of ions resulting from loss of acyl chain as ketene (RCH=C=O), CH_3 and acetate from precursor ion and also by neutral loss of RCOOH group, loss of CH_3 and acetate from precursor ion, where the abundance of ions losing $R_2'CH{=}C{=}O$ and R2COOH is greater than the abundance of ions resulting from the loss of $R_1'CH{=}C{=}O$ and R_1COOH. Conversely, two other commercially available standards, i.e., 16:0/14:0-PC, respectively 15:0/15:0-PC, have mass spectra that show differences, although the ion [M-H]$^-$ at *m/z* 764.5447 is identical for both molecular species. For this reason, further analyses of the mixture were conducted. It was performed mainly for enzymatic

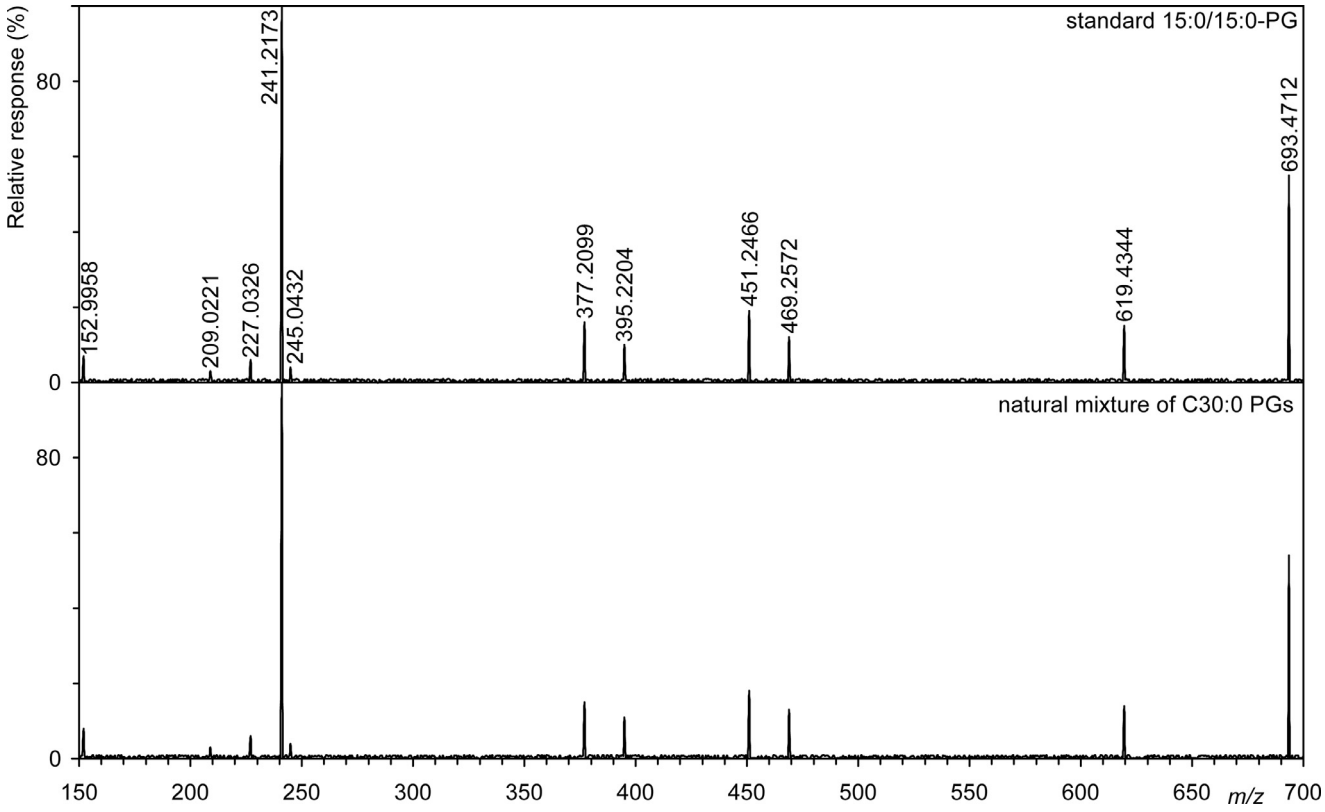

Fig. 2. The tandem MS of standard (commercial sample) 15:0/15:0-PG and natural mixture of PGs C30:0 from *K. rhizophila* CCM 4950. For experimental details, see Experimental conditions and explanation of chromatographic behavior, in all figures, including mass spectra, see Results.

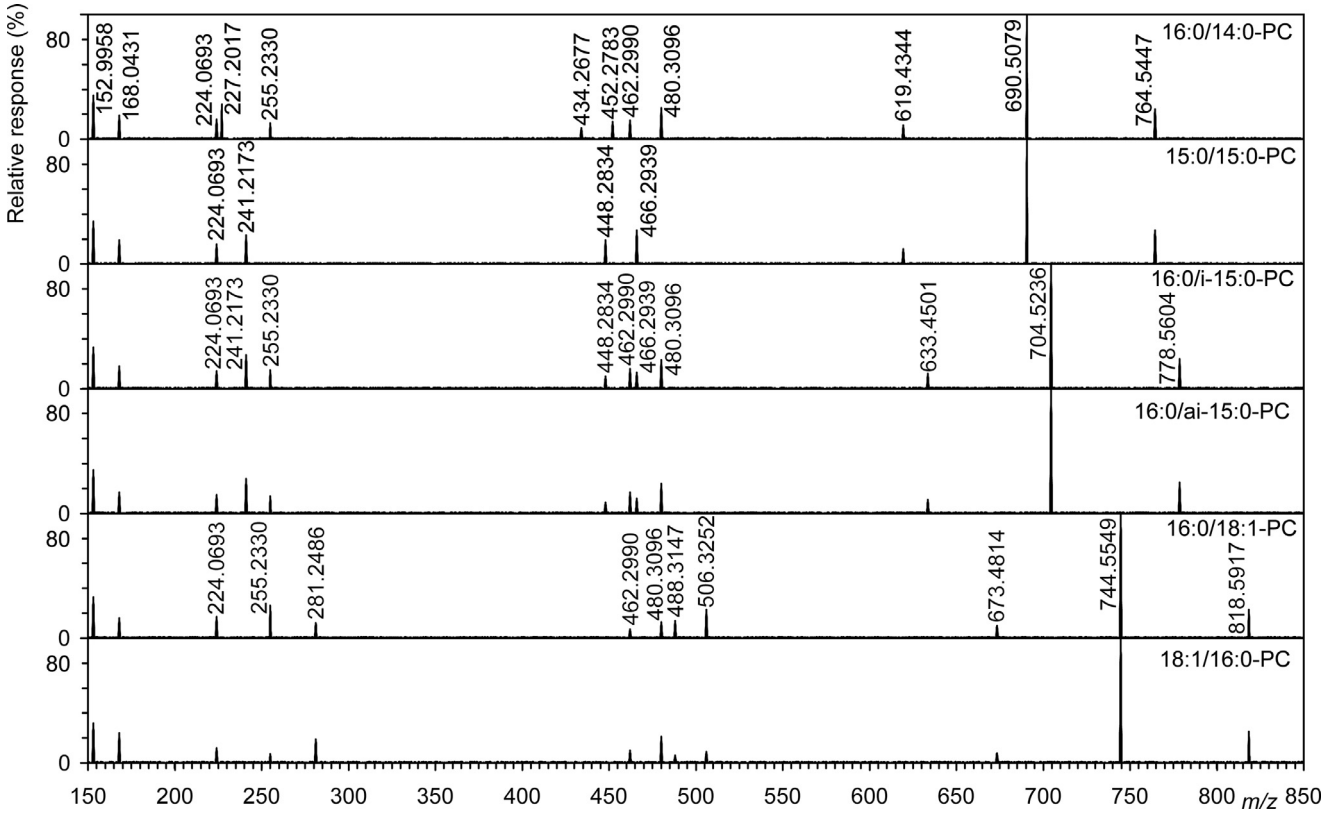

Fig. 3. The tandem MS of standards (commercial samples) different PCs. The tandem MS of three pairs of compounds are presented in this figure. Although the pair 16:0/14:0-PC and 15:0/15:0-PC has the same precursor ion $[M+acetate]^-$ value, the spectra differ. In the case of the second pair (16:0/i15:0-PC and 16:0/ai15:0-PC), although they have different structures, the spectra are identical (no distinction can be made between iso and anteiso FAs). In the addition of the third pair (16:0/18:1-PC and 18:1/16:0-PC), regioisomers can be distinguished by tandem MS.

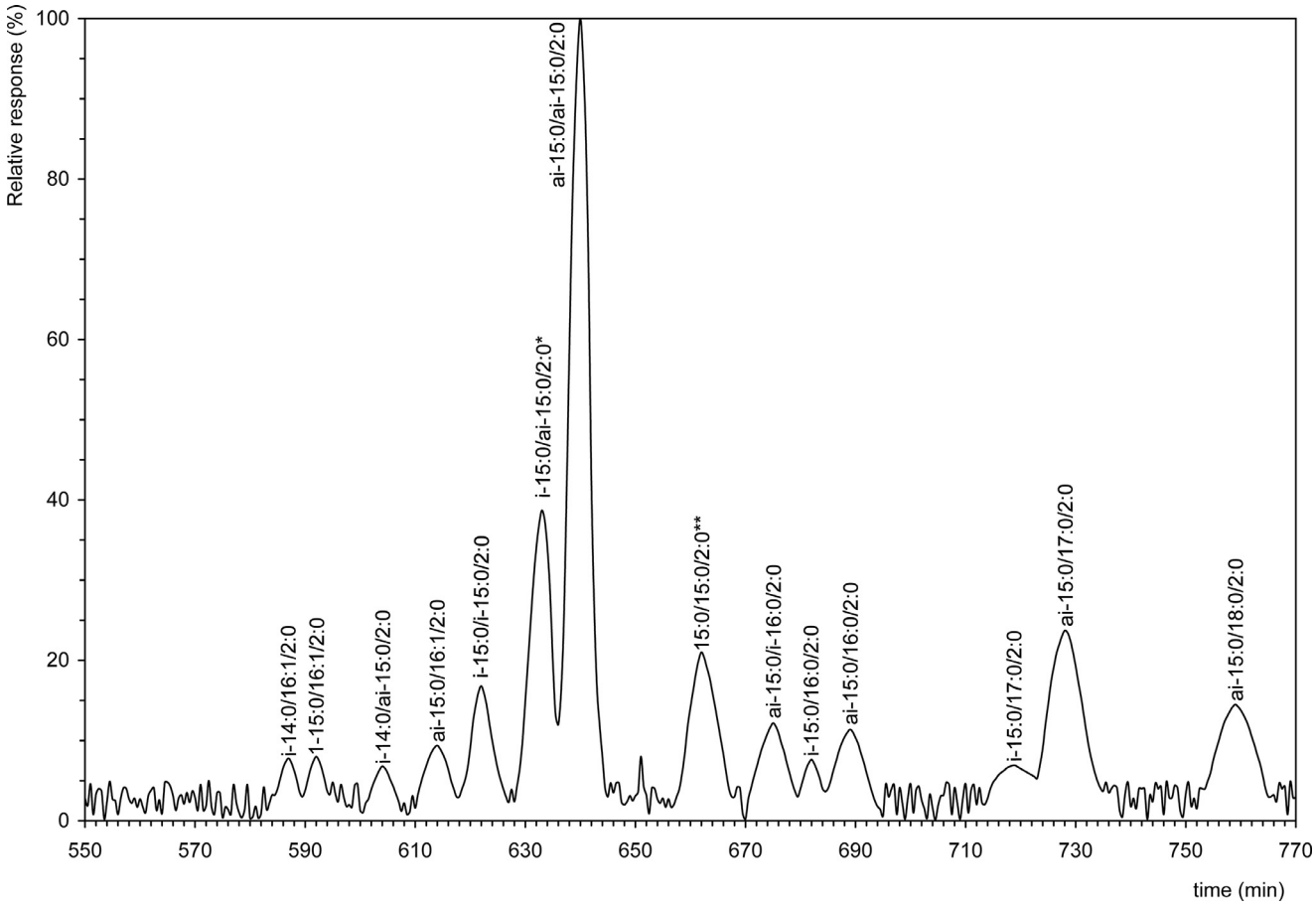

Fig. 4. Separation of 1,2-diacyl-3-acetyl TAGs (AcTAGs) on three reversed phase columns connected to series. The peak marked with an asterisk (*) is a non-separable mixture of two regioisomers (i-15:0/ai-15:0/2:0 vs. ai-15:0/i-15:0/2:0), the peak marked with two asterisks (**) was added as an internal standard.

cleavage of the polar head group from PGs and after derivatization of the resulting DAGs, AcTAGs were analyzed on reversed phase columns.

3.3. Stereospecific analysis of TAG (acetyldiacyl-TAGs)

3.3.1. Enzymatic hydrolysis by phospholipase C

Phospholipid dephosphorylation was accomplished by enzymatic hydrolysis with phospholipase C (EC 3.1.4.3), which is commercially available e.g. from *Bacillus cereus*. The 1,2-diacyl-*sn*-glycerols thus obtained were immediately derivatized to avoid acyl migration during the subsequent separation processes.

3.3.2. RP-HPLC analysis of acetyldiacyl-TAGs and their tandem MS

Acetyldiacyl-TAGs (AcTAGs) were prepared from phospholipids using phospholipase C and subsequent acetylation in the *sn*-3 position, as is described in the Experimental methods.

The separation of AcTAGs into three reversed phase columns connected in the series is shown in Fig. 4. It is apparent from the chromatogram that molecular species containing branched and unbranched FAs, such as i-15:0/i-15:0/2:0 vs. 15:0/15:0/2:0, have been separated. This connection yielded ~75.000 theoretical plates, measured by 15:0/15:0/2:0 molecular species (internal standard, marked with * in the chromatogram), i.e. AcTAG obtained from a commercially available 15:0/15:0-PC. Unfortunately, due to the length of the connected columns in series, the retention times (tR) have reached very high values and, as shown in Fig. 4, exceeded 770 min. On the contrary, it should be noted that even structurally similar molecular species such as ai-15:0/i-16:0/2:0, i-15:0/16:0/2:0, ai-15:0/16:0/2:0, etc., were well separated. The only two regioisomers that failed to separate were i-15:0/ai-15:0/2:0 vs. ai-15:0/i-15:0/2:0 (marked with *).

The Fig. 3 showed tandem MS of commercially available standards of PCs, in Fig. 5 are showed tandem MS of AcTAGs obtained after enzymatic hydrolysis of these PCs and esterification of DAGs. Tandem MS of ammonium adduct $[M+NH_4]^+$ (600.5198 Da) produced a ion $[M+NH_4\text{-}NH_3]^+$ at *m/z* 583.4932 with low intensity. In contrast, the $[M+NH_4\text{-}NH_3\text{-}H_2O]$+ type ion was not identified, see Fig. 5. The most abundant ions were $[DAG]^+$ ions, $[M+NH_4\text{-}NH_3\text{-}FA]^+$, i.e. ions resulting from neutral loss of *sn*-3 $RCOOH+NH_3$ from $[M+NH_4\text{-}NH_3]^+$ at *m/z* 523.4721 and ion at *m/z* 341.2686, resulting from neutral loss of *sn*-1 or *sn*-2 $RCOOH+NH_3$ from $[M+NH_4\text{-}NH_3]^+$. Another commonly occurring product ions for TAG (15:0/15:0/2:0) were four ions at *m/z* 299.2581 (*sn*-1 or *sn*-2 acyl chain ($[RC{=}O+74]^+$)), *m/z* 281.2475 (*sn*-1 or *sn*-2 acyl chain ($[RC{=}O+74]^+$) loss of H_2O)), *m/z* 225.2213 *sn*-1 or *sn*-2 acyl chain ($[RC{=}O]^+$), and *m/z* 207.2107 (*sn*-1 or *sn*-2 acyl chain ($[RC{=}O\text{-}H_2O]^+$). The tandem MS of the second of the standards, i.e., 16:0/14:0/2:0, is different, even if the value of $[M+NH_4]^+$ is identical. The ion at *m/z* 523.4722 formed by neutral loss AcOH and NH_3 from $[M+NH_4]^+$ is again the base peak. The other two ions at *m/z* 355.2843 and at *m/z* 327.2530 are formed by the loss of the corresponding acyl (myristic or palmitic acid) from $[M+NH_4]^+$. Another pair of TAGs containing branched FAs, i.e. i-15:0 and/or ai-15:0, has a tandem MS completely identical. Again, two regioisomers (18:1/16:0/2:0 and 16:0/18:1/2:0) could be distinguished from each other by the abundance of ions in the $[DAG]^+$ type.

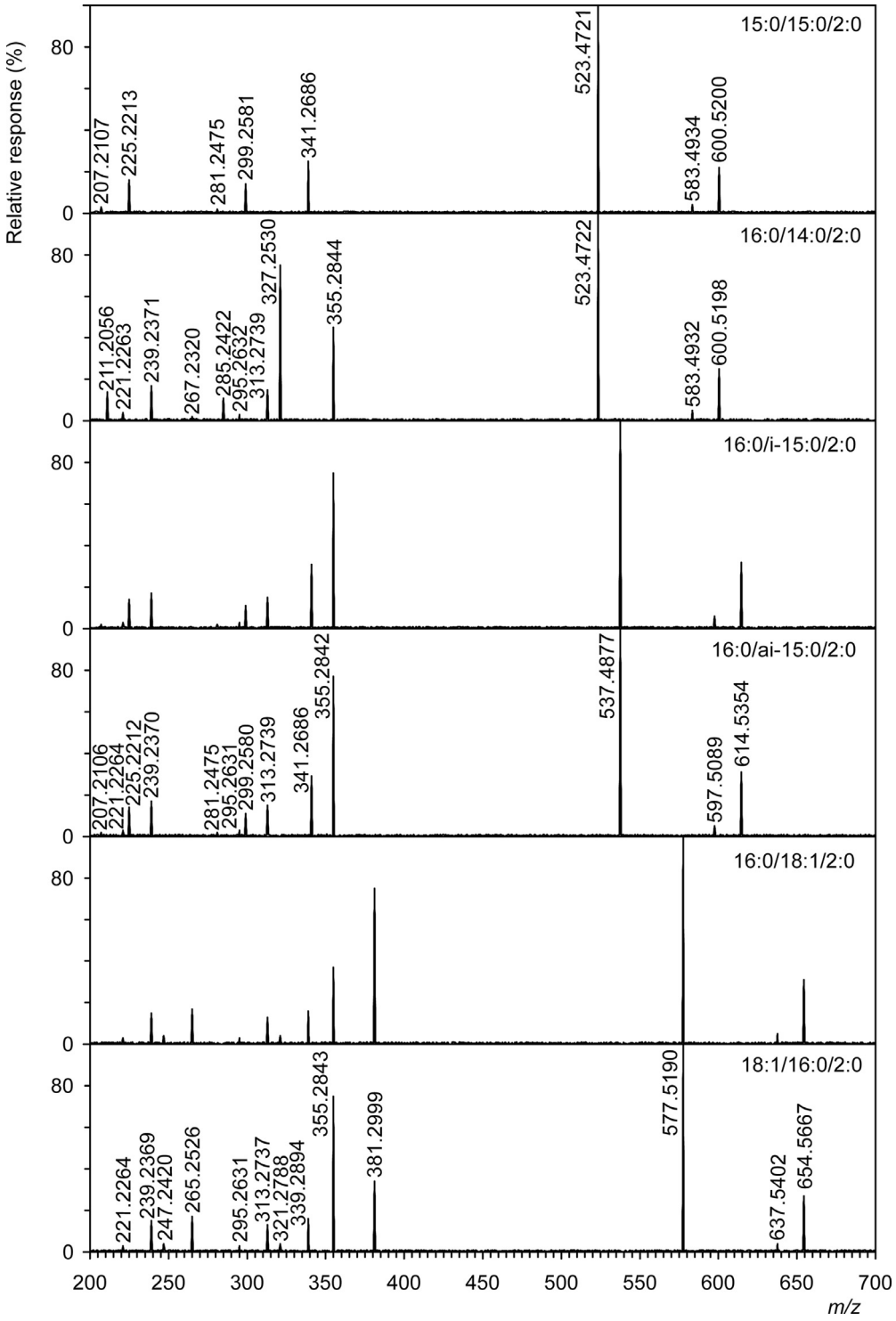

Fig. 5. The tandem MS of AcTAGs prepared from commercial standards of PCs. The tandem MS of three pairs of compounds are presented in this figure. The pair 15:0/15:0/2:0 and 16:0/14:0/2:0 has the same value of $[M+NH_4]^+$ ions, but the spectra of AcTAGs, i.e. ions at *m/z* 355.2844, 341.2686, 327.2530, and 299.2581 allow seamless identification. Conversely, at 16:0/i-15:0/2:0 and 16:0/ai-15:0/2:0 again having the same value of $[M+NH_4]^+$ ions, their spectra are identical and there is no difference between AcTAGs containing i-FAs and/or ai-FAs. Regioisomers 16:0/18:1/2:0 and 18:1/16:0/2:0 can be easily identified based on abundances of ions resulting in a neutral loss of appropriate $R_xCOOH + NH_3$ from $[M+NH_4]^+$.

This fully confirmed the structure of the standard as well as the structure of the natural mixture, i.e., TAGs 32:0, see below. Unfortunately, there is again the well-known fact that there is no difference between tandem MS of TAGs containing straight or branched chains of fatty acids. Unfortunately, as can be seen from tandem MS of both standards, i.e., 16:0/i-15:0/2:0 and 16:0/ai-15:0/2:0 and of course from real samples, i.e., i-15:0/i-15:0/2:0, ai-15:0/ai-15:0/2:0, and 15:0/15:0/2:0 (standard) the ion abundances are almost identical (Fig. 6).

3.3.3. Enzymatic hydrolysis by pancreatic lipase

Therefore, the marked peak with * (tR in the interval 628-634.5 min) was manually collected and after hydrolysis by pancreatic lipase (glycerol ester hydrolase, EC 3.1.1.3), a mixture of free fatty

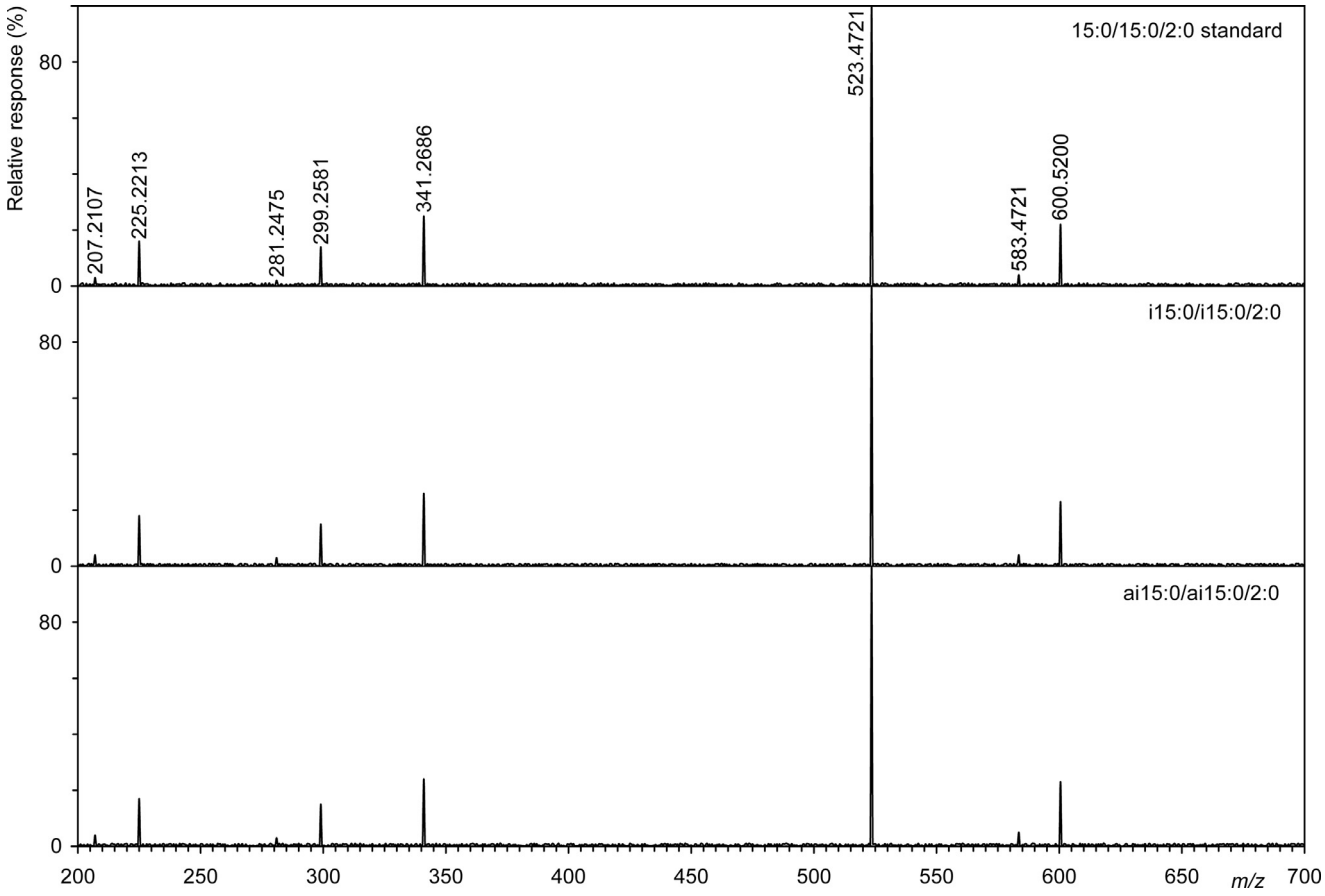

Fig. 6. The tandem MS of three AcTAGs containing straight- and branched-chains FAs. The abundances of all ions are identical. Therefore, individual molecular species can be identified only based on retention times, see Fig. 4.

Table 3
Molecular species ratio of AcTAGs containing saturated C15 acyl chains.

Ratio isomers	CCM 552 20°C	CCM 552 28°C	CCM 552 37°C	CCM 4950 20°C	CCM 4950 28°C	CCM 4950 37°C
i/i	5.7	9.3	12.3	5.4	7.5	11.0
i/ai[a]	**6.5**	**4.5**	**3.3**	**6.2**	**4.3**	**3.2**
ai/i	**10.3**	**11.8**	**15.6**	**8.4**	**11.4**	**13.4**
ai/ai	34.7	28.0	22.2	35.2	28.4	23.5
n/n	3.9	6.3	8.1	4.8	7.5	9.0

a bold, ratio of molecular species determined by pancreatic lipase.

acids (acetic, i-15:0, and ai-15:0) from both positions (*sn*-1 and *sn*-3) and a mixture of two *sn*-2-MAGs (*sn*-2-i-15:0 and *sn*-2-ai-15:0) was separated by TLC, see Supplements. After esterification and/or transesterification to FAMEs, the ratios were determined by capillary GC-MS. The results are shown in Table 3. It is apparent from this table that the ratio of the two molecular species plays an essential role in changing the fluidity of the membranes of cells cultured at different temperatures.

4. Discussion

The content of FAs in both our strains of *Kocuria* (Table 1) was practically not different from the sum of FAs that was previously published [34,35,36,37]. The structures of branched FAs (mainly i-15:0 and ai-15:0 FAs), were confirmed by GC-MS of the 3-pyridylcarbinol esters.

Therefore, so far, all analyses of the genus *Kocuria* have shown that PG and DPG were always present as major phospholipids, in addition to many, mostly not fully identified phospholipids, aminophospholipids, aminolipids, and unknown lipids. Most authors were satisfied with identifying two or three polar lipids by comparing R_f by two-dimensional TLC. For example, Tuo et al. [35] described six spots, Braun et al. [36], eight spots, and Jiang et al. [4] did not identify eight of the ten spots.

The analysis of branched TAGs not only from bacteria but also from all different sources, i.e. from bacteria to mammals (subcutaneous fat or milk) can be divided into two parts. First, it is a separation of unbranched TAGs from branched TAGs by RP-HPLC. The second and integral part of the analysis is their identification by mass spectra.

The separation of TAGs having both straight-chains and branched chains in the molecule has so far been done predominantly in reports describing the separation of straight- and branched chains of TAGs from milk lipids. Myher et al. [37] used a polarizable capillary column and separated the odd AcTAGs, e.g. i-15:0/16:0/2:0, ai-15:0/16:0/2:0, and 15:0/16:0/2:0. Later analysis [38] of the same material by RP-HPLC allowed the separation of straight- and branched-chain regioisomers of several odd-chain TAGs. For example, the molecular species 4:0/12:0/br-15:0 had a tR more than half a minute lower than the molecular species

4:0/12:0/15:0 (14.41 vs. 14.95 min). Unfortunately, Spanos et al. [39] and Mottram and Evershed [40] by means of HPLC/desorption chemical ionization tandem MS, or RP-HPLC/atmospheric pressure chemical ionization (APCI-MS) would not describe isobaric AcTAGs, and in only a few cases odd isobaric butyrates of TAGs. Lísa et al. [41] identified TAGs containing branched FAs in ruminant fat. Molecular species of TAGs containing three straight-chains versus two straight-chains and one branched-FAs were separated. Furthermore, it was confirmed that TAGs containing branched FAs had smaller tR in the NARP-HPLC/APCI-MS system, e.g. br-SSP (78.7 min), whereas SSP had 79.8 minutes. Unfortunately, it was again confirmed that the APCI mass spectra of TAGs containing both branched and straight-chains were completely identical and no diagnostic fragment of ions was found and no differences in ion abundance were observed either.

TAGs containing i-FAs, ai-FAs, and n-FAs have been identified in the bacterium *Rhodococcus* [42]. These TAGs were separated by isocratic elution, unfortunately with a retention time of several hours. Our results describing the separation of the individual AcTAGs were in agreement with previous report, see Fig. 4. This separation was achieved by means of three columns connected in series with a theoretical number of plates of about 75,000. Unfortunately, the tR was again several hours, see Fig. 4, where the separation of the real sample is shown.

The practical use of the analysis of branched (iso- or anteiso-TAGs) and unbranched (straight chain TAGs) is seen mainly in the analysis of phospholipids from Gram-positive bacteria and/or TAGs in mammalian products. It is known that branched TAGs were found in mammalian milk or in depot fat from bacteria living in the digestive tract [43,44]. Unfortunately, their analysis is very difficult.

For example, Oursel et al. [45] wrote that isobaric forms of phospholipids from *Escherichia coli* are co-eluted and could not be distinguished. Additionally, the article describing an analysis of phospholipids from *Bacillus licheniformis* states that it was not possible to distinguish phospholipids with linear C15 and C17 from their branched isomers by LC-MS and MS/MS analysis [46].

Using two different types of chromatography, i.e., HILIC and RP-HPLC and at the same time two enzymatic reactions, i.e., cleavage of the polar head of phospholipids by phospholipase C and further enzymatic cleavage by pancreatic lipase could also quantify regioisomers such as i-15:0/ai-15:0/2:0 vs. ai-15:0/i-15:0/2:0.

The effect of temperature on the membrane fluidity of Gram-positive bacteria of the genus *Kocuria* was chosen as one of many examples. Bacteria of the two species were cultured at three different temperatures (20, 28, and 37°C) and their phospholipids, respective AcTAGs, were analyzed. The high content of iso- and anteiso-FAs is characteristic of phospholipids of Gram-positive bacteria. Both iso- and anteiso-FAs have a lower melting point than straight chain FAs with the same number of carbon atoms, which causes greater membrane fluidity. At temperatures above the melting point, the acyl chains are more disordered, while at temperatures below the melting point they are arranged regularly. The correlation between the melting point of FAs and the phase transition of phospholipids has been described [47]. Bacteria of the genus *Kocuria* generally contain a maximum of a few percent of unsaturated FAs, based on total FAs. Therefore, they are a suitable model organism for which the effect of culture temperature on the composition of phospholipids can be investigated. The change in transition temperature (Tm) is thus caused only by the different proportions of saturated FAs, better said the ratio of phospholipids to straight-, iso, and anteiso-FAs. For the correct functioning of the membrane, the fluidity of the bilayer lipid is important, which is influenced by a change in the surrounding environment, e.g., temperature.

As an example of a change in the abundance of PG, molecular species having a composition of two pentadecanoic acids were selected, regardless of whether they were straight chain or branched. Their qualitative representation is given in Table 2, i.e., in tandem MS they have an ion of the type $[M\text{-}H]^-$ at *m/z* 693.4712. Table 3 lists five compounds, including two regioisomers. Minor molecular species, i.e., 14:0/16:0-PG and/or 16:0/14:0-PG are not listed because their abundance is so low that they were not detected by RP-HPLC. The change in temperature significantly affected the content of all five molecular species. With increasing temperature, the content of PGs containing iso- and straight-chains of FAs increased. In contrast, the content of PGs decreased with ai-FAs. Thanks to the enzymatic hydrolysis of the peak collected in the interval 628-634.5 min (Fig. 4), the mixture of regioisomers (i-15:0/ai-15:0/2:0 and ai-15:0/i-15:0/2:0) was also quantified. It is also clear from Table 3 that the ratios of individual regioisomers have changed from 10.3:6.5 to 15.6:3.3 (CCM 552), respectively in the second strain (CCM 4950) from 8.4:6.2 to 13.4:3.2. The result clearly indicates that individual regioisomers must have different Tm, as is the case, for example, with two molecular species of PC (14:0/16:0-PC, vs. 16:0/14:0-PC) [48]. It has been reported that the difference in that case is 7.5 °C. From these data, it is apparent that the replacement of a few percent of the total FAs, e.g., replacing straight chain FAs with anteiso FAs, will cause a significant decrease in Tm and thereby increase fluidity.

5. Conclusion

The use of four independent methods, i.e., two chromatographic (HILIC and RP-HPLC) and two enzymatic (cleavage of the polar head of phospholipids by phospholipase C and further enzymatic cleavage by pancreatic lipase) made it possible to identify many molecular species of PGs. The above procedure allowed removing the polar head of PGs with phospholipase C, and after derivatization, the resulting TAGs were separated and identified by RP-LC/tandem ESI^+. Unfortunately, based on tandem MS of both commercially available standards and isolated natural molecular species, it has been shown that the mass spectra of both PGs and AcTAGs having both branched (iso and/or anteiso FAs) and straight-chain FAs are identical. Their identification is possible only based on previous separation by RP-HPLC, but the retention times are of the order of hundreds of minutes. Nevertheless, it was possible to identify the abundance ratios of such similar molecular species as the two regioisomers i-15:0/ai-15:0/2:0 and ai-15:0/i-15:0/2:0. The use of these methods has made it possible to prove that the fluidity of membranes depends on the ratio of molecular species of PGs containing both branched (iso and/or anteiso FAs) and straight-chain FAs. The methods are not limited to bacterial lipids, but as mentioned above, e.g., to mammalian milk lipids or their depot fats.

Declaration of Competing Interest

The authors declare no competing financial interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461708.

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