Příloha #1

Analysis of phosphate and phosphate containing headgroups enzymatically cleaved from phospholipids of *Bacillus subtilis* by capillary electrophoresis.

Podíly spoluautorů na článku

„Analysis of phosphate and phosphate containing headgroups enzymatically cleaved from phospholipids of *Bacillus subtilis* by capillary electrophoresis“

- Doc. RNDr. Radomír Čabala, Dr.

Konzultace při výběru elektrolytu a textových formulacích.

Datum: 8. 10. 15

Podpis: [Signature]

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Příprava reálného vzorku pro otestování metody, korektura mikrobiologii se zabývající části textu.

Datum: [Signature]

Podpis: [Signature]

- RNDr. Martina Riesová, Ph.D.

Použití pufru s neupraveným pH (váženého pufru). Optimalizace separačního elektrolytu pomocí počítačového programu.

Datum: 5. 40. 15

Podpis: [Signature]

- Mgr. Lukáš Taraba

Úprava podmínek průplachu kapiláry, jež vedly k lepší reprodukovatelnosti migračních částí.

Datum: [Signature]

Podpis: [Signature]
Analysis of phosphate and phosphate containing headgroups enzymatically cleaved from phospholipids of *Bacillus subtilis* by capillary electrophoresis

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Received: 20 April 2015 / Revised: 19 June 2015 / Accepted: 26 June 2015
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**Abstract** A new, fast, selective, and reliable capillary electrophoresis method has been developed for analysis of selected phosphoryl (phosphoserine, phosphoethanolamine, phosphoglycerol) and phosphate. The method is based on separation of specific phosphophat containing headgroups (phosphoesters) which are separated from the glycerol skeleton of a phospholipid by a regiospecific enzyme (phospholipase C). Analysis of intact phospholipids with the same polar headgroup but different fatty acid composition has a high impact on separation of phospholipids, so analysis of separated polar headgroups, which avoids this influence, represents a much more suitable approach for phospholipid class research. Optimization of method parameters results in running buffers of relatively narrow pH interval (pH about 10) where all phosphoesters are separated. Further validation has shown that direct UV detection has a sufficient detection limit and all analytes to perform suitable analyses of cell membrane lipids. The optimized method was tested on the lysate of cell membrane of *Bacillus subtilis*, where all analytes were determined.

**Keywords** Phospholipids · Phosphoesters · Phosphoethanolamine · Phosphoglycerol · Phosphoserine · *Bacillus subtilis*

**Introduction** Glycero phospholipids (PL) belong to one of the most important groups of polar lipids, and they are composed of glycerol and two fatty acids (FA) bonded at sn-1 and sn-2 positions. Usually, fatty acid at sn-1 position is saturated while at sn-2 position is unsaturated. When unsubstituted inorganic phosphate is bonded at sn-3 position, it forms the simplest PL, phosphatidic acid. This phosphate can be further substituted by low-mass molecular structures forming the so-called “polar headgroup” such as phosphocholine, phosphoethanolamine, phosphoserine, phosphoinositol, phosphoglycerol, etc. According to these headgroup substituents, PL are sorted into respective classes independent on their acyl composition. Cardiolipins, which is a diphasphatidylglycerol, constitutes a separate PL class. PL are amphiphilic compounds, which are not only substantial constituents of cell membranes, but they define membrane biophysical properties as well as have an active role in cell function. The length and degree of saturation of the lipid acyl chains determine the thickness and fluidity of the inner core of the membrane. The polar part of PL participates on membrane chemical properties, act surface charge of the membrane, membrane curvature, and influence specific interaction with some membrane proteins. PL help proteins and lipoproteins to form the complex membrane bilayer and ensure indirect communication between inner part of cell and its environment. Importantly, it was also shown that phospholipid composition is a key factor in the selectivity and effectiveness of antibiotics targeting the cytoplasmic membrane.
and can also be a resistance determinant towards membrane-active antibiotics [1,2]. Therefore, analysis of PL polar headgroups exhibits continuously growing importance for cytology, microbiology, and biotechnology [3,4].

Nowadays, current PL analyses are performed by various instrumental methods (HPLC, CE); nevertheless, thin layer chromatography (TLC) is still widely used [5-12]. Since each PL class is a group of different chemical individuals that differs in their FA composition, and since polar headgroups are chemically very different to one another, there is no standard, simple, fast, and robust instrumental method to assay them [13].

Although several authors show successful separation of intact PL according to their polar headgroups [5-8], they have separated pure standard chemicals [3] so the influence of variable FA composition in each PL class could not be clearly seen, in contrast to the situation when isolated PL, i.e., PL of natural origin would be used [6]. PL could be separated according to their FA composition by electromigration methods [9,14,15], showing that this effect cannot be generally neglected. Apart from intact PL analysis, which can be carried out by either non-aqueous capillary electrophoresis [6,9,16,17] or micellar electrokinetic chromatography [7,18,19], analysis of cleaved polar headgroups, which are chemical individuals, by any analytical electromigration method has not been published yet. These phospholipids can be obtained by hydrolysis of PL by regiospecific enzyme phospholipase C (see Fig. 1) which splits preferentially the bond between polar headgroup and sn-1 position on glycerol backbone. PL headgroups can exist in the anionic form at suitable pH; therefore, they can be separated in the electric field according to the difference in their ion mobility.

Single phospholipids such as phosphatidyl ethanolamine [20], phosphatidyl ethanolamine [21], and phosphoserine [22], which are important protomic analyses [23], have been already analyzed by capillary electrophoresis; however, these analyses were primarily focused toward the amino acids. Moreover, phospholipids are very different compared to amino acids, so optimization of separation conditions have to follow another rule. Detection of phospholipids is also complicated by the fact that they do not significantly absorb in UV/VIS range, neither emit fluorescence. They might be detected indirectly [24] as it was demonstrated recently on intact PL [5,12]. Some amino phospholipids could be derivatized with fluorescence reagents [25-28], which are, however, not suitable for all the phospholipids. Mass spectrometric (MS) detection was used for some intact PL analyses [9,10], but it cannot be used with nonvolatile buffers. Another universal approach, namely contactless conductivity detection (CCD) of phospholipids, has not been published yet.

The goals of this work are (a) the development of fast electromigration method for reliable determination of phospholipids that correspond to PL polar headgroups, and (b) the comparison of spectrophotometric and contactless conductivity detection of phospholipids. The presented method was optimized and tested on enzymatically hydrolyzed isolated membrane lipids of Bacillus subtilis, a common soil microbe. B. subtilis produces lipopeptide surfactin, which is a potent surface active compound and potential antimicrobial. Its activity depends on the phospholipid composition of the target membrane. Therefore, the knowledge about the composition of the cell membrane of B. subtilis should help to understand how the microbe protects itself against the antibacterial action and, indirectly, to study the general mechanism of bacterial resistance to membrane-targeting antibiotics.

Materials and methods

Instrumentation

Analyses were carried out on CE Agilent 7100 equipped with contactless conductivity detector (CCD) and diode array detector (DAD). All measurements in non-aqueous solutions were performed on capillary CE instrument equipped with CCD [26], commercial UV spectrophotometric detector Spectra 100 (Spectra-Physics), and high-voltage source HCN 35-3500 (Fag Elektronik Gmbh, Rosenheim). On both the instruments, analyses were carried out in bare silica capillaries of 75 μm diameter, 50 cm length, and 50 cm effective length. Separation voltage of 20 kV and detection wavelength of 265 nm were used in both cases.

Chemicals

Analytical standards phosphoglycerol (85%), phosphoserine, phosphoehanolamine, phosphatidylethanolamine (97%), phosphatidylglycerol (98%), phosphatidylserine (70%), cardiolipin (98%), phosphatidic acid (98%) with acetate (99.7%) and formic acid (98%), and acetonitrile (HPLC grade) were supplied by Sigma-Aldrich (Prague). Preparation, sodium hydroxide (both p.a.), and phosphoric acid (85%),
Analysis of phosphate and phosphate containing headgroups

were purchased from Penta Chemicals (Czech Republic). Disodium tetraborate (99.5 %), lithium hydroxide (53.5 %), hydrazine, and citric acid were supplied by Lachema (Neratovice, Czech Republic); ammonium acetate (p.a.) and NaCl (p.a.) were purchased from Lachner (Neratovice, Czech Republic) and CHES was supplied by Merck and TRIS (p.a.). Lab-Lemco powder, bacto-yeast extract, and bactopeptone for broth preparation were purchased from Oxoid (Czech Republic).

Buffers and stock solutions

Non-aqueous electrolyte was ammonium acetate, 60 mmol L⁻¹, and 0.3 % (v/v) acetic acid, both in mixture of acetonitrile and propan-2-ol (3:2, v/v). Aqueous buffers were as follows: pH 10–0.1 mol L⁻¹ CHES and 0.06 mol L⁻¹ LiOH; pH 9–0.1 mol L⁻¹ disodium tetraborate; pH 8–0.1 mol L⁻¹ TRIS; pH 6–0.1 mol L⁻¹ citric acid; pH 3.3–0.1 mol L⁻¹ formic acid; pH 3.8–0.1 mol L⁻¹ acetic acid. Stock solutions of 1 mg mL⁻¹ of phosphoesters and PL were prepared for each pH in corresponding buffer solution.

Real samples

Culture of *B. subtilis* ATCC 2/332 (American Type Culture Collection) was grown aerobically (120 rpm) in nutrient broth (1 g Lab-Lemco powder, 2 g Bacto-yeast extract, 5 g Bactopeptone, 5 g NaCl per liter) at 30 °C. Culture growth was monitored turbidimetrically at 420 nm until it reached exponential phase of growth. The cells were harvested by rapid filtration through a Sypmor no. 5 filter (Pragochema, Czech Republic). Membrane phospholipids were extracted from cell biomass with hexane/propan-2-ol (3:2, v/v) mixture. After evaporation of the solvent in vacuum at 40 °C, the phospholipids were dissolved in chloroform, filtered through glass fiber filter (Whatman), and concentrated under a stream of nitrogen. Phospholipase C, 1 mg, was added to dry sample (about 1 mg of PL mass), and 100 µL of water and 10 µL of methanol were added to leave the ester bonds. Reaction mixture was kept at 37 °C for 2 h in ultrasonic bath. Clear hydrolysate was used for the analysis without any further treatment.

Results and discussion

Analysis of intact phospholipids

At the beginning, intact PL from *B. subtilis* were separated in non-aqueous electrolyte. For peak identification, the real sample was spiked with PL standards of phosphatidylethanolamine and phosphatidylglycerol, each spike was 100 µL of 1 mg mL⁻¹ stock solution (Fig. 2). Both PL standards have long fatty acids whereas *B. subtilis* membranes contain mostly branched-chain FA with odd number of carbon atoms, which are shorter than the FA in used standard PL [4]. Approximately, two thirds of all PL in *B. subtilis* are formed by phosphatidylglycerol and phosphatidylethanolamine [38], so the major peaks should correspond to these PL but the added PL standards migrate with different velocity than any other analyte from the presented real sample. When migration times of PL of the same class but differing in FA composition differ, identification of PL in electropherogram is troublesome. Without determination of FA composition, prediction of migration order of PL classes is impossible because PL are separated not only according to their classes migration times but also according to the FA composition. The impact of FA on the ion mobility of intact PL molecule cannot be simply stated, so FA composition of all PL in the hypothetic sample should have been identical to allow analysis in described way.

This problem could be solved, at least partially, by analysis of polar headgroups cleaved by stereospecific hydrolysis from PL. Main advantage of this approach is that it reduces the number of PL to be identified to a few phosphoesters. Routine analysis in microbiology often do not require determination of individual PL but a relative abundance of FA and PL classes only [30]. Therefore, analysis of polar headgroups would be sufficient for majority of research work except for specialized lipidomics.

Electrolyte selection for phosphoester analysis

Phosphoesters were analyzed separately in aqueous electrolyte to eliminate disadvantageous impact of FA on their mobility. Depending on applied pH, these molecules could bear 1 to 3 negative charges. Successful separation of all phosphoesters needs such a pH in which all the analytes
would be changed in such a way that the differences in their effective electrophoretic mobilities would be sufficient for their proper resolution.

It is impossible to use phosphate buffer for the analysis because inorganic phosphate is one of analytes. Therefore, mostly organic buffers can be used. Various buffers were tested for separation in a relatively broad pH range (4–11.5). Low pH buffers (formate and acetate, pH<4) were not suitable to maintain a stable electrophoretic flow (EOF) and to ensure reproducible analyses. Electrolyte buffered by citric acid was not a suitable solvent for phosphoesters, which are almost insoluble at pH 6; moreover, the resolution of the analytes was very low. Analyses performed at pH 8 (MES) and pH 9 (disodium tetraborate) suffered from low resolution too. More basic CHES+Li+ buffer at pH 10 gave far better results, and the resolution of all the analytes was sufficient (at least 1.8) for their determination. An important aspect of the selected method is a constant ionic strength of the electrolyte, therefore, the buffer was designed by a special software [31], and it was prepared by dissolution of defined amounts of LiOH and CHES in water without any pH adjustment by the base or acid. Slight variations of ionic strength caused irreproducibility of EOF. High resolution and short time of analysis allow the using of the method for real samples. Major disadvantage of the selected method is the asymmetry of long-time migrating peaks; however, electrophoretic mobility of the analytes is sufficient for efficient resolution. At higher pH values (11.5), all the dissociable moieties are totally deprotonated, so the differences in the mobilities of analytes were too low to be separated.

Method validation

Analyses of equimolar mixture of all four phosphoesters by selected method (pH 10, 0.1 mol L⁻¹ CHES and 0.06 mol L⁻¹ LiOH, 40 kV m⁻¹) were performed to evaluate limit of detection (LOD), resolution, repeatability, reproducibility, and linearity. Resolution of critical pair of analytes, phosphoserine, and phosphoglycerol is 1.8; therefore, applied electric field intensity seems to be satisfactory. Limit of quantification was measured for all the analytes using UV–vis detection at 205 nm, whereas LOD for CCD was higher, consequently, spectrophotometric detector was used for further testing only. Repeatability (n=7) was calculated from both peak areas and heights, whereas reproducibility (n=6) was calculated at different weeks. Analyte peaks are asymmetrical, therefore, there is a considerable difference between the results obtained either from peak areas or peak heights (see Table 1). Repeatability calculated from peak height is lightly better than the one calculated from areas, probably because the integration of asymmetrical peaks is more difficult than estimation of their heights. On the contrary, there is no significant difference in reproducibility.

Real sample analysis

The selected method was evaluated on hydrolyzed isolated lipids of _B. subtilis_. All the supposed analytes were detected; however, phosphoethanolamine and phosphate were below the limit of quantification. Ratio between concentrations of phosphoglycerol (0.208 mg mL⁻¹) and phosphoserine (0.077 mg mL⁻¹) and low concentration of inorganic phosphate corresponds with PL composition of _B. subtilis_.
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Conclusion

New capillary electrophoresis method was developed for analysis of phospholipids, which correspond to PL headgroups. Analysis of separated PL polar headgroups was necessary because intact PL of the same PL class but different acyl composition migrated with different electrophoretic mobilities. Phospholipase C regioselectively cuts polar PL parts and allows analysis of released phospholipids. The separation conditions of the presented method allow analysis of PLs headgroups independently of the origin of biological sample. Many experimental conditions were tested and the optimum electrolyte CHES/Li of pH 10 with defined ionic strength was selected. The method was validated, and the results were evaluated statistically. Limit of detection of all the analytes is sufficient to analyze real biological samples. The method was tested on hydrolyzed isolated lipids of B. subtilis.

Acknowledgments

This work was supported by SVV263025 and 13-18051P from the Czech Science Foundation and authors declare no conflict of interest.

References

Příloha #2

Gas chromatography with mass spectrometry analysis of phosphoserine, phosphoethanolamine, phosphoglycerol, and phosphate.


- Doc. RNDr. Radomír Čabala, Dr.

Parametry teplotního gradientu, upřesnění formulaci závěrů a celkové vedení výzkumu

19. 5. 2015

datum

podpis

- RNDr. Robert Kubiček, CSc.

Derivatizace slabším z použitých činidel za velmi nízké teploty po dlouhou dobu

25. 2. 2015

datum

podpis

- Mgr. Peter Podolec, Ph.D.

Použití dvou derivatizačních činidel (slabšího a silnějšího)

24. 2. 2015

datum

podpis

- Mgr. Alexandra Hengerics Szabó, Ph.D.

Úprava vnější formy článku dle chromatografických tradic nad rámec požadavků časopisu

29. 1. 2016

podpis

- Mgr. Martin Ston

Poradenství ohledně GC-MS instrumentace

4. 5. 2015

datum

podpis
Research Article

Gas chromatography with mass spectrometry analysis of phosphoserine, phosphoethanolamine, phosphoglycerol, and phosphate

A new, rapid, sensitive, robust, and reliable method has been developed for the qualitative analysis of phosphoserine, phosphoethanolamine, phosphoglycerol, and phosphate using gas chromatography with mass spectrometry and two-step trimethylsilylation. The method employs hexamethyldisilazane for silylation of the phosphate and hydroxyl groups in the first phase and bis(trimethylsilyl)trifluoroacetic anhydride for silylation of the less reactive amino groups in the second phase. This order is of key importance for the method because of the different reactivities of the two reagents and the mechanism of derivatization of the active groups of the analytes. Trimethylsilylated derivatives of the analytes were identified on the basis of their retention times and mass spectra. The probable structures of the major fragments were identified in the spectra of the trimethylsilylated derivatives and characteristic m/z fragments were selected for each analyte. Fragments with m/z 73 and 29 occurred in the spectra of all the analytes. The characteristic retention data were employed to calculate the retention indices of the individual silylated phosphorylated substances in the hydrocarbon range C₁₂–C₉ for the DB-5ms column. The method was employed to measure the polar fraction of the hydrolysate of the crotalusplanic membrane of Bactias nubilis. The detection limits vary between 5 μg/ml (trimethylsilylated phosphate) and 72 μg/ml (trimethylsilylated phosphoethanolamine).

Keywords: Gas chromatography with mass spectrometry / Phosphoethanolamine / Phospholipids / Phosphoserine / Trimethylsilylation

DOI: 10.1002/jssc.201400687

Additional supporting information may be found in the online version of this article at the publisher’s web-site.

1 Introduction

Phospholipids (PLs) form part of the nonpolar fraction of the biological samples and are mostly investigated from the viewpoint of the contents of fatty acids together with nonpolar lipids [1]. PLs are formed of a glycerol skeleton with two ester-bonded fatty acids (hydrophobic part), to which the phosphate functional group is bonded, which is frequently further modified by an additional low-molecular-weight structure (hydrophilic part, e.g. serine, ethanolamine, glycerol, choline, etc.). Analysis of PLs is generally based on a wide range of separation techniques (HPLC, GC, CE) with various detection systems. Before the actual analysis of the fatty acids, PLs are commonly separated into the relevant classes according to their polar groups [2] using a separation technique suitable for polar substances [3]. From the viewpoint of their chemical properties, these classes are relatively inhomogeneous and the ratio of the individual classes of PLs is not generally determined by GC. Instead of this ratio, the individual PLs are determined in the total hydrophobic phase [4,5], or the contents of the fatty acids are determined in the individual PLs classes [6]. While analyzing PLs by GC, it is necessary to reduce their polarity and increase their volatility using a suitable derivatization agent. If the PLs contain a quaternary ammonium salt (phosphocholine), a demethylation agent must also be used [7]. Next to methylation [8,9], trimethylsilylation [10] is preferred today because of its advantages, not only in the hydrophilic group normally occurring in PLs, but also for derivatization of rarely occurring lipids, frequently isolated from microorganisms living under extreme conditions [11,12,13]. The study of PLs generally falls in the field of lipidomics, which is currently an important and rapidly evolving field.

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Abbreviations: BSTFA, N,O-bis(trimethylsilyl)trifluoroacetic anhydride; HMDS, hexamethyldisilazane; PLs, phospholipids; TMS- Phosphate, trimethylsilyl-phosphate; TMS- phosphoethanolamine, trimethylsilylated phosphoethanolamine; TMS- phosphoglycerol, trimethylsilylated phosphoglycerol; TMS- phosphoserine, trimethylsilylated phosphoserine

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Microorganisms are a very important group of organisms for lipidomics and are used in an enormous range of research and testing applications. From the viewpoint of bacterial lipidomics, PI's are present in higher concentrations in cell membranes and thus sensitive and accurate analysis of the lipid composition of cell membranes can be used for study, characterization, and identification of microorganisms. In the study of metabolic processes, it is more important to determine the content of individual classes of PI's than their acyl composition. The acyl composition of PI's can affect the determination of the individual classes of PI's or their mutual ratios because the total polarity of a PI molecule depends strongly on both parts, and when fatty acid composition is not homogeneous enough separation is carried out mostly into fractions instead of PI classes. High-performance separation techniques such as normal-phase HPLC or hydrophilic interaction chromatography can be used for separation, however, detection is a serious problem because PI's cannot be detected by UV-VIS. So MS or evanescent light-scattering detection, which is expensive and not available for standard HPLC systems, is necessary. Electrophoretic migrations are as suitable as HPLC. They can be used to separate PI's. According to their polar headgroups, separation of PI's in an electric field is also influenced by fatty acid chains. Different length and shape of acyl chains may affect the migration time of analytes, so the determination is sometimes troublesome. This problem could be solved by determining not the individual PI's, but only their polar groups without their glycerol skeletons and hydrophilic acyls. Although these are polar substances, their suitable derivatives can be determined by GC. For example, phosphoserine was determined by GC before the extensive use of MS detectors. Because this is an important analyte, not only for lipomics, but also for proteomics, the metabolite is now used in connection with some degenerative changes in the organism. The combination of GC with MS permits the determination of phosphoserine in a mixture of chemically very similar substances, for example its metabolites, and it is frequently determined together with other phosphorylated amino acids.

The work was performed to develop a sensitive, robust, and reliable GC–MS method for the determination of phosphoserine together with phosphoethanolamine, phosphoglycerol, and phosphate, following derivatization with silylation agents. This method can be used as a faster alternative to current methods in cases where they are not available, are too expensive, or are too tedious.

2 Materials and methods

2.1 Chemicals

N-O-Bis[(trimethylsilyl)] trifluoroacetamide (BSTFA: Supelco, Bellefonte, PA, USA) and hexamethyldisilazane (HMDS: Sigma–Aldrich, Steinheim) were used to silylate the analytes. Phosphoethanolamine (purity not declared), phosphoserine (standard purity not declared), 1-phosphoglycerol (85%), and acetaminole (HPLC grade) were supplied by Sigma–Aldrich, Prague. He (99.9995%, Linde Gas, Prague) was used as the carrier gas. Phosphoric acid (85%, f.a., Penta, CZ), TFA (99.5%, Romil, Cambridge, UK), and a mixture of standard hydrocarbons C<sub>0</sub>–C<sub>20</sub> (Spectra-Physics) were used as supplied.

2.2 Sample preparation

Phosphoserine, 1-phosphoglycerol, phosphoethanolamine, and phosphoric acid were weighed individually in 2 mg amounts into 2 mL vials and then dissolved in 600 μL of a mixture of acetaminole and HMDS (1:1, v/v): 2 μL of TFA was added to each vial. Then the samples were maintained at a temperature of 40°C for 60 min in open vials to allow ammonia, formed as a side product of trimethylsilylation, to escape. In the next step, 400 μL of BSTFA was added and the samples were closed with a cap with a septum and maintained for 30 min at 80°C. These samples were then analyzed by GC-MS.

2.3 Preparation of the calibration series

A stock solution was prepared from a mixture of standards with concentrations of 2000 μg/mL (phosphoethanolamine), 1500 μg/mL (phosphoserine), 1000 μg/mL (phosphoglycerol), and 100 μg/mL (phosphate), which were silylated (see Section 2.2) and then diluted 2-, 5-, 10-, 20-, 50-, 100- and 200-fold with acetaminole.

2.4 Validation

The linearity was tested first in the same range as the calibration series, although the value of the correlation coefficient across three orders of magnitude was greater than 0.99 only for trimethylsilyl phosphate (TMS-phosphate); r<sup>2</sup> was between 0.95 and 0.98 for the other analytes, while the correlation coefficients for all the analytes were greater than 0.99 when testing the linearity across two orders of magnitude (concentrations of a hundred times of LOQ). The curve of the dependence of the concentration over three orders of magnitude obeys a quadratic dependence; in this case, the correlation coefficients for a quadratic dependence were far better than 0.99. The detection and quantification limits were determined from the calibration series and varied between 5 or 18 μg/mL (TMS-phosphate) and 72 or 240 μg/mL (TMS-phosphoethanolamine, more detail in Supporting Information Table S1). The reproducibility was measured on a set of ten measurements, where the SD was measured for each analyte with a value between 2% (TMS-phosphoethanolamine) and...
9% (TMS$_2$-phosphate). The reproducibility was tested on a series of ten measurements, repeated after one day and one week. The differences between the relative contents of the individual analytes did not change after one day; the greatest change was observed for TMS$_2$-phosphorylcer (by 3%), but this is an unsuitable substance that almost completely decomposed after one week; thus, it is advisable to perform routine analysis during a single day.

### 2.5 Real sample

The method was tested on the aqueous fraction of the hydrolysate of the cell membrane lysate of the bacteria Bacillus subtilis, which remains unused after transphosphorylation of membrane lipids, to determine the fatty acid fraction. Approximately 1 mL of the sample, containing <1000 μg of P, was obtained from the Department of Genetics and Microbiology of the Faculty of Science of Charles University, where this microbe is studied [26] with respect to production of poerta anabiotic CYCLO-ALCOHOLIC.

### 2.6 Instrumentation

The GC-MS analysis was performed using a gas chromatograph coupled with a GC-MS QP 5050A mass spectrometer (Shimadzu, Japan). Separation was performed using a DB-5ms capillary column (30 m × 0.25 mm ID, stationary phase thickness 0.25 μm) from Agilent Technologies (USA). The linear velocity of He was set at 35 cm/s. A sample (2.5 μL) was injected using an AOC-20 automatic sampler (Shimadzu) at an injection temperature of 250°C in both the splitless mode (0.5 min) and split (1:100) mode. The mixture of hydrocarbons was measured for calculation of the retention indices and analysis in the SIM regime at a split of 1:250. The analyses were performed with the following temperature program: initial temperature 60°C, 2 min, then 9°C min$^{-1}$ to 250°C, 5 min. The transfer line was maintained at a temperature of 250°C and the detector solvent cut time was 5 min. In an analysis of the mixed samples in the SIM mode, responses were measured for m/z 73, 209, 174, 138, 116, and 87, characteristic for the individual analytes. The chromatograms were processed using the GC Solution v. 1.21 (Shimadzu) software. The NIST 2011 database was used to evaluate the mass spectra.

### 3 Results and discussion

#### 3.1 Trimethylsilylation of phosphates

It was found in preliminary experiments that single-step derivatization of all the analytes is not possible because of the great differences in reactivity between the hydroxyl and amine groups. Thus, the investigated phosphates were separated into two groups for the trimethylsilylation: (i) phosphates containing only hydroxyl groups (phosphoric acid, phosphoglycerol) and (ii) phosphates also containing amine groups (phosphoethanolamine, phosphoserine). While phosphates containing only hydroxyl functional groups can be fully trimethylsilylated using relatively inexpensive HMDS, amine groups can practically not be trimethylsilylated using this reagent. Trimethylsilylation of the hydroxyl groups in the first group of phosphates proceeds according to the scheme in Fig. 1.

The amine groups of the second group of phosphates must be subsequently trimethylsilylated using relatively expensive BSTFA that, however, has lesser reactivity. It is not suitable to use only BSTFA for overall trimethylsilylation in a single step because the reagent of BSTFA with the hydroxyl groups is too violent and the reaction products are frequently precipitated, leading to completely unrepeatable results. Consequently, two-step derivatization was employed, where the hydroxyl groups are trimethylsilylated in the first step using HMDS (weaker silylation reagent) and the amine groups are derivatized in the second step together with the remaining hydroxyl groups using BSTFA and the reaction is thus not too violent. This two-step trimethylsilylation using reagents with different reactivities has not yet been described in the literature. To ensure a standard procedure, two-step trimethylsilylation was used for all the samples. Figure 2 depicts a scheme for the second step in the trimethylsilylation. While phosphoethanolamine is completely trimethylsilylated on the amine group, the amine group of phosphoserine is trimethylsilylated only once, probably for steric reasons.

#### 3.2 Identification of TMS derivatives of the analytes

Trimethylsilylated phosphorylated substances were measured by GC-MS and their mass spectra were compared with the spectra in databases and the literature. The characteristic fragments of TMS$_2$-phosphate correspond to trimethylsilyl (TMS) for m/z 73 and desmethyl-TMS$_2$-phosphate for m/z 209 (MS spectrum in Supporting Information Fig. S1).
In addition to fragments for m/z 73 and 299, the mass spectrum of trimethylsilylated phosphoglycerol (TMS$_2$-phosphoglycerol; see Supporting Information Fig. S2) also exhibits a very intense fragment at m/z 357, corresponding to trimethylsilylated phosphoglycerol. Theoretically, phosphoglycerol can exist in two positional isomers, as 1-phosphoglycerol and 2-phosphoglycerol. 2-Phosphoglycerol practically does not exist in biological matrices; however, it may be present in small amounts in synthetic chemicals. Although the mass spectra of the two isomers are very similar (both contain m/z 357), they have different retention times (see Fig. 5) and thus this isomer practically does not affect the analysis of the mentioned phosphorylated substances.

In contrast to TMS$_2$-phosphoserine, TMS$_2$-phosphoethanolamine contains a fully trimethylsilylated amino group, as it does not contain a carboxyl group that would prevent complete trimethylsilylation. The mass spectra of TMS$_2$-phosphoethanolamine (Supporting Information Fig. S3) and TMS$_2$-phosphoserine (Supporting Information Fig. S4) are very similar and thus m/z 174 and 188 were selected for their measurement, where the differences between the spectra of these substances are greatest. All the more intense fragments of TMS$_2$-phosphoethanolamine also occur in the spectrum of TMS$_2$-phosphoserine. The mass spectrum of TMS$_2$-phosphoethanolamine contains fragment m/z 174, which can be assumed to correspond to the bis(trimethylsilyl)glycerol fragment. It is surprising that the same fragment m/z 174 also appears in the spectrum of TMS$_2$-phosphoserine even though it theoretically cannot yield the bis(trimethylsilyl)glycerol fragment. As the intensity of m/z 174 for TMS$_2$-phosphoserine is much higher than for TMS$_2$-phosphoethanolamine, it can be assumed that the first is a different (minor) fragment in the latter case.
As the NIST 2011 database does not contain the spectrum of TMS₂-phosphoserine, the measured mass spectrum was compared with the published results [18], where agreement was found both in the contents of the fragments and their relative intensities. The intense and characteristic fragment \( m/z \) 356, corresponding to the \( \text{H}_2\text{O}_{2}\text{O} \) tetra(trimethylsilyl)ethanolamine-O-phosphate fragment was selected for measurement in the SIM mode, where this fragment contains a monotrimethylsilylamine group and thus TMS₂-phosphoethanolamine and the other analytes do not contain it.

The above-mentioned fragments 73, 174, 188, 299, 356, and 357 were employed for MS detection of the analytes in the SIM regime with their separation, where their retention times were measured and the occurrence of the characteristic fragments was compared (see Fig. 3). It can be observed in Fig. 3 that the separation of the analytes is adequate and there is sufficient reserve in the resolution for the occurrence of accompanying substances in real samples. The structures, monoisotopic masses, and occurrence of characteristic fragments suitable for determination of the individual analytes are given in the Supporting Information.

### 3.3 Retention indices

The results of the GC-MS analyses for calculation of the retention indices were used for more thorough characterization of the TMS derivatives of the phosphorylated substances. The measured retention times were used to calculate the elution temperatures, which were further used to calculate the retention indices for the individual analytes (Table 1). It is apparent from the results that analytes are eluted between hydrocarbons \( C_{12} \) and \( C_{18} \) under the experimental conditions.

As the MS response of the TMS₂-phosphate in the equimolar mixture of all four monitored analytes is in order of magnitude higher than that of the other components, split mode 1:100 was used to measure its retention time in the scan mode, while all the other measurements were performed in the splitless mode. For validation of the retention times, the equimolar mixture was also measured in the SIM mode for \( m/z \) 73 (Fig. 4), where large differences can be observed between the response factors of the analytes.

### 3.4 Analysis of a real sample

TMS₂-phosphate (5.7 μg/ml) a TMS₂-phosphoglycerol below the detection limit was found by the described procedure in a sample of the aqueous fraction of the hydrolysate of the cell membrane lysate of \( B. \) subtilis. The other two analytes were not detected. Earlier research on this bacterium indicates a high content of phosphophethanolamine [27] and relatively lower content of phosphoglycerol. As the detection limit of TMS₂-phosphoethanolamine is the highest of the monitored analytes, it can be assumed that a major part of the ammonium phosphorylated analytes was destroyed during alkaline hydrolysis of the membrane lysate. We are working on less destructive and more selective enzymatic hydrolysis yielding intact polar groups following splitting off of the acyl groups.

### 4 Conclusion

A new method has been developed for simultaneous analysis of phosphoserine, phosphoethanolamine, phosphoglycerol, and phosphate by GC-MS with two-stage trimethylsilylation using two elution agents with different reactivities. The method enables effective derivatization of compounds.
important from the viewpoint of lipid metabolism. The problem related to the large difference in the signals of time-resolved phospholipid substances in their simultaneous analysis was resolved using the scan and SIM mode in combination with split and splitless injection. The determined retention indices indicate that the derivatives are eluted between hydrocarbons C11 and C25. The described method is sufficiently effective and the use of the MS detector ensures sufficient selectivity against any potential interference in real samples. The measured spectra agree with the spectra in the commercial database and the spectrum of TMS phosphate acylates with the formerly published results. Characteristic fragments were selected for the analysis of real samples, where their probable structures were determined. Their retention indices were calculated from the TMS retention times. Only phospho- and phosphoglycerol were found in the aqueous fraction of the cell membrane hydrolysate, probably because of the too radical procedure for the hydrolysis of membrane lysate.

The work was performed at the Department of Analytical Chemistry of the Faculty of Science of Charles University in Prague in the 2013/2014 academic year with support from grants SVV-260684, APVV-084411, APVV-0415-10, APVV-0861-11, and APVV-0861-12.

The authors have declared no conflict of interest.

5 References

Příloha #3

Simultaneous analysis of polar and non-polar components of cell membrane phospholipids by GC-MS.

Podíly práce spoluautorů na článku

„Simultaneous analysis of polar and non-polar components of cell membrane phospholipids by GC-MS“

- Mgr. Miroslava Bursová

Statistické určení optimálních parametrů derivatizace.

Datum: 19.5.2016

Podpis:

- Mgr. Martin Ston

Konzultace týkající se programu EZGC.

Datum: 24.5.2016

Podpis:

- Doc. RNDr. Radomír Čabala, Dr.

Struktura a celková režie článku včetně odborné korekce vlastního textu.

Datum: 725.2016

Podpis:

- RNDr. Gabriela Seydlová, Ph.D.

Příprava reálného vzorku pro otestování metody.

Datum: 23.6.16

Podpis:
Simultaneous analysis of polar and non-polar components of cell membrane phospholipids by GC-MS

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Received 17 September 2015; Revised 19 March 2016; Accepted 11 March 2016

A rapid and selective GC-MS method was optimised for the simultaneous analysis of fatty acids, phosphoethanolamine, phosphorylglycerol and phosphoserine, which comprise the polar headgroups of phospholipids. Enzymatic hydrolysis was used for sample preparation to selectively release fatty acids and prevent cleavage of the phosphoester bonds of the polar headgroups. A two-step consecutive derivatisation of the fatty acids and polar headgroups was applied to enable the simultaneous analysis of the selected analytes. The method was tested on samples of cell membrane phospholipids of Bacillus subtilis, a microbe with a broad spectrum of fatty acids. This approach can accelerate and simplify the technological research and quality control. The experimental conditions were optimised using chemometric approach denoted as experimental design.

Keywords: bacterial membrane, branched-chain fatty acids, GC-MS, phospholipids, lipidomics, silylation

Introduction

Phospholipids (PL) are a class of lipids, a major component of all cell membranes, forming the cell lipid bilayer. The physicochemical properties of cell membranes are jointly determined by the fatty acyl chains and polar component (headgroups) of the PLs' composition (Boumann et al., 2006). Knowledge of cell membrane composition is indispensable in many scientific and industrial applications, e.g. lipidomics (Caboni & Lercker, 1984), or foodomics (García-Cañas et al., 2012). An important aspect of cell membrane analysis is the study of microorganisms, especially with regard to their potential medical and industrial uses.

Most phospholipids are esters of glycerol, fatty acids and phosphoric acid or phosphoric acid modified with other polar moieties (phosphoserine, phosphoethanolamine, phosphorylglycerol). The phosphoester represents the polar headgroup of PL, which makes it partially soluble in water and enables all the interactions with the rest of the environment, which is important in cell communication and membrane transport.

Bacillus subtilis, which is a common soil microbe, is a typical Gram-positive bacterium; it has been thoroughly researched and frequently used as a model microbe and for fermentation. Some of its wild-type strains produce the promising detergent and antibiotic lipopeptide surfactin (Kawai et al., 2004). The membrane lipids of the surfactin producer strain contain five phospholipid classes:

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phosphatidic acid, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine and cardiolipin. The majority of fatty acids (FA) (about 90%) are branched-chain FA and some common straight-chain FA (Seydlová et al., 2012).

Branched-chain fatty acids (BCFA) are classified into two different types according to the position of the terminal methyl groups of the aliphatic chain (iso- and anteiso-). In the iso-conformation, the penultimate carbon atom of the aliphatic chain (ω-2 carbon) is branched with a methyl group, while in the anteiso-conformation the third last carbon (ω-3) atom is branched. The production of BCFA is typical of Gram-positive bacteria, so their presence can contribute to bacteria identification (Li et al., 2010).

The identification and determination of intact phospholipids are carried out using thin-layer chromatography (TLC) or liquid chromatography-mass spectrometry (LC-MS) but these methods have their disadvantages. TLC offers only a limited sensitivity and quantification of certain PL groups, and is often not sufficiently selective (Fuchs et al., 2007). In addition, TLC is time- and solvent-demanding. On the other hand, LC-MS is an advanced but expensive instrumental technique (Butovitch, 2007). Due to the variability of the fatty acid composition of PLs, it is impossible to designate unique m/z values corresponding to the respective PL classes. However, PL membranes are composed of broad spectra of FA and PL classes. They can be measured by LC-MS, but the spectra of the individual peaks are relatively complex and often difficult to evaluate because the PL classes are not chemical individuals (Ostrowski et al., 2008).

Fatty acids are commonly analysed by GC or GC-MS after PL hydrolysis (Seppinen-Laukko et al., 2002). Alkaline/acid-catalysed transesterification is commonly used, which cleaves bonds between glycerol and fatty acids and transforms FAs into methyl esters (Yousef et al., 2012). This method is not suitable for polar headgroups, because it cleaves the phosphate group from the polar headgroup. Stereospecific cleavage of the bond between the polar headgroup and glycerol backbone can be provided by phospholipase C (Rastogi et al., 2005).

The present study-group recently published a method for the GC-MS analysis of PL polar headgroups after a two-step derivatisation (Bierhandl et al., 2015). The analysis of individual phospho-esters by GC is needed for the simultaneous analysis with fatty acids. Since hydrolysed PLs contain moieties with different silylation reactivities, they need to be derivatised in two steps. A weaker agent (hexamethyldisilazane, HMDS) is used in the first step to silylate the more reactive groups (hydroxyl, carboxyl), whereas the stronger one (N,O-bis(trimethylsilyl)trifluoracetamide, BSTFA) is used for amine. The stronger agent cannot be used in a one-step procedure because it reacts too vigorously and produces varied reaction products (Podolec et al., 2014).

The optimal conditions for the derivatisation method have been found using a chemometric approach known as response surface methodology (RSM). This approach is taking the place of the common one-factor-at-a-time (OFAT) procedures because they are time-consuming and do not take into account the possible interactions among the individual parameters (Table 1). The RSM procedure consists of four principal steps: (i) selection of parameters and responses; (ii) screening of parameters (e.g. temperature, volume, rate) exerting a statistically significant influence on the response studied (e.g. extraction efficiency, peak area, etc.); (iii) modelling, to find a mathematical description of the system response as a function (usually a polynomial) of the selected parameters; and (iv) optimisation, to specify the combination of parameters yielding the optimum response (Hibbert, 2012).

In terms of statistical methods, fractional factorial design was employed for screening, face-centred central composite design for modelling and the desirability function for optimisation.

The present work sought to develop and optimise a rapid, sensitive and robust GC-MS method suitable for the simultaneous analysis of both polar PL headgroups and non-polar fatty acids in one single measurement, hence saving on the time and material required for two sets of analyses, using advanced statis-
Table 2. Experimental parameters, their levels and experimental plan used in screening and modelling by fractional factorial design

<table>
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<th>Volume of trifluoroacetic acid (µL)</th>
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Theoretical

Optimisation of derivatisation conditions

The eight silylation parameters were selected based on the prior experience of the authors (Bierhand et al., 2015) and the results of the preliminary experiments. They are listed in Table 1 together with their levels. The maximum response was sought, defined as the sum of the absolute peak areas of fatty acids and polar headgroups (phosphoserin, phosphoethanolamine, phosphoglycerol).

To screen the significance of the parameters, fractional factorial design was used, based on a determination of the potential main effects and interactions between the parameters (Ryan, 2007). Three values were attributed to each parameter (Table 1), where only the low and the high values were used for screening. A table was created using the program Minitab 16, and a set of 16 random measurements was performed accordingly (Table 2).

The results were evaluated using the ANOVA test, determining the main effects at a significance level of 95 % (Armstrong et al., 2002). The result for the sum of the absolute peak areas can be seen in the Pareto graph (Fig. 1).

This test of parameter significance indicated all the selected parameters, apart from the volume of the reaction mixture of HMDS/ACN, to have statistical significance. In addition, the following interactions between parameters could not be omitted from the statistical influence on the selected response: (i) derivatisation temperature (1) – volume of BSTFA; (ii) derivatisation temperature (1) – derivatisation time (2); (iii) volume of trifluoroacetic acid – derivatisation time (1); (iv) volume of trifluoroacetic acid – time of derivatisation (1); and (v) time of derivatisation (2) – derivatisation temperature (2).

To optimise the operational parameters referred to above, face-centred central composite design (Myers et al., 2009) was employed. Table 3 summarises the 14
Table 3. Experimental parameters, their levels and experimental plan used in screening and modelling by face-centred central composite design

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<th>Volume of trifluoroacetic acid (μL)</th>
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random combinations of the low, central and high parameter levels, created in accordance with the CCD plan by Minitab 16. Functional polynomial dependence was used to evaluate this system by the ANOVA method. After treating the results for the sum of the analyte peak absolute areas using Minitab 16 and excluding insignificant contributions to the response, the following quadratic equation was obtained (Table S1). The lack-of-fit test was insignificant at the 5 % level and the coefficient of determination ($R^2 = 0.8399$) indicates sufficient agreement between the experimental data and the model created. The response surface plot (Fig. S1, in Supplementary data) illustrates the inter-relationship of statistically significant parameters with the response data.

The system was optimised deploying Minitab 16, using a desirability function to find the combination of the selected parameters which yielded the maximum response value (Costa & Pereira, 2010). The following optimum parameters were obtained: volume of BDMS/ACN = 200 μL, volume ratio of reaction mixture of BDMS/ACN = 1 : 1, volume of trifluoroacetic acid = 2.5 μL, temperature and time of derivatisation (1) = 100°C for 60 min, volume of BSTFA = 40 μL and temperature and time of derivatisation (2) = 100°C for 15 min.

Experimental

BDMS, BSTFA, (99 %), acetonitrile, (HPLC grade), hexane (97 %, HPLC grade), chloroform (99 %), phosphoethanolamine, phosphoserine, phosphoglycerol (85 %), phosphatidyglycerol (99 %), phosphatidylethanolamine (97 %), phosphatidylinerine (70 %), palmitoleic (98.5 %) and oleic (99 %) acid, 12- and 13-methylmyristic (98 %) and 14- and 15-methylpalmitic (98 %) acids and phospholipase C, isolated from Bacillus cereus, were all purchased from Sigma–Aldrich (USA). Stearic, palmitic and myristic acids (p.a.) were supplied by Lachema (Czech Republic). Propan-2-ol (LC-MS grade) was provided by Fluka (Sigma-Aldrich). Trifluoroacetic acid (99.9 %; Romil, UK) was used as the catalyst for silylation. Phosphoric acid (85 %, p.a.) was supplied by Penta (Czech Republic). Lab-loceno powder, baco-yeast extract, and bacopetone for broth preparation were purchased from Oxoid (Czech Republic); NaCl (p.a.) was purchased from Lachner (Czech Republic).

Standards (0.5 mg) or real samples (20 μL) were poured into a 2 mL vial and derivatised using 200 μL of HMDS/acetonitrile (2 : 1 : 1) reaction mixture and 2.5 μL of trifluoroacetic acid, and the mixture was maintained at 100°C for 60 min. Next, 40 μL of BSTFA was added to derivatised the amino groups (phosphoethanolamine and phosphoserine), and the reaction was maintained at 100°C for a further 15 min.

The culture of Bacillus subtilis ATCC 21332 (American Type Culture Collection) was grown aerobically (120 min⁻¹) in nutrient broth (1 g Lab-Loceno powder, 2 g Bacto yeast extract, 5 g Bactepepton (all Oxoid; Czech Republic), 5 g NaCl (Lachner; Czech Republic) per litre) at 30°C in the Department of Genetics and Microbiology, Faculty of Science, Charles University in Prague, Czech Republic. The culture growth was monitored turbidimetrically at 420 nm until it attained the exponential phase. The cells were harvested by rapid filtration through a Sproor no. 5 filter (Pragocema, Czech Republic). The membrane
Table 4. Method validation parameters

<table>
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<tr>
<th>TMS derivative</th>
<th>Calibration Intercept</th>
<th>Equationa Slope</th>
<th>R²</th>
<th>LOD (µg mL⁻¹)</th>
<th>LOQ (µg mL⁻¹)</th>
<th>Repeatability (%)</th>
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<td>70522269</td>
<td>0.9948</td>
<td>3.6</td>
<td>12.1</td>
<td>3</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>173818</td>
<td>109573854</td>
<td>0.9940</td>
<td>4.5</td>
<td>16.5</td>
<td>4</td>
</tr>
<tr>
<td>Palmitic</td>
<td>113712</td>
<td>116858696</td>
<td>0.9952</td>
<td>2.9</td>
<td>9.8</td>
<td>4</td>
</tr>
<tr>
<td>15-Methylpalmitic</td>
<td>105510</td>
<td>68954573</td>
<td>0.9941</td>
<td>4.6</td>
<td>15.4</td>
<td>4</td>
</tr>
<tr>
<td>14-Methylpalmitic</td>
<td>181723</td>
<td>666246946</td>
<td>0.9951</td>
<td>3.6</td>
<td>11.9</td>
<td>4</td>
</tr>
<tr>
<td>Oleic</td>
<td>2696424</td>
<td>122405098</td>
<td>0.9936</td>
<td>7.1</td>
<td>23.7</td>
<td>5</td>
</tr>
<tr>
<td>Stearic</td>
<td>138413</td>
<td>983593635</td>
<td>0.9948</td>
<td>4.3</td>
<td>14.3</td>
<td>5</td>
</tr>
</tbody>
</table>

a) Calibration equation in form: slope × concentration – intercept.

Table 5. Abundances, m/z values and retention times of TMS derivatives measured on Bacillus subtilis membrane lipid sample

<table>
<thead>
<tr>
<th>TMS derivative</th>
<th>m/z</th>
<th>Retention time (min)</th>
<th>Derivative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myristic</td>
<td>53, 73, 75, 117, 129</td>
<td>07.69</td>
<td>04.3</td>
</tr>
<tr>
<td>13-Methylmyristic</td>
<td>59.0714</td>
<td>08.128</td>
<td>12.3</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>09.072</td>
<td>09.281</td>
<td>26.4</td>
</tr>
<tr>
<td>Palmitic</td>
<td>09.87112</td>
<td>10.963</td>
<td>10.2</td>
</tr>
<tr>
<td>15-Methylpalmitic</td>
<td>11.095</td>
<td>11.400</td>
<td>09.0</td>
</tr>
<tr>
<td>14-Methylpalmitic</td>
<td>11.095</td>
<td>11.400</td>
<td>09.0</td>
</tr>
<tr>
<td>Stearic</td>
<td>11.400</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Polar headgroups:

- Phosphoglycerol: 73, 299, 357, 06.814
- Phosphoethanolamine: 73, 299, 174, 188, 07.072
- Phosphoserine: 73, 299, 356, 07.191

Phospholipids were extracted from the cell biomass using a hexane/propan-2-ol (2:3) mixture, which was a standard extraction procedure in accordance with Seydlová and Svobodová (2008). After evaporation of the solvent in a vacuum at 40 °C, the phospholipids were dissolved in chloroform, filtered through glass fibre filters (Whatman) and dried under a stream of nitrogen. Phospholipase C, 1 mg, was added to the dry sample, and 100 µL of water and 10 µL of methanol were added to cleave the ester bonds. The reaction mixture was maintained at 37°C for 2 h in an ultrasonic bath, then allowed to stand for 10 min and the clear solution used for the analysis.

All GC-MS analyses were carried out on a GC-17A gas chromatograph with a QP 5000A single quadrupole MS (Shimadzu, Japan), using electron ionisation at 70 eV. A DB-5ms capillary column (30 m × 0.25 mm ID, stationary phase thickness 0.25 µm, Agilent Technologies, USA) was used for chromatographic separation. Helium (99.999 %, Linde Gas, Czech Republic) was used as the carrier gas. The linear velocity of the carrier gas was set to 35 cm s⁻¹, and 1 µL of the sample was injected using an AOC-20i autosampler (Shimadzu) at an injector temperature of 250 °C (splitless mode, 1 min). The transfer line temperature was maintained at 250°C. The results were evaluated using the software GC Solution v.1.21 (Shimadzu) and the NIST (2011) mass spectra database.

The temperature programme was optimised separately using EZGC v.2.20 gas chromatography modelling software (Analytical Innovations, Inc.) to shorten the analysis time. The software calculated almost 16 million combinations of the parameters and recommended the following conditions: initial temperature 150°C for 1 min, then 14°C min⁻¹ to 250°C for 5 min. The solvent cut time was 2.5 min. Optimised analyses were performed in selected-ion monitoring (SIM) mode (for selected m/z values see Table 5).

Results and discussion

Enzymatic hydrolysis was first tested on an artifi-
cial mixture of PL standards (see Experimental) and the results revealed that the enzyme, whose level is determined purely by its activity, was able to provide a reliable cleavage of phosphoester bonds, which was necessary for polar headgroups analysis. Linearity, repeatability, reproducibility and limits of detection and quantification were estimated for trimethylsilylated fatty acids. Standard solutions of TMS-fatty acids in acetonitrile were diluted 10-, 20-, 50-, 100-, 200-, 500- and 1000-fold. Method linearity was tested over this concentration range and the correlation coefficients of all analytes were approximately 0.99, reflecting sufficient linearity. Repeatability was tested on the set of 7 parallel measurements of a single mixture sample, affording a relative standard deviation of 3-6%. All the limits of detection and quantification of FA derivatives were below 10 μg mL⁻¹ and 30 μg mL⁻¹, respectively (Table 4). However, the limit of detection (LOD) and limit of quantification (LOQ) of the polar headgroups were higher by two orders of magnitude.

After the selection of fatty acids and polar headgroups in accordance with the results from a previously published paper (Seydlová & Svobodová, 2008) on Bacillus subtilis membrane, the retention times of single TMS-fatty acid and TMS-polar headgroup derivative standards were measured. For both TMS-modified fatty acids and polar headgroups, characteristic m/z values were selected to determine their identity in SIM measurements together with the retention times (Table 5).

Next, a hydrolysate of membrane lipids from Bacillus subtilis was trimethylsilylated and measured under optimised conditions (Fig. 2).

The fatty acids used, which are listed in Table 5, were selected according to their abundance (higher than 1%) in the previously analysed samples (Seydlová et al., 2013), and they were also detected in the real sample by the method presented here. The polar headgroups were also reliably detected in the real sample, despite their intensities being comparatively low. A positive feature is that all the TMS-derivatives of the polar headgroups elute before the fatty acid TMS-derivatives of the Bacillus subtilis sample.

The major peaks corresponded to palmitic and 12-methylmyristic (anteiso-) TMS-derivatives. The abundance of palmitic acid was found to be much higher than in the previously published article (Seydlová et al., 2013) for Bacillus subtilis (ATCC 21332). Slight differences in the relative abundance of FA are dependent on the strain and living conditions and they can also vary between individuals of one subspecies. Therefore, within the biological variability of the FA composition of the Bacillus subtilis membrane at various periods of the growth curve, the order of the FA abundances are in full agreement with the previously published results (Seydlová & Svobodová, 2008). The sole exception was palmitic acid, which was found to be more abundant.

Iso- and anteiso-isomers of the methylmyristic and methylpalmitic TMS-derivatives are relatively difficult to distinguish due to the similarity of their MS spectra (Ran-Resler et al., 2012), because there is no significant marker (e.g. ratio between two fragment intensities, etc.). Therefore, the confirmation of peak identity was achieved by comparing both the retention times and the MS spectra of the standards. The relative ratio of iso- and anteiso- fatty acids was found to be 0.6 in the present case, and this value is in full agreement with that previously published (0.5-1.1) (Seydlová et al., 2013), so it is probably characteristic of this Bacillus subtilis strain.

Conclusions

A new, sensitive instrumental method was developed and optimised for the simultaneous analysis of PL polar headgroups and fatty acids in a single measurement. To the best of our knowledge, no such analysis has previously been reported. The optimal derivatisation conditions were evaluated statistically using the central composite design method, and the chromatographic parameters were selected by the software out of almost 16 million options. Emphasis was placed on the resolution of BCFA isomers, which are considered to be the most difficult to resolve using MS, hence it confirmed the suitability of this method for lipid analyses in general whilst also saving time. Enzymatic hydrolysis, which is necessary to avoid the destruction of polar headgroups, ensures the clearing of specific
ester bonds, whereas two-step silylation provides the derivatisation of hydroxyl, carbonyl and amine groups to facilitate their determination in a non-polar GC column. Analysis of the membrane PL of Bacillus subtilis confirmed the reliability of the method, and all the species’ typical and major fatty acids were analysed together with its polar headgroups.

Acknowledgements. This work receive financial support from grant nos. SVV2603517 and 17-18015P from the Czech Science Foundation.

Supplementary data

Supplementary data associated with this article can be found in the online version of this paper (DOI: 10.1515/chempar-2016-0073).

References


Comparison of direct injection mass spectrometry, thin layer chromatography and gas chromatography in analysis of phospholipids.

Podělky práce spoluautorů na článku „Comparison of direct injection mass spectrometry, thin layer chromatography and gas chromatography in analysis of B. subtilis phospholipids“

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  Datum: 17. 5. 2015
  Podpis: [signature]

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- Mgr. Peter Kotora
  Administrativa a organizace spolupráce s pracovištěm s ESI-APCI-MS.
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- Mgr. Viktória Ferenczy
  Příprava analytických metod intaktních fosfolipidů.
  Datum: 9. 10. 2015
  Podpis: [signature]
Direct injection mass spectrometry, thin layer chromatography, and gas chromatography of *Bacillus subtilis* phospholipids

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Received: 3 December 2015 / Accepted: 9 March 2016 © Springer-Verlag Wien 2016

Abstract Direct injection mass spectrometry was amended with chromatographic methods in analysis of membrane phospholipids of *Bacillus subtilis* mutant strain, which contains four polar headgroups only (phosphatidylglycerol, phosphatidylyethanolamine, phosphatidylserine, and phosphaticid acid). Phospholipid classes were determined by thin layer chromatography as the basic method. Analysis of fatty acids by gas chromatography determined relative abundances of saturated, unsaturated, linear, and branched C₁₄ to C₁₉ fatty acids in phospholipids. Experimental data measured by direct injection mass spectrometry in the *m/z* range of 590–790 were used to estimate theoretical phospholipid composition which was compared with the data of chromatographic methods. The phospholipid class relative abundances measured by mass spectrometry did not agree fully with those of thin layer chromatography and the probable explanation was proposed. The direct injection mass spectrometry approach seems to be fast and easy procedure suitable for analyses of proportional changes in phospholipid composition and can be used as a complementary method for trend analyses and in process monitoring.

Graphical abstract

![Graphical abstract](image_url)

Keywords *Bacillus subtilis* · Lipids · Membranes · Direct injection mass spectrometry

Introduction

Phospholipids compose major part of cell membranes, which are responsible for amphiphilic properties of cells and allow life and existence of cells in aqueous environment. The nature of membrane lipids is a key prerequisite for proper membrane structure and function. Phospholipids are a heterogeneous group comprising several hundreds of chemical substances, which differ in two major moieties: fatty acyls (FA) and polar headgroups. Fatty acyls are routinely analyzed in the form of respective esters by gas
chromatography (GC), whereas polar headgroups are analyzed by suitable instrumental methods including classical thin layer chromatography (TLC). On-line or off-line separated phospholipids are often identified and quantified by mass spectrometry utilizing its high specificity and sensitivity. Combination of GC and TLC gives the desired information about the composition of phospholipids but one single method to analyze both fatty acids and polar part in one step is still not used for routine measurements. Despite disadvantages of TLC as a high time demand, low sensitivity, and limited quantitation precision, it is still routinely used for robust classification of phospholipids according to their polar parts. Apart from TLC a number of modern chromatographic and other separation techniques have been developed, however, neither high-performance liquid chromatography (HPLC) or electro-migration method have acquired appropriate popularity to replace TLC. Since phospholipids do not absorb sufficiently at any wavelength of UV or visible spectrum the use of mass spectrometry (MS) seems inevitable for unquestionable identification [1, 2]. Majority of phospholipids differ in molecular weight so they can be analyzed according to their m/z values. Direct injection of phospholipids on mass spectrometer is an approach, which should allow not only phospholipid classification according to respective polar headgroups but, additionally, identification according to the individual composition. Several techniques using direct injection mass spectrometry (DI-MS) without separation have been already published. Fast atom bombardment has been replaced with more suitable ionization techniques [3]. Identification of phospholipid molecules requires weak ionization technique to avoid destruction of molecular ion so the most popular one, which was tested, is electrospray (ESI), which was successfully used for all phospholipid classes in both positive and negative polarities [4]. Some phospholipid classes (p.e. phosphatidylcholine, which is absent in Bacillus subtilis, demands positive mode) have different responses at negative resp. positive modes [5]. Nevertheless, convenience of ESI for phospholipid analysis has been showed in coupling with hydrophilic interaction chromatography (HILIC)-HPLC [6] where several species of phospholipid classes together with other polar lipids had been successfully separated. The other used soft techniques are atmospheric pressure photoionization (APPI), which can be used for several diluents only [7] and atmospheric pressure chemical ionization (APCI) which can be an alternative to ESI, but this ionization technique produces not only deprotonation but several other molecule losses too [8]. The last soft technique is matrix assisted laser desorption ionization (MALDI) [9, 10]. The main disadvantage of MALDI is off-line based setup, which rule out LC/MS analyses in real time [11]. However, MALDI-MS was used several times for identification of phospholipids that had been separated by TLC [12, 13]. Analysis of TLC spot by MALDI-MS is troublesome, because the ionization at various spot locations could yield different results [14], the presence of phosphatidylcholine, which has high detection sensitivity [15], may influence identification of other phospholipids [16] and stationary phase of TLC has to absorb UV radiation to serve as an ionization matrix [17]. Beside usual matrices for proteins and macromolecules, strong basic matrix called proton sponge can be used as a matrix for low molecular weight phospholipids [18]. Analysis of phospholipid composition by direct injection mass spectrometry (without pre-separation) has been used for identification of alimentary products [10, 19], different animal cells [20, 21], and different plant pollen [22]. Likewise, human medicine could use changes in phospholipid composition [23, 24] in diagnostic procedures for bacterial identification [25]. Together with cell membrane proteins, phospholipid composition [26] could probably lead to microbe identification without classical microbial or biochemical techniques [27]. Last but not least, in case of bacterial pathogens, the cell membrane is the target of several newly launched antibiotics and antimicrobial peptides and the lipid composition of the membranes is crucial for the antibiotic-membrane interaction, its efficiency and selectivity for bacteria [28]. Therefore, determination of membrane lipid composition should help antibiotic research in targeting the microbes with a suitable drug [29].

Goal of this paper was to simplify analysis of FA and phospholipid classes with elemental laboratory equipment (single quad MS), to provide the suitability on isolated lipid fractions of Bacillus subtilis SDB206 by direct injection mass spectrometry method and to correlate its results with those of TLC and GC–MS. B. subtilis is an ideal experimental object as it is thoroughly studied model of gram-positive bacterium, its membrane is normally composed of five [30] phospholipid classes and contains branched-chain fatty acids with odd number carbon atoms. Therefore, its phospholipid composition avoids the overlap of phospholipids with the same molecular weight.

Results and discussion

Fatty acids determination by GC–MS

Fatty acids, which are present in Bacillus subtilis membrane lipids are mostly branched-chain fatty acids (BCFA) with odd number of carbons. Generally, their acyls are not typical in phospholipids and this fact simplifies identification of phospholipid molecular fragments during direct injection mass spectrometry (DI–MS; see chapter Direct injection mass spectrometry). Abundance of respective FA
methyl ester was calculated as a ratio between its peak area and the sum of peak areas of all identified FA methyl esters. Relative abundances determined by GC–MS are presented in Table 1. It can be presumed that majority of phospholipid molecules contain mostly C₁₅ (59.4 %) and C₁₇ (24.1 %) fatty acid isomers. The ratio of the branched to straight FA was found 87.7 %/12.3 % and the fraction of the unsaturated FA represents 3.1 %, which facts correspond with the published data [31]. It can be derived from the data that the fractions of the odd and even carbon number FA correspond to 83.5 and 16.5 %, respectively.

**Phospholipids class ratio**

Phospholipid class analysis by TLC (see Table 2) showed that the majority (almost 90 %) of membrane phospholipids are composed of only two phospholipid classes, namely phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). High content of PG can be attributed to the absence of cardiolipin, which is reduced by cardiolipin synthesis from two PG molecules in other *Bacillus subtilis* strains. A small portion of phosphatidylinerine (PS) was detected too. The last phospholipid class, which should be present in membrane of the mutant strain, phosphatidic acid (PA), could not be detected reliably because of the high detection limit (10 μg for a spot) of TLC method. Fifth class—cardiolipin which should not be present at all because of genetic mutation [32] was not detected.

**Direct injection mass spectrometry of membrane lipids**

According to mutant strain technical specification *Bacillus subtilis* SDB206 should contain only PA, PE, PG, and PS. FA analysis showed that only C₁₄ to C₁₈ isomers including C₁₆,₄ and C₁₈,₁ FA are present. Theoretically, if all possible FA combinations (couples) occurred in all phospholipid classes, there would be hundreds of phospholipid individuals. Accordingly, the lightest phospholipid would have monoisotopic mass of 591 whereas the heaviest would have 790. In this range of m/z = 200 hundreds distinguishable masses should be detected. According to the monoisotopic masses of *Bacillus subtilis* phospholipid headgroups (lack of phosphatidylcholine), there would not be two different phospholipids of the same monoisotopic mass. Differences in monoisotopic masses of the headgroups are sufficiently large to distinguish between the corresponding phospholipid classes independently on their FA composition. Mass spectrum of direct injection of membrane lipids showed three major deprotonated molecules (721, 707, and 693), and almost 20 other fragments which overpassed the limit of 1 % of relative abundance (Fig. 1).

Although all phospholipid molecules probably do not yield identical detector response, the summed abundances could serve as an important raw information. Sum of abundances of respective phospholipid classes are as follows: PG 65.1 %, PE 12.2 %, PS 6.8 %, and PA 15.9 %. The amount of nitrogen containing phospholipids (PE and PS) is lower in results of DI-MS than in those from TLC analysis whereas the content of anionic phospholipids (PA and PG) is higher. This difference in abundance between major phospholipids is probably caused by the higher ionization efficiency of anionic phospholipids in negative polarity ionization mode of MS. The mixtures of different ratio of phospholipid standards have been measured and the results confirmed that phospholipids of one class could not give false positive signal of other class.

All of three major peaks in Fig. 1 correspond to phosphatidylglycerol class, which should be the most abundant one, with one C₁₂ fatty acid and either C₁₇ (m/z = 721), or C₁₅ (m/z = 693) and C₁₆ (m/z = 707). FA composition of

| FA fractions: C₁₄ 13.8 %, C₁₅ 59.41 %, C₁₆ 9.42 %, C₁₇ 24.09 %, C₁₈ 5.70 % | Springer | 65 |

---

**Table 1 Abundance of fatty acids in R. subtilis phospholipids determined by GC–MS**

<table>
<thead>
<tr>
<th>Methyl ester</th>
<th>Fatty acid group</th>
<th>Abundance/%</th>
<th>Monoisotopic mass</th>
<th>Molecular formula</th>
</tr>
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<tbody>
<tr>
<td>Icosanoylsteatate</td>
<td>C₁₄</td>
<td>0.87</td>
<td>242.4</td>
<td>C₁₄H₂₃O₂</td>
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<tr>
<td>Myristate</td>
<td>C₁₄</td>
<td>0.51</td>
<td>242.4</td>
<td>C₁₄H₂₃O₂</td>
</tr>
<tr>
<td>13-Methylmyristate</td>
<td>C₁₅</td>
<td>16.34</td>
<td>256.4</td>
<td>C₁₅H₂₅O₂</td>
</tr>
<tr>
<td>12-Methylmyristate</td>
<td>C₁₅</td>
<td>43.07</td>
<td>256.4</td>
<td>C₁₅H₂₅O₂</td>
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<tr>
<td>Palmitoleate</td>
<td>C₁₆</td>
<td>3.31</td>
<td>270.5</td>
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<tr>
<td>Palmitate</td>
<td>C₁₆</td>
<td>0.72</td>
<td>268.4</td>
<td>C₁₆H₃₇O₂</td>
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<tr>
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<td>C₁₆</td>
<td>5.39</td>
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<td>C₁₆H₃₇O₂</td>
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<td>15-Methylpalmitate</td>
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<td>284.5</td>
<td>C₁₇H₃₉O₂</td>
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<tr>
<td>14-Methylpalmitate</td>
<td>C₁₇</td>
<td>15.23</td>
<td>284.5</td>
<td>C₁₇H₃₉O₂</td>
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<td>Oleate</td>
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<td>2.36</td>
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<td>C₁₈H₃₇O₂</td>
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<tr>
<td>Stearate</td>
<td>C₁₈</td>
<td>3.34</td>
<td>298.5</td>
<td>C₁₈H₃₇O₂</td>
</tr>
</tbody>
</table>
Owing the unknown exact weight of the sample (approximately 1 mg), the detection limit of the DI-MS was estimated to be approximately 45 ng of PL in 5 mm³ of injected sample volume.

**Conclusion**

Direct injection mass spectrometry of membrane phospholipids has identified all present phospholipid classes, whereas TLC could not identify PA owing to its low sensitivity. Phospholipids classes’ ratio from DI-MS results is different from TLC results. One of the possible reasons could be different ionization efficiency of MS towards phospholipid classes. Mass spectrometry can distinguish reliably between phospholipids containing saturated and unsaturated FA and determine the sum of carbons in both phospholipid acyls, however, isomeric FA cannot be distinguished properly by DI-MS only. Combination of direct injection mass spectrometry and chromatographic analyses of phospholipids gives sufficient information to determine both fatty acids and polar headgroup ratio.

The fast and easy DI-MS approach could be recommended for routine analyses of samples where relative composition of FA and phospholipids is monitored. FA and phospholipids analyses should be done only once and then DI-MS is sufficient so the issue can be simplified and shortened. DI-MS method eliminates partially the application of commercially unavailable phospholipid standards also.

**Experimental**

Phosphatidic acid (98 %), phosphoethanolamine (97 %), phosphoserine (70 %), phosphoglycerol (99 %), acetonitrile (HPLC grade), hexane (97 %, HPLC grade), pentane (98 %), and chloroform (99 %, anhydrous) were supplied by Sigma Aldrich. He (99,999 %) which was used as the carrier gas was purchased from Linde Gas (Czech Republic). Propan-2-ol (LC–MS grade), heptane (99 %), and methanol (LC–MS grade) were delivered by Fluka. NaCl (for analysis) was delivered by Lach-Ner (Czech Republic). Lab-lexo powder, bacto-yeast extract and hactopepton for broth preparation were supplied by Oxoid (Czech Republic).

**Real sample: Bacillus subtilis cell membrane phospholipids**

*Bacillus subtilis* SDB206 used in this study was a mutant strain with disruption in cardiolipin synthase genes, and was a kind gift of Kouji Matsumoto [32]; it is cultivated as
Table 3: Abundance of monoisotopic ions corresponding to phospholipids of Bacillus subtilis determined by DI-MS

<table>
<thead>
<tr>
<th>m/z</th>
<th>Proposed composition</th>
<th>PA</th>
<th>PG</th>
<th>PS</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>591</td>
<td>C14 x C14</td>
<td></td>
<td></td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>647</td>
<td>C15 x C17</td>
<td></td>
<td></td>
<td></td>
<td>2.6</td>
</tr>
<tr>
<td>671</td>
<td>C16:1 x C18:1</td>
<td></td>
<td></td>
<td></td>
<td>3.2</td>
</tr>
<tr>
<td>673</td>
<td>C16 x C18:1</td>
<td></td>
<td></td>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td>690</td>
<td>C15 x C15</td>
<td></td>
<td>C16 x C16:1</td>
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<td>2.6</td>
</tr>
<tr>
<td>699</td>
<td>C18:1 x C18:1</td>
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</tr>
<tr>
<td>701</td>
<td>C18 x C18:1</td>
<td></td>
<td></td>
<td></td>
<td>2.6</td>
</tr>
<tr>
<td>707</td>
<td>C15 x C16</td>
<td></td>
<td></td>
<td></td>
<td>9.3</td>
</tr>
<tr>
<td>718</td>
<td>C16 x C16:1</td>
<td></td>
<td>C16 x C18:1</td>
<td></td>
<td>1.3</td>
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<td>719</td>
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<td>720</td>
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<td>6.6</td>
</tr>
<tr>
<td>721</td>
<td>C15 x C16:1</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>722</td>
<td>C15 x C17</td>
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<tr>
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<td>C15 x C16:1</td>
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<tr>
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<td>C18 x C18:1</td>
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</table>

Table 4: Abundance of FA combinations in phospholipid classes of B. subtilis determined by DI-MS

<table>
<thead>
<tr>
<th>FA combination</th>
<th>Abundance/%</th>
<th>Phospholipid class</th>
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<tr>
<td></td>
<td>PA</td>
<td>PG</td>
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<tr>
<td>C15 x C16:1</td>
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<td>C15 x C17</td>
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<td>2.6</td>
</tr>
<tr>
<td>C15 x C18</td>
<td>1.8</td>
<td>-</td>
</tr>
<tr>
<td>C15 x C18:1</td>
<td>6.1</td>
<td>-</td>
</tr>
<tr>
<td>C16 x C16:1</td>
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<td>C16 x C18:1</td>
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<td>C17 x C17</td>
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<td>C18 x C18:1</td>
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<tr>
<td>C18C1 x C18:1</td>
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</table>

- Absent

a surfactin producer so its monitoring is demanded for production optimization [33]. Lipids were extracted with hexane-propan-2-ol mixture (3:2, v/v) from bacterial cell biomass (400 cm³ of culture in nutrient broth) harvested in the exponential phase of growth by rapid filtration (Sypor no. 5 filter, Pragogehma, Czech Republic). After vacuum evaporation of the solvent at 40 °C, phospholipids were dissolved in chloroform, filtered through GF/C glass fiber filters (Whatman) and concentrated under a stream of nitrogen. The lipid samples were stored at −80 °C. Dry
sample (about 1 mg) was dissolved in 1 cm³ of methanol/chloroform (1:1 v/v) and directly injected to mass spectrometer or analyzed by thin layer chromatography.

Gas chromatography of fatty acids

Membrane lipids were transesterified to FA methyl esters by incubation in sodium methoxide at room temperature according to previously published procedure [31]. After neutralization by the addition of methanolic HCl (2 mol dm⁻³), the fatty acid methyl esters (FAMEs) were extracted three times with 200 mm³ of pentane and dried under the nitrogen flow. Methyl esters were dissolved in 100 mm³ of heptane. Gas chromatograph with mass spectrometer detector GCMS-QP 5050A (Shimadzu, Japan) was used for fatty acid analysis. Separation capillary column DB-5MS (30 m x 0.25 mm ID, stationary phase thickness 0.25 μm) was purchased from Agilent Technologies (USA). Linear velocity of carrier gas was set at 39.2 cm s⁻¹. Automatic sampler AOC-20i (Shimadzu, Japan) was used for sample injection (1 mm³) in split mode (1:20). Injection temperature was held at 270 °C. The measurements were carried out with the following temperature program: initial temperature 60 °C, 2 min, then 7.5 °C min⁻¹ to 250 °C, 5 min. The transfer line was maintained at 255 °C. Detector was operated in scan mode and solvent cut time was 5 min. The chromatograms were evaluated with GC Solution v. 1.21 software (Shimadzu) and mass spectra were compared with NIST 2011 database.

Thin layer chromatography

Phospholipids were separated by thin layer chromatography (TLC, silica gel 60 G plates; Merck) in chloroform-methanol-water (65:25:4, v/v/v) as the mobile phase. The spots were detected with iodine vapor and to localize the amino groups, the plates were treated with a 0.2 % ninhydrin solution (butanol-acetic acid 95:5, v/v). The spots were collected from the plates and then phospholipids quantified spectrophotometrically, as previously published [34]. The average results from three independent experiments including standard deviations are presented.

Mass spectrometer (direct injection)

Direct injection was performed at single quadrupole LC/MS 6110 (Agilent technologies) Agilent G1978B multimode (ESI+APCI) ion source (simultaneous ESI and APCI, for details see [35]). Multimode ion source was utilized to achieve a balanced response for analyzed compounds in a single analysis. All analyses were carried out with negative polarity, drying gas temperature was 300 °C, and drying gas flow was 5 dm³ min⁻¹. Degasser temperature was set at 200 °C, voltage on capillary was 2000 V, and corona current 2 μA. The direct injection of 5 mm³ was done by autosampler of the LC/MS system (no separation column installed).

Acknowledgments The work was performed at the Institute of Chemistry of the Faculty of Natural Science of Comenius University with support from SVV203317 and Mobility Fund of Charles University in Prague, further support was provided by project I3-18051P from the Czech Science Foundation and Grants APVV-0660-11, APVV-0446-10, APVV-0661-11 and APVV-0286-12 from Slovak Research and Development Agency.

References

Direct injection mass spectrometry, thin layer chromatography, and gas chromatography of...


References: