Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 199–206
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Analysis of the phospholipid composition of bronchoalveolar lavage (BAL) fluid from man and minipig by MALDI-TOF mass spectrometry in combination with TLC

Daniela Sommerer [a], Rosmarie Süß [a], Stefan Hammerschmidt [b], Hubertus Wirtz [b], Klaus Arnold [a], Jürgen Schiller [a,*]

[a] *Institute of Medical Physics and Biophysics, Medical Department, University of Leipzig, Liebigstrasse 27, D-04103 Leipzig, Germany*
[b] *Medical Clinics I, Johannisallee 32, D-04103 Leipzig, Germany*

Received 4 November 2003; received in revised form 20 December 2003; accepted 20 December 2003

Abstract

Surfaces of lungs are covered by the surfactant, an aqueous mixture of different phospholipids (PL) and proteins. Although the surfactant represents a relatively simple mixture of only a few PL (primarily phosphatidylcholine (PC) and phosphatidylglycerol (PG)), reliable methods of routine lipid analysis of the surfactant are still lacking. It will be shown that matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry (MALDI-TOF MS) represents a suitable technique for the differentiation of the apolar components of the surfactant of different species.

Samples of man and minipig are used in this study since both are known to vary in their PL composition. PL of surfactant were separated by thin-layer chromatography (TLC) and the obtained subfractions subjected to MALDI-TOF MS analysis in order to monitor the presence of even minor PL species. It will be shown that besides PG and PC, also phosphatidylethanolamine, -inositol and sphingomyelin can be detected in surfactant of man, whereas only sphingomyelin could be detected in the minipig sample.

Keywords: Bronchoalveolar lavage (BAL); Surfactant; Phosphatidylglycerol; Phosphatidylcholine; Phospholipid composition; MALDI-TOF mass spectrometry; TLC

1. Introduction

Lung surfactant, a mixture of different phospholipids (PL), cholesterol and proteins [1], is present at the air/liquid interface in the pulmonary alveoli of the lung. The most important task of the surfactant is to decrease the surface tension and, therefore, to reduce the tendency of alveoli to collapse during expiration [2].

Abbreviations: LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; SM, sphingomyelin; TAG, triacylglycerol

* Corresponding author. Tel.: +49-341-9715733; fax: +49-341-9715709.
E-mail address: schij@medizin.uni-leipzig.de (J. Schiller).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2003.12.016

Extracellular surfactant can be harvested from the lungs by bronchoalveolar lavage (BAL) [1]. It is well known that lipids (cholesterol, phosphatidylglycerol (PG) and phosphatidylcholine (PC)) constitute the majority (about 90%) of the non-volatile compounds of pulmonary surfactant [3]. PC make up the largest moiety (70–80%) of the PL of the surfactant and diversity of the fatty acid composition of PL is low: about 50–60% of the PC molecules contain two saturated palmitic acid (16:0) chains [1].

The second abundant PL species in adult surfactant is phosphatidylglycerol that constitutes about 10% of the overall surfactant PL. All further PL contribute only to a very low extent [1]. The lipid composition of BAL was investigated in the past primarily by thin-layer chromatography (TLC) or HPLC. Unfortunately, these methods have a number of limitations [4]. For instance, it is difficult to determine the fatty acid composition of the individual PL classes. Methods allowing PL analysis in a single step were so far only scarcely used. Although ^{31}P NMR spectroscopy has the considerable advantage that all individual PL can be differentiated in a single experiment, ^{31}P NMR was so far only two times applied to BAL analysis [5,6]. Using ^{31}P NMR, it turned out that plasmalogens, i.e. PL with alkenyl-ether-linked fatty acid residues represent an important constituent of bovine BAL [5] and that the composition of BAL from different species (e.g. man, rat, rabbit and minipig) can be easily differentiated [6]. However, it is a serious disadvantage that at least 25–50 μg of each PL are required to record ^{31}P NMR spectra in an acceptable time [6].

Because of its much higher sensitivity, mass spectrometry (MS) was more often applied [4–6]. Since the surfactant is composed of many different PL, the yield of fragmentation products should be as low as possible. There are currently two “soft-ionisation” techniques, namely electrospray (ESI) and matrix-assisted laser desorption and ionisation (MALDI) that fulfil this requirement [4]. So far, ESI MS was more often used for the evaluation of the PL composition of BAL [7,8].

However, MALDI-TOF MS has considerable advantages in comparison to ESI [6]. For instance, PL analysis can be faster performed and a higher content of impurities is tolerated by MALDI while the sensitivity of both techniques is comparable [9].

In this paper the organic extracts of BAL of man and minipig are compared. These species were chosen because of their known considerable differences in PL composition [6]. It will be shown that subsequent to TLC separation, even very minor PL species are detectable by MALDI-TOF MS in the surfactant of man. In contrast, the minipig sample was exclusively characterised by the presence of PC, PG, and SM.

2. Experimental procedures

2.1. Chemicals

Chemicals for BAL preparation, solvents (chloroform and methanol), 2,5-dihydroxybenzoic acid (DHB) and trifluoroacetic acid (TFA) were obtained in highest commercially available purity from Fluka Feinchemikalien GmbH (Taufkirchen, Germany).

2.2. Bronchoalveolar lavage (BAL) in experimental studies

Details of BAL were already previously described [6]. Briefly, 5 ml lavage was centrifuged and the supernatant was used for extraction. Lipid extraction was performed according to the Bligh and Dyer procedure using a ratio of 1:1:0.9 (v/v/v) between chloroform/methanol and the lavage [10].

2.3. MALDI-TOF mass spectrometry

For the majority of samples a 0.5 mol/l 2,5-dihydroxybenzoic acid (DHB) solution in methanol containing 0.1% trifluoroacetic acid was used. In selected cases, in order to identify the potential presence of plasmalogens, TFA was omitted. Under these conditions, measurements were also possible but at the cost of worse signal-to-noise ratio. Tenfold concentrated, organic PL extracts were mixed 1:1 (v/v) with the matrix solution and deposited onto the sample plate.

All MALDI-TOF mass spectra were acquired on a Voyager Biospectrometry DE workstation (PerSeptive Biosystems, Framingham, MA, USA) and details of these measurements are given elsewhere [6,9].

2.4. Separation of phospholipids by thin-layer chromatography

Tenfold concentrated BAL extracts were subjected to TLC prior to MALDI-TOF MS. 5 μl PL samples per spot were applied on HPTLC silica gel 60 plates (10 cm × 10 cm in size) (Merck, Darmstadt, Germany) and developed in TLC chambers (CAMAG, Switzerland) using chloroform, ethanol, water and triethylamine (30:35:7:35, v/v/v/v) as elution solvent.

PL were visualised by spraying with a solution of PRIMULINE (Direct Yellow 59) according to [11]. Upon irradiation by UV light (254 nm), individual PL become detectable as violet spots. These spots were scratched off and the individual PL classes eluted by intense vortexing with a mixture of 75 μl $CHCl_3$, 75 μl methanol and 75 μl 0.9% aqueous NaCl. Afterwards, samples were centrifuged (2500 rpm) to allow phase separation. The organic layer was evaporated to dryness and the residual material redissolved in 20 μl matrix solution.

The following R_f values were used for spot identification: cholesterol (0.84), phosphatidylglycerol (0.64), phosphatidylethanolamine (0.47), phosphatidylinositol (0.40), phosphatidylcholine (0.15), and sphingomyelin (0.08).

3. Results and discussion

In Fig. 1 typical positive (trace (a)) and negative (trace (b)) ion MALDI-TOF mass spectra of an organic extract of human BAL are shown. Since an organic matrix (DHB) is used, there are in both spectra

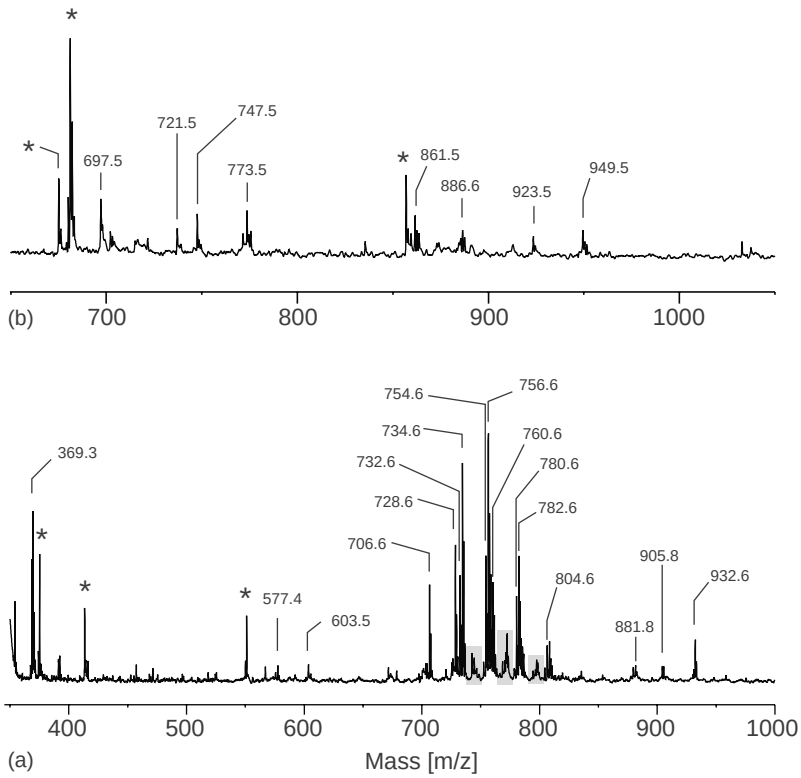

Fig. 1. Positive (a) and negative ion (b) MALDI-TOF mass spectra of an organic extract (obtained according to Bligh and Dyer) of human surfactant. Prior to measurements, the sample was tenfold concentrated. Peaks are marked according to their *m*/*z* ratios and peaks caused by the applied DHB matrix are labelled with asterisks (for details see text). Peaks labelled with grey bars in trace (a) correspond to PG species that can be detected as positive as well as negative ions.

also intense peaks caused by matrix oligomerisation (marked with asterisks) [12]. In agreement with the known PL composition of human BAL [3], the positive ion spectrum (trace (a)) indicates primarily the presence of PC 16:0/16:0 ($m/z = 734.6$ for the proton and $m/z = 756.6$ for the sodium adduct). A second, completely saturated but less abundant compound is PC 16:0/14:0 ($m/z = 706.6$ and 728.6).

It is evident that PCs with an unsaturated fatty acid residue are also detectable, e.g. PC 16:0/16:1 ($m/z = 732.6$ and 754.6), PC 16:0/18:2 ($m/z = 758.6$ and 780.6), PC 16:0/18:1 ($m/z = 760.6$ and 782.6), and to a smaller extent PC 16:0/20:4 ($m/z = 782.6$ and 804.6).

In cases where peak assignment was ambiguous, spectra were recorded in the presence of CsCl. This method is described in more detail in [13].

The assignment of all peaks detectable in the positive as well as the negative ion mass spectra of organic extracts of BAL is given in Table 1. The reader should note that the peaks at $m/z = 881.8$ and 905.8 are most likely caused by triacylglycerols (TAG 52:2 and TAG 54:4, respectively) and the small peaks at $m/z = 577.4$ and 603.5 correspond to TAG subsequent to the elimination of one fatty acid residue [14]. The assignment of the peak at $m/z = 932.6$ is not yet completely clear but is most probably caused by a matrix adduct of PC (16:0/16:0). Additionally, there are also smaller peaks corresponding to PG. These peaks are marked with grey bars and their assignment will be discussed later.

In the lower mass region (left end of trace (1a)) the most intense peak is caused by cholesterol ($m/z = 369.3$ as a consequence of water elimination from the proton adduct) [15]. Besides cholesterol, there are—at least in the absence of TFA—no major peaks in this mass region besides matrix peaks at $m/z = 375$, 413 and 551 [12]. The presence of TFA leads to the generation of very small peaks of lysophosphatidylcholines (data not shown). This might indicate the presence of (highly acid-labile) plasmalogens [5].

In the negative ion spectrum (trace (1b)), only small peaks of PC (as cluster ions with negatively-charged matrix molecules) are detectable [16], whereas negatively-charged PL (e.g. PG and PI) are detectable with higher intensities. For instance, the peak at $m/z = 886.6$ corresponds to a DHB adduct of PC

Table 1
Assignment of the *m/z* ratios of the most intense peaks of matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectra of organic extracts of human surfactant

Peak position	Assignment of molecular mass
Positive ion MALDI-TOF mass spectra	
369.3	Cholesterol + H^+ − H_2O
577.4	Fragmentation product of TAG
603.5	Fragmentation product of TAG
703.6	SM 16:0 + H^+
706.6	PC 14:0/16:0 + H^+
725.6	SM 16:0 + Na^+
728.6	PC 14:0/16:0 + Na^+
732.6	PC 16:0/16:1 + H^+
734.6	PC 16:0/16:0 + H^+
740.6	PE 16:0/20:4 + H^+
745.5	PG 16:0/16:0 + H^+
754.6	PC 16:0/16:1 + Na^+
756.6	PC 16:0/16:0 + Na^+
758.6	PC 16:0/18:2 + H^+
760.6	PC 16:0/18:1 + H^+
762.6	PE 16:0/20:4 + Na^+
767.5	PG 16:0/16:0 + Na^+
771.5	PG 16:0/18:1 + H^+
780.6	PC 16:0/18:2 + Na^+
782.6	PC 16:0/18:1 + Na^+ or PC 16:0/20:4 + H^+
793.5	PG 16:0/18:1 + Na^+
797.5	PG 18:0/18:2 + H^+ (or PG 18:1/18:1 + H^+)
804.6	PC 16:0/20:4 + Na^+
819.5	PG 18:0/18:2 + Na^+ (or PG 18:1/18:1 + Na^+)
881.8	TAG 52:2 + Na^+
885.6	PI 18:0/18:2 + H^+
887.6	PI 18:0/18:1 + H^+
905.8	TAG 54:4 + Na^+
907.6	PI 18:0/18:2 + Na^+
909.6	PI 18:0/18:1 + Na^+ (or PI 18:0/20:4 + Na^+)
932.6	PC 16:0/16:0 + DHB-Na + Na^+
Negative ion MALDI-TOF mass spectra	
721.5	PG 16:0, 16:0 − Na^+
745.5	PG 16:0, 18:2 − Na^+
747.5	PG 16:0, 18:1 − Na^+
773.5	PG 18:0, 18:2 − Na^+ (PG 18:1, 18:1 − Na^+)
835.5	PI 16:0, 18:1 − Na^+
861.5	PI 18:0, 18:2 − Na^+
863.6	PI 18:0, 18:1 − Na^+
885.5	PI 18:0, 20:4 − Na^+
886.6	PC 16:0/16:0 + DHB-H^+
897.5	PG 16:0, 16:0 − Na^+ + DHB-Na
923.5	PG 16:0, 18:1 − Na^+ + DHB-Na
949.5	PG 18:0, 18:2 − Na^+ + DHB-Na (or PG 18:1, 18:1 − Na^+ + DHB-Na)

Both, positive and negative ion spectra are listed. The calculation of the molecular weight of the neutral form of acidic PL like PG and PI is based on the assumption that charge compensation is warranted by one sodium ion.

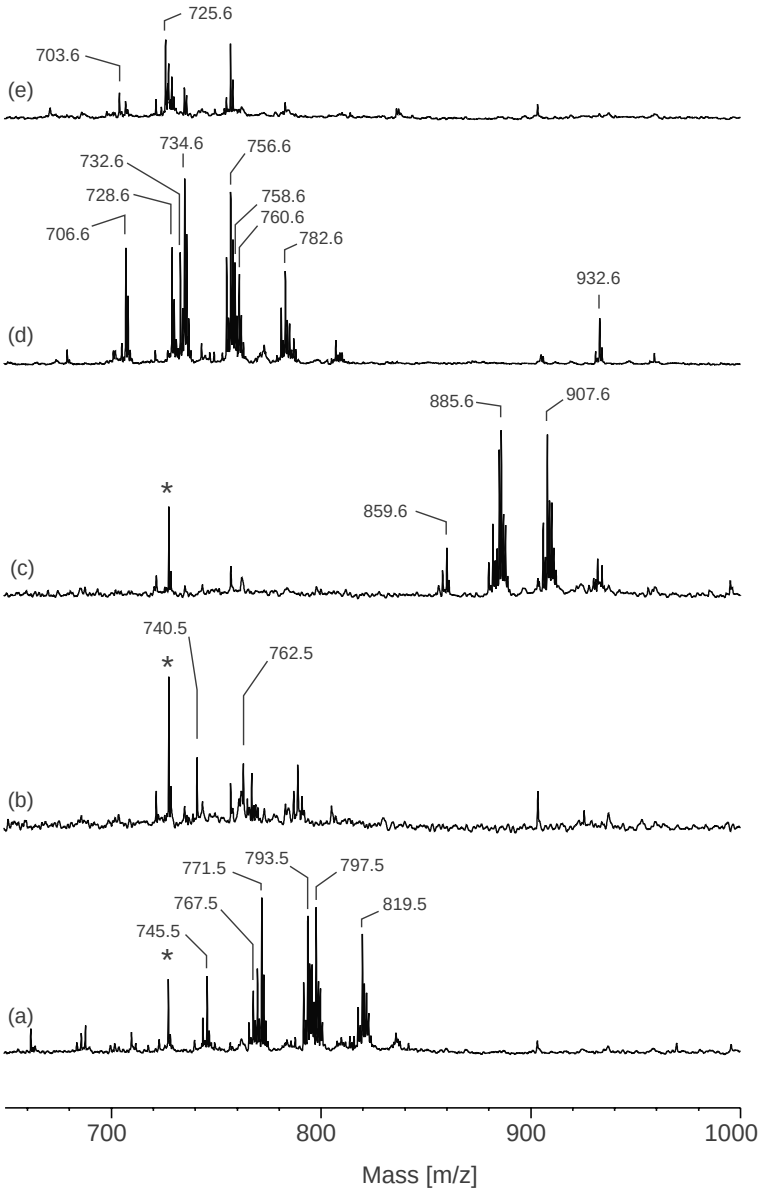

Fig. 2. Positive ion MALDI-TOF mass spectra of previously TLC-separated PL subfractions of human surfactant. The same sample as in Fig. 1 was subjected to TLC and the obtained lipid fractions afterwards analysed by MALDI-TOF MS. Individual spectra correspond to PG (a); PE (b); PI (c); PC (d); and SM (e). Individual PL classes were assigned by comparison with the known reference samples. Peaks are marked according to their *m*/*z* ratios and peaks caused by the matrix are labelled with an asterisk (for details see the text).

16:0/16:0. Since this is the most abundant PC, it is also detectable as negative ion.

Although it is an advantage that DHB can be used as matrix for the positive as well as the negative ion mass spectra [4], one disadvantage is that DHB yields intense negative ion signals [12]. The corresponding peaks at $m/z = 675$, 681 and 857 are marked with asterisks in trace (1b). May be, the significant contribution of the matrix signals is the main reason for the reduced sensitivity of the negative detection mode [17].

Mainly PGs are detectable in the negative ion spectrum and this is in good agreement with the known PL composition of BAL [1]. Peaks at $m/z = 721.5$, 747.5 and 773.5 correspond to PG 16:0/16:0, 16:0/18:1 and 18:0/18:2, respectively. At least two PG (16:0/18:1 and 18:0/18:2) are also detectable as cluster ions with the DHB matrix (at $m/z = 923.5$ and 949.5).

In order to investigate the PL composition of human BAL in more detail and because of the known detection differences of individual PL classes [12], PL were additionally separated by TLC and the obtained subfractions subjected to MALDI-TOF MS analysis. It was shown previously that this is especially necessary for the detection of PE [18] since PE is otherwise completely obscured by the simultaneous presence of PC.

In Fig. 2 the positive ion MALDI-TOF mass spectra of individual subfractions of human BAL obtained by TLC separation are shown. Trace (a) corresponds to the PG fraction; (b) to PE; (c) to PI; (d) to PC; and (e) to SM. The cholesterol fraction was not further analysed.

The *m/z* values of all identified compounds are listed in Table 1. Since PC and PG were already discussed for Fig. 1 their spectra shall not be discussed here but one additional comment is necessary: from the *m/z* values alone, it is not possible to assign unambiguously the fatty acid composition of a certain PL. For instance, the peak at $m/z = 797.5$ was assigned to the proton adduct of PG 18:0/18:2. Obviously, this peak might also correspond to the proton adduct of PG 18:1/18:1. However, this assignment problem may be overcome by enzymatic digestion with phospholipase A_2. This enzyme cleaves selectively the fatty acid residue in *sn*-2 position and the resulting lysophospholipids allow clear conclusions on the original fatty acid composition [15].

Using the individual subfractions, the assignment of PI (trace (2c)) is much more simple than in the PL mixture: the most intense peaks at $m/z = 885.6$ and 907.6 are caused by the proton and the sodium adduct of PI 18:0/18:2, respectively, while the signal at $m/z = 859.6$ is caused by PI 16:0/18:1. A more detailed assignment of the PI fraction is provided in Table 1.

The least abundant fraction, phosphatidylethanolamine (PE, trace (2b)), is primarily composed of PE 16:0/20:4 ($m/z = 740.6$ and 762.6) and contained also significant amounts of LPE 16:0 ($m/z = 454.3$ for the proton adduct, data not shown). The mechanism leading to LPE generation is so far unknown.

Finally, the sphingomyelin (SM) fraction (trace (2e)) of human BAL consists primarily of SM 16:0 ($m/z = 703.6$ and 725.6). Most probably, the SM contribution must be regarded as an artefact since no SM should be present in pure lavage [1]. Therefore, SM is most likely stemming from a cellular contribution that must be regarded as an impurity [6].

Although the majority of PL classes discussed so far does not give intense negative ion signals, this does not hold for PG and PI, and the corresponding negative ion spectra are shown in Fig. 3. Trace (3a) corresponds to PG and trace (3b) to PI. The same peaks as in the positive ion spectrum of PG are found, but all peaks are shifted by 24 Da to lower masses in comparison to the positive ions. This mass difference corresponds to the loss of one proton (−1) and one sodium ion (−23) according to two charges (from the charge state (−1) to (+1)) [17]. The same PGs are also detectable as cluster ions with the matrix at $m/z = 897.5$ (PG 16:0/16:0), 923.6 (PG 16:0/18:1) and 949.5 (PG 18:0/18:2). The same explanation holds for the peak pattern of the PI (trace (3b)) that is explained in more detail in Table 1.

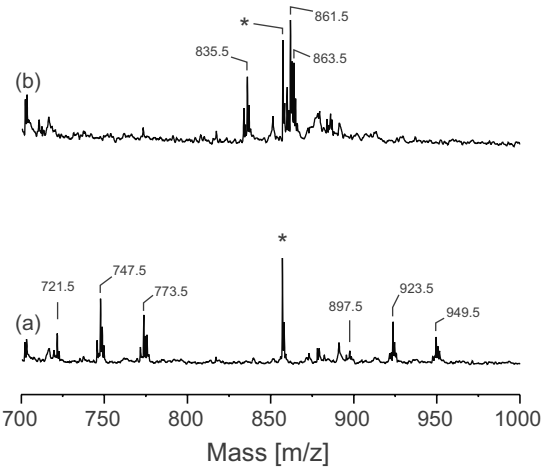

Fig. 3. Negative ion MALDI-TOF mass spectra of previously TLC-separated PL subfractions of human surfactant. The same sample as in Fig. 1 was used, but only PL that yield intense negative ion signals are shown, i.e. trace (a) corresponds to PG; while trace (b) represents PI. Peaks are marked according to their *m/z* ratios and peaks caused by the matrix are labelled with an asterisk.

From these results it is obvious that a detailed qualitative analysis of all PL species in the surfactant of man can be easily performed by MALDI-TOF MS. In order to have a second BAL sample from another species, the lavage from minipig was also investigated. Minipig was chosen since the BAL fluid of this animal is known to differ from that of humans [6]. MALDI-TOF mass spectra of most relevant subfractions (obtained by TLC) of an organic extract of minipig BAL are shown in Fig. 4. Trace (4a) corresponds to the negative ion spectrum of the PG fraction while all other spectra are positive ones. Trace (4b) corresponds to PG; (4c) to PC; and (4d) to SM. In contrast to the human BAL there were only very small amounts of PE and PI in the minipig sample and, accordingly, these spectra are not shown. Although this is the most obvious difference between man and minipig, there are also smaller differences in the fatty acid compositions of the individual subfractions. For instance, the contribution of PC (14:0/16:0) is much lower in the minipig in comparison to the human sample.

MALDI-TOF mass spectra were so far only qualitatively analysed although there are growing indications that quantitative analysis is possible [19]. It was already shown that MALDI-TOF mass spectra of lipids can be quantified in three different ways: (a) by the use of a known internal standard [20]; (b) by using the signal-to-noise ratio [17]; or (c) by comparing the

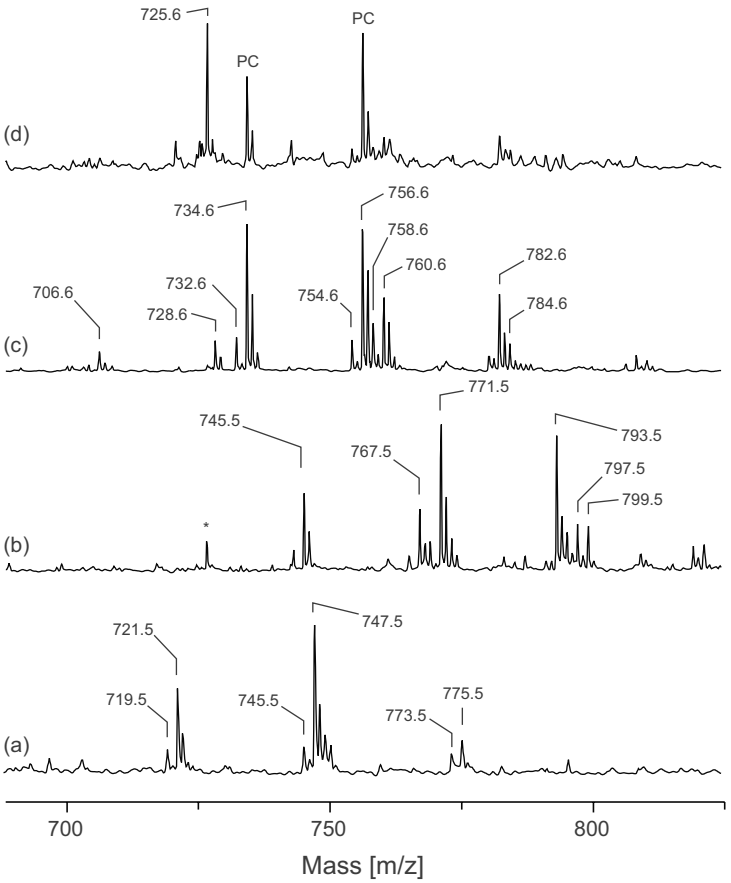

Fig. 4. MALDI-TOF mass spectra of previously TLC-separated PL subfractions of BAL of minipig. Spectrum (a) corresponds to the negative ion spectrum of PG while all further spectra were recorded in the positive ion detection mode. Individual spectra correspond to PG (b); PC (c); and SM (d). Individual PL classes were assigned by comparison with the known reference samples. Peaks are marked according to their *m*/*z* ratios and peaks caused by the matrix are labelled with an asterisk. Please note that in contrast to the human sample, the sample of the minipig did not contain major amounts of neither PE nor PI.

intensity of the lipid peak with a known matrix peak. Method (c) was successfully applied to triacylglycerol mixtures [14] and has the special advantage that the sample is not altered at all.

According to our so far obtained results, the quantitative analysis of both main components of BAL, PG and PC is most accurately performed by comparing the peak intensities of both compounds. Since peak intensities are strongly influenced by the ion contents, it is advisable to combine the intensities of the proton and sodium adducts for each PL class. However, there are considerable deviations between individual measurements with respect to BAL, although artificial PG and PC mixtures can be easily analysed. Therefore, we suggest to use MALDI-TOF MS for the evaluation of the fatty acid composition of BAL and, for instance, ^{31}P NMR for quantitative PL analysis. First aspects of this approach were already successfully used in BAL analysis [21].

Acknowledgements

This work was supported by the Bundesministerium für Bildung und Forschung (BMB + F), Interdisciplinary Centre for Clinical Research (IZKF) at the University of Leipzig (01KS9504, Project A 17). We thank Dr. Haferburg for his co-operation and many fruitful discussions.

References

[1] J.J. Batenburg, H.P. Haagsman, Prog. Lipid Res. 37 (1998) 235–276.
[2] J. Goerke, Biochim. Biophys. Acta 344 (1974) 241–261.
[3] R. Veldhuizen, K. Nag, S. Orgeig, F. Possmayer, Biochim. Biophys. Acta 1408 (1998) 90–108.
[4] J. Schiller, K. Arnold, in: R.A. Meyers (Ed.), Encyclopedia of Analytical Chemistry, Wiley, Chichester, 2000, pp. 559–585.
[5] F.R. Rana, J.S. Harwood, A.J. Mautone, R.A. Dluhy, Biochemistry 32 (1993) 27–31.
[6] J. Schiller, S. Hammerschmidt, H. Wirtz, J. Arnhold, K. Arnold, Chem. Phys. Lipids 112 (2001) 67–79.
[7] E.L. Heeley, J.M. Hohlfeld, N. Krug, A.D. Postle, Am. J. Physiol. Lung Cell Mol. Physiol. 278 (2000) L305–L311.
[8] R. Paananen, A.D. Postle, G. Clark, V. Glumoff, M. Hallman, J. Lipid Res. 43 (2002) 99–106.
[9] J. Schiller, J. Arnhold, S. Benard, M. Müller, S. Reichl, K. Arnold, Anal. Biochem. 267 (1999) 46–56.
[10] E.G. Bligh, W.J. Dyer, Can. J. Biochem. Physiol. 3 (1959) 911–917.
[11] T. White, S. Bursten, D. Frederighi, R.A. Lewis, E. Nudelman, Anal. Biochem. 10 (1998) 109–117.
[12] M. Petković, J. Schiller, M. Müller, S. Benard, S. Reichl, K. Arnold, J. Arnhold, Anal. Biochem. 289 (2001) 202–216.
[13] J. Schiller, R. Süß, M. Petković, N. Hilbert, M. Müller, O. Zschörnig, K. Arnold, Chem. Phys. Lipids 113 (2001) 123–131.
[14] G.R. Asbury, K. Al-Saad, W.F. Siems, R.M. Hannan, H.H. Hill, J. Am. Soc. Mass Spectrom. 10 (1999) 983–991.
[15] J. Schiller, J. Arnhold, H.-J. Glander, K. Arnold, Chem. Phys. Lipids 106 (2000) 145–156.
[16] J. Schiller, R. Süß, M. Petković, O. Zschörnig, K. Arnold, Anal. Biochem. 309 (2002) 311–314.
[17] M. Müller, J. Schiller, M. Petković, W. Oehrl, R. Heinze, R. Wetzker, K. Arnold, J. Arnhold, Chem. Phys. Lipids 110 (2001) 151–164.
[18] J. Schiller, R. Süß, B. Fuchs, M. Müller, O. Zschörnig, K. Arnold, Chromatographia 57 (2003) S297–S302.
[19] L.H. Cohen, A.I. Gusev, Anal. Bioanal. Chem. 373 (2002) 571–586.
[20] S. Benard, J. Arnhold, M. Lehnert, J. Schiller, K. Arnold, Chem. Phys. Lipids 100 (1999) 115–125.
[21] S. Hammerschmidt, J. Schiller, C. Gessner, M. Meybaum, K. Arnold, H. Wirtz, Biochim. Biophys. Acta 1639 (2003) 17–26.