Comparison of Different Procedures for the Lipid Extraction from HL-60 Cells: A MALDI-TOF Mass Spectrometric Study

Marijana Petković*, Andreas Vocks, Matthias Müller, Jürgen Schiller, and Jürgen Arnhold

Institute of Medical Physics and Biophysics, Medical Faculty, University of Leipzig, Liebigstraße 27, D-04103 Leipzig, Germany. Fax: +49 341 97 15 709.
E-mail: petm@medizin.uni-leipzig.de

* Author for correspondence and reprint requests

Z. Naturforsch. **60 c**, 143–151 (2005); received August 13/September 17, 2004

A human leukaemia cell line – HL-60 – can be differentiated into neutrophils or macrophages and both differentiation processes are accompanied by changes of the lipid composition. Various methods were described for the extraction of lipids from cellular systems, but only two of them were applied to the HL-60 cell line so far. In this study we compared five selected extraction methods for the lipid extraction from HL-60 cells with regard to their qualitative analysis by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS): chloroform/methanol at volume ratios 2:1 and 1:2, isopropanol/chloroform, isopropanol/hexane and butanol. In addition, the cholesterol and phospholipid concentrations in organic extracts were measured by colorimetric assays. Results can be summarized as follows: For the analysis of polar phospholipids obtained from HL-60 cells by MALDI-TOF MS, a chlorofom/methanol (1:2) or isopropanol/chloroform mixture or butanol can be applied as extraction systems. On the other hand, if one would like to analyze changes in triacylglycerols, then chloroform/methanol (2:1) would be the method of choice.

Key words: HL-60 Cells, Lipid Composition, MALDI-TOF MS

Introduction

Cell growth and differentiation, as well as various intracellular signalling processes are accompanied by changes in the lipid and particularly phospholipid (PL) composition. In order to investigate these changes, lipids are extracted from the cells and analyzed subsequently to their fractionation. Methods that were mostly used for the analysis of lipids are thin-layer (TLC) and high-performance liquid chromatography (HPLC), as well as a combination of gas chromatography and HPLC (GC/HPLC) (Lennartz, 1999; Singer *et al.*, 1997; Marinetti and Cattieu, 1982).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been increasingly applied for the analysis of lipids and PLs for a few years (Petković *et al.*, 2001a, 2002a, b; Schiller *et al.*, 1999, 2001a, b; Leßig *et al.*, 2004). The method possesses a number of advantages over other more time-consuming methods in the field of lipid research because MALDI-TOF MS does not require the use of radioactively-labelled lipids or any derivatization prior to their analysis. The spectra acquisition and the data interpretation are fast and easy (Schiller *et al.*, 2001b). High reproducibility of MALDI-TOF MS and low yield of fragmentation products (Hillenkamp *et al.*, 1991) enables also the quantification of lipids by the addition of internal standards and/or by using the signal-to-noise ratio (Petković *et al.*, 2001b). In addition, for some lipid classes, the ratio between the analyte peak and a characteristic matrix peak was also found useful for quantification (Asbury *et al.*, 1999). Although there are some limitations of MALDI-TOF MS for its application to biological systems (Petković *et al.*, 2001a), this method is extremely useful for the initial screening of changes in the lipid composition in various biological samples (Petković *et al.*, 2002a; Leßig *et al.*, 2004; Schiller *et al.*, 2001a).

A human leukaemia cell line, HL-60, can be differentiated into neutrophil-like cells, by treatment with DMSO or retinoic acid (RA) (Collins *et al.*,

Abbreviations: PL phospholipid; MALDI-TOF MS matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry; TAG triacylglycerol; DAG diacylglycerol; PI phosphatidylinositol; PC phosphatidylcholine; PS phosphatidylserine; PE phosphatidylethanolamine; LPL lysophospholipid; SM sphingomyelin; DHB 2,5-dihydroxybenzoic acid; TFA trifluoroacetic acid.

0939–5075/2005/0100–0143 $ 06.00

1978), or into macrophages by phorbol esters (Manning *et al.*, 1995). The differentiation of HL-60 cells by phorbol ester is accompanied by a significant increase in triacylglycerols (TAGs) and decrease in the PL content (Cabot *et al.*, 1980) as well as by alterations in the fatty acids metabolism (Cabot and Welsh 1981). Moreover, changes in PL composition occur prior to changes, or the appearance, of typical protein markers of the cell differentiation (Naito *et al.*, 1987). Therefore, it is of interest to find out what method is best suited for the extraction of lipids from this cell line in order to monitor their alterations.

The question addressed in this study was what lipids can be recovered – and thereafter analyzed by MALDI-TOF MS – from HL-60 cells by various extraction procedures. For that purpose, five selected extraction procedures were used in this work.

The first two methods are the most often used extraction systems and in both a chloroform/methanol mixture is applied. Chloroform/methanol in a 2:1 ratio (Folch *et al.*, 1957) was originally established for the isolation of brain lipids, but it could also be used for other tissues. On the other hand, chloroform/methanol in a 1:2 ratio was usually applied to cells in suspension *i.e.* water-rich samples (Bligh and Dyer, 1959). In both methods the washing step – either with water or salt solution – is included and lipids are recovered in the chloroform layer. Isopropanol/chloroform (Rose and Oklander, 1965) as extraction system – applied for the lipid extraction from human erythrocytes – was reported to work well and yields high amounts of cholesterol and PLs but low yields of heme from these cells. Extraction of lipids by low-toxicity solvents – isopropanol and hexane – was also described (Hara and Radin, 1978), but it was applied exclusively to brain. With this extraction procedure the complete liquid phase is used for analysis. This procedure uses less aggressive solvents instead of chloroform and, therefore, also plastic material can be used for handling and subsequent MALDI-TOF MS analysis. The last and the simplest extraction system used in this study is the butanol extraction (Bremer, 1963). A single solvent is applied and lipids are recovered after centrifugation in the upper layer. This method was also exclusively applied to tissues, and no application to cell lines could be found so far.

Material and Methods

Materials

The HL-60 cell line was obtained as frozen cell culture from "German Collection of Microorganisms and Cell Cultures" (Braunschweig, Germany). Cell culture medium, RPMI 1640, as well as other components for maintaining of the cell culture – L-glutamine, penicilline/streptomycine solution and fetal calf serum – were purchased from Sigma. Trypane blue solution for the cell viability test was also a product of Sigma. Pancreatic phospholipase A_2 and the 1 M solution of $CaCl_2$ were also products of Sigma.

Solvents used for the lipid extraction, as well as for MALDI-TOF MS – chloroform, methanol, isopropanol, butanol and hexane – were obtained in highest commercially available purity from Fluka (Taufkirchen, Germany). The matrix for MALDI-TOF MS (2,5-dihydroxybenzoic acid, DHB), trifluoroacetic acid (TFA) and triethylamine were also purchased from Fluka.

Amplex® Red Cholesterol Assay Kit was obtained from Molecular Probes (USA). Other chemicals – $FeCl_3 \times 6\ H_2O$ and NH_4SCN – used for the measurement of PL concentration were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Cell culture

HL-60 cells were maintained in the continuous cell culture (0.5×10^6 cells/ml) in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 nM L-glutamine, 10 mg/ml streptomycine and 100 U/ml penicillin. The cell viability was determined by the trypan blue exclusion test prior to the lipid extraction. Afterwards, the cells were washed twice with 10 mM phosphate buffered saline (PBS), pH 7.4, followed by centrifugation.

Extraction of lipids from HL-60 cells

Extraction of lipids was carried out in all cases from 10×10^6 cells collected in the logarithmic growth phase. The following procedures were applied to extract lipids from the cells:

a) Chloroform/methanol (2:1, v/v) was used according to the original procedure described by Folch *et al.* (1957). Briefly, 2 ml of the extraction mixture (chloroform/methanol, 2:1) was added directly to the cell pellet (10×10^6 cells) obtained after centrifugation. The suspension

was vortexed and incubated at room temperature with agitation for 20 min. After addition of 0.9% NaCl (400 μl) the mixture was vortexed and centrifuged (500 × *g*, 10 min) to separate the organic from the aqueous phase. The chloroform (lower) layer was collected for the analysis.

b) The second extraction was done as originally described by Bligh and Dyer (1959). The cell suspension (10×10^6 cells/ml, 1 ml) in PBS was extracted by the addition of 3.75 ml of chloroform/methanol (1:2, v/v) and vortexed for 2 min. Chloroform (1.25 ml) was then added and the suspension vortexed for additional 30 s. After that, the mixture was washed with 1.25 ml of 1.5 M NaCl by vortexing for further 30 s. Centrifugation was done under the conditions described in the previous procedure. Also in this case, the chloroform layer was used for analysis.

c) The next procedure uses an isopropanol/chloroform (11:7, v/v) system and was usually applied for isolation of lipids from erythrocytes (Rose and Oklander, 1965). Isopropanol (11 ml) was applied to 1 ml of the cell suspension in PBS (10×10^6 cells/ml) that was further incubated for 1 h at room temperature with agitation. Agitation was continued after the addition of 7 ml of chloroform followed by centrifugation under the same conditions as described above. The chlorofom layer was then used for further analysis.

d) An isopropanol/hexane mixture was also used for the lipid extraction (Hara and Radin, 1978). A mixture of isopropanol/hexane (2:3, v/v) was added to the cell pellet (10×10^6 cells/ml, 1.8 ml of the extraction mixture/cell pellet) that was further left with magnetic stirring at room temperature for 1 h. The mixture was filtered and the pellet washed three times with the double volume of the extraction mixture. After filtration the combined liquid phases were used for analysis.

e) The last extraction procedure uses a single extraction step with butanol (Bremer, 1963). The extraction was attempted as originally described (0.5 vol. of butanol/1 vol. of the cell suspension), but in this case a voluminous protein layer was formed preventing the harvesting of the organic phase. Therefore, the moiety of the extraction solvent was increased and at the end it was twice the volume of the cell suspension. After centrifugation under the conditions described above, the upper butanol layer was taken for further analysis.

In all cases, the organic phase was evaporated to dryness in a vacuum evaporator and the residual lipid film was resuspended in 300 μl of chloroform. The lipid suspension was stored in the refrigerator (– 20 °C) until use.

Digestion of lipids with pancreatic phospholipase A_2 (PLA_2)

All lipid extracts obtained from HL-60 cells were subjected to the digestion with pancreatic PLA_2. After chloroform removal, the residual lipid film was resuspended in 10 mM PBS containing 1 mM $CaCl_2$ (pH 7.4). After vigorous vortexing, PLA_2 (1 mg/ml final concentration) was added to the suspension and the mixture was left at room temperature for 4 h. The reaction was terminated by the addition of chloroform/methanol (chloroform/methanol/water phase 1:1:0.5, v/v/v) and after vortexing and centrifugation the chloroform phase was used for MALDI-TOF MS.

Measurement of phospholipid concentration

The PL concentration was measured by a colorimetric assay as described by Stewart (1980). The chloroform solutions of lipids extracted from HL-60 cells were mixed with a solution containing 0.1 M $FeCl_3 \times 6\ H_2O$ and 0.4 M NH_4SCN in an 1:1 ratio (v/v). After vortexing for 15 s, the mixture was centrifuged at 500 × *g* for 10 min. The absorbance of the chloroform layer was measured at 485 nm with chloroform as blank. The PL concentration was calculated from the standard curve obtained with PC 16:0, 16:0.

Measurement of cholesterol concentration

After removing the chloroform by vacuum evaporation the concentration of cholesterol in the lipid extracts of HL-60 cells was measured by the Amplex® Red Cholesterol Assay kit, purchased from Molecular Probes (USA). The assay – except a very few modifications – was performed according to the manufacturer's instructions.

Preparation of samples for MALDI-TOF MS

Samples obtained by various extraction procedures were evaporated to dryness in a vacuum evaporator and redissolved with the same volume

of the matrix solution (0.5 M DHB with 0.1% TFA in methanol). After vigorous vortexing, the sample was applied onto the sample plate (1.5 μl) and immediately dried under a moderate warm stream of air.

MALDI-TOF MS

MALDI-TOF mass spectra were acquired on a Voyager Biospectrometry workstation (Perseptive Biosystems, Framingham, MA, USA) that utilizes a pulsed nitrogen laser, emitting at 337 nm. The pressure in the ion chamber was maintained between 1.33×10^{-5} and 5.33×10^{-5} Pa. The delayed extraction mode applied improves both mass resolution and mass accuracy (Hillenkamp *et al.*, 1991). The ions that were generated were accelerated with 20 kV voltage within the ion source. In order to enhance the mass resolution, the device was used in the reflectron mode, so that the total field free time-of-flight distance was 2 m. An internal calibration with the peak of DHB matrix (155.034 Da) was performed. This procedure was found to be sufficiently accurate for the determination of individual molecular masses. Each acquired spectrum represents an average of 128 single laser shots.

Results and Discussion

Changes in the lipid and PL metabolism of HL-60 cells after induction of their differentiation either into neutrophil-like or macrophage-like cells are well documented (Manning *et al.*, 1995; Cabot *et al.*, 1980; Cabot and Welsh 1981; Naito *et al.*, 1987). However, these studies involve the extraction of lipids from the cells and in most cases the use of radioactively-labelled lipids and/or their precursors. In most of the reports dealing with this topic two procedures for the lipid extraction from HL-60 cells were applied and both use chlorofom/methanol as extraction system, however in different ratios (Folch *et al.*, 1957; Bligh and Dyer, 1959).

In order to answer the question what is the best method for the extraction of lipids from HL-60 cells for the purposes of their analysis by MALDI-TOF MS, five different solvent mixtures were employed. MALDI-TOF MS analysis of individual lipids is accurate, fast and simple, what was demonstrated on a number of various lipid species (cf. Schiller *et al.*, 2001b). Although this method possesses some limitations for the analysis of complex lipid and PL mixtures (Petković *et al.*, 2001a) it is suitable for initial screeening of the lipid composition of a given sample. This approach has been already successfully applied to various biological systems (Leßig *et al.*, 2004; Schiller *et al.*, 2001a; Petković *et al.*, 2002a).

The suitability of various extraction systems for the recovery of different lipids from HL-60 cells with respect to their analysis by MALDI-TOF MS is briefly summarized in Table I. For the analysis of polar phospholipids obtained from HL-60 cells by MALDI-TOF MS, a chlorofom/methanol (1:2) or isopropanol/chloroform mixture or butanol can be sucessfully applied. The analysis of changes in the triacylglycerol composition could be achieved if lipids are extracted by the chloroform/methanol (2:1) system.

The phospholipid and cholesterol concentration

Before the composition of lipids obtained by various extraction procedures was analyzed by MALDI-TOF MS, PL and cholesterol concentrations in the organic extracts were determined and the results are summarized in Fig. 1. In all cases lipids were extracted from 10×10^6 cells that were either resuspended in PBS or used as pellet (cf. Materials and Methods).

The PL concentration obtained by different extraction mixtures was quite similar, except when chloroform/methanol in a 2:1 ratio or particularly isopropanol/hexane mixtures were applied (Fig. 1) when much lower PL concentrations were measured. PLs – that are comparably polar com-

Table I. Overview of the suitability of different extraction procedures applied in this study for the recovering of the individual lipids: "+" indicates the presence and "–" the absence of an individual lipid/PL in the extract. With the number of "+"-es, the amount of an individual lipid and/or PL obtained by the corresponding procedure is rated. This was introduced for means of comparison between individual extraction systems and not for quantification of lipids.

Extraction procedure	SM+PC+PE	Cho	TAGs
Chloroform/methanol (2:1,v/v)	++	+	++
Chloroform/methanol (1:2, v/v)	++	+	+
Isopropanol/chloroform (7:11, v/v)	++	+	–
Isopropanol/hexane (2:3, v/v)	+	++	–
Butanol	+	++	–

Cho, cholesterol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerol.

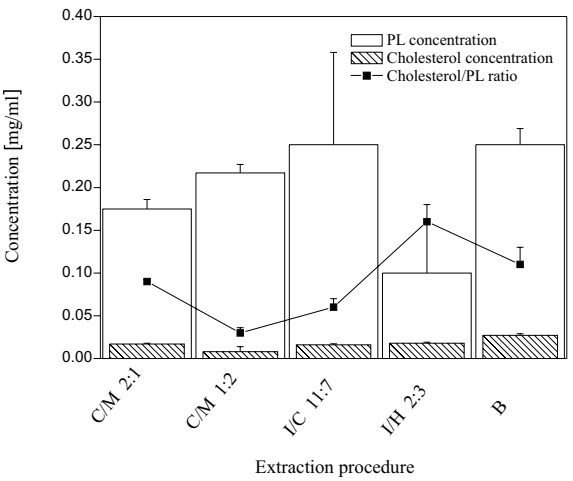

Fig. 1. The phospholipid and cholesterol concentrations measured in the extracts of HL-60 cells obtained by various extraction procedures: chloroform/methanol (2:1), chloroform/methanol (1:2), isopropanol/chloroform (11:7), isopropanol/hexane (2:3) and butanol. The cholesterol/phospholipid ratio is also presented in the figure. Lipids were always recovered from 10×10^6 cells (either in the suspension or in the pellet) that were previously grown in the suspension. The phospholipid concentration was measured in the chloroform layer by a colorimetric assay and the cholesterol concentration was determined by a commercially available fluorescence assay. Briefly, the lipid extracts are incubated with cholesterol oxidase that lead to generation of H_2O_2 and ketone from cholesterol. H_2O_2 is detected using the corresponding reagent as described by the manufacturer. For other experimental details see Materials and Methods. Abbreviations: C/M, chloroform/methanol; I/C, isopropanol/chloroform; I/H, isopropanol/hexane; B, butanol; PL, phospholipid.

pounds – are most probably not easily extractable by these apolar solvent systems.

The cholesterol concentration in the organic extracts of HL-60 cells was measured by a fluorescence assay obtained commercially from Molecular Probes (USA). The cholesterol concentration determined by this assay represents in fact the summary of the free cholesterol concentration together with very low amounts of cholesterol esters present in the cells. The highest cholesterol concentration was obtained when the butanol extraction procedure was applied, even if butanol is a quite polar solvent, whereas the lowest one was measured in the extract obtained by chloroform/methanol 1:2.

The cholesterol/PL ratios (Cho/PL) calculated for the individual extraction systems are also given in Fig. 1. The highest Cho/PL ratio was obtained when isopropanol/hexane was used (Cho/PL = 0.16), whereas the lowest ratio was calculated in the case of the chloroform/methanol – 1:2 (v/v) – mixture (Cho/PL = 0.03). This result indicates that the first extraction procedure applied (chloroform/methanol 2:1) and the isopropanol/hexan system are better suited for the extraction of apolar lipids than for the extraction of polar PLs. On the other hand, it seems that butanol, although a relatively polar organic solvent, could meet the requirements for the extraction of both polar and non-polar lipids, at least from HL-60 cells.

MALDI-TOF mass spectrometric characterization of organic extracts of HL-60 cells

The qualitative lipid composition of organic extracts of HL-60 cells obtained by various extraction methods was analyzed further by MALDI-TOF MS with DHB as matrix.

Fig. 2 shows selected positive ion MALDI-TOF mass spectra of the organic extracts of HL-60 cells obtained by chlorofom/methanol (2:1) (a), chlorofom/methanol (1:2) (b), isopropanol/chloroform (11:7) (c) and isopropanol/hexane (2:3) (d). For the spectrum given in (e) butanol was used for extraction. The mass region between m/z = 350 and m/z = 1150 is presented, comprising the molecular mass range of the most expected cellular lipids and PLs. Main lipid and PL classes are indicated by their m/z ratios and their identity is given in Table II. Due to the high number of peaks and considerable peak overlap only the most intense peaks are labelled in the mass spectra.

The intensity of an individual peak in the MALDI-TOF mass spectra of a complex lipid mixture depends on many factors, including the number of lipid species and the distribution of ions used for cationisation (*i.e.* H^+, Na^+ or K^+). Although the proton adducts are favoured by the addition of TFA to the matrix (cf. Materials and Methods) solution, peaks corresponding to the sodium, as well as the potassium (in particular in the samples of biological origin) adducts cannot be completely suppressed.

In all spectra given in Fig. 2, cholesterol could be detected at the characteristic m/z ratio of 369.3 that corresponds to the ion generated by the loss of water subsequent to protonation. The corresponding sodium adduct is also detectable at m/z = 391.3 (cf. Table II). In this mass region, a number

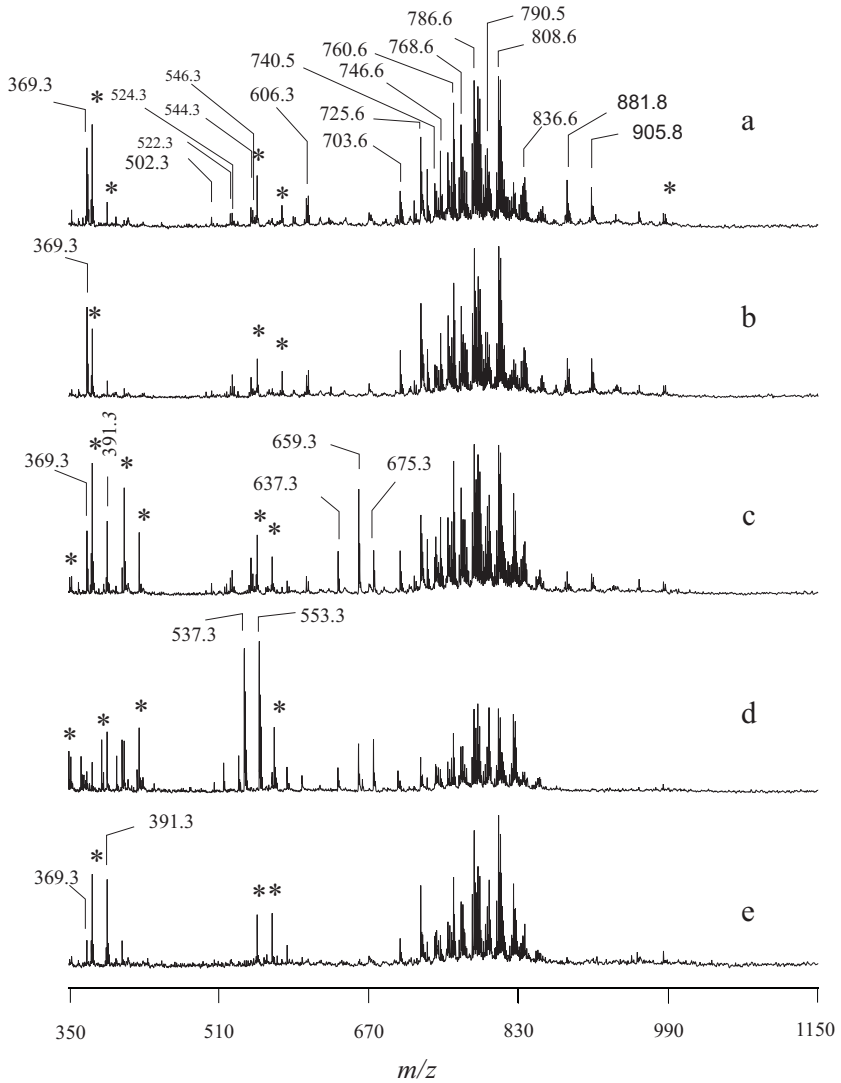

Fig. 2. Positive ion MALDI-TOF mass spectra of the lipid extracts of HL-60 cells obtained by various extraction procedures: (a) chloroform/methanol (2:1), (b) chloroform/methanol (1:2), (c) isopropanol/chloroform (11:7) and (d) isopropanol/hexane (2:3) were used; in (e) lipids from HL-60 cells were obtained by butanol extraction. Main peaks are exclusively indicated in trace (a) according to their *m/z* ratio, whereas additional peaks are indicated in other spectra. Peaks labelled with asterisks correspond to typical matrix (DHB) products and are present in all spectra. The identity of the peaks is given in the text and in Table II. Spectra were recorded under delayed extraction conditions and with a reflectron to increase mass resolution and mass accuracy. For other details see Materials and Methods.

of matrix peaks (labelled with an asterisk in the spectra) are also detected.

In the mass region between *m/z* ~ 420 and *m/z* ~ 600 there is a number of peaks corresponding to lyso-compounds detectable: Peaks at *m/z* = 502.3, *m/z* = 522.3 and *m/z* = 544.3 correspond to LPE 18:1 + Na^+, LPC 18:1 + H^+ and LPC 18:1 + Na^+, respectively; peaks at *m/z* = 524.3 and 546.3 are also detectable and correspond to the proton and the sodium adduct of LPC 18:0, respectively. These LPL peaks were, however, absent in the spectra of butanol extracts (Fig. 2e). LPLs might principally also be generated during sample preparation. The presence of small amounts of TFA in the matrix solution could trigger the hydrolysis of PLs (*e.g.* alkenyl-acyl species) leading to the LPL generation. To check that, spectra were recorded also without TFA but no significant changes in this mass region were detected (data not shown).

Moreover, alkenyl ether-linked PLs (plasmalogens) are known to be more susceptible to acidic hydrolysis (even in the presence of trace amounts of TFA) than acyl-linked lipids. The absence of significant differences in the spectra recorded in the presence and in the absence of TFA (in the LPL region) indicates also that alkenyl ether-linked PLs do not contribute to the PL of HL-60 cells. This is in accordance with previously pub-

Table II. Assignment of peaks detected in positive ion MALDI-TOF mass spectra of organic extracts of HL-60 cells obtained by various extraction procedures. Peak position is indicated by the *m/z* ratio and peak identity is also given in the table. For TAGs both the summarized fatty acid composition as well as potential fatty acid combination is indicated. The table comprises all spectra presented in this work.

Peak position	Peak assignment
369.3	Cholesterol-H_2O + H^+
391.3	Cholesterol-H_2O + Na^+
502.3	LPE 18:1 + Na^+
522.3	LPC 18:1 + H^+
524.3	LPC 18:0 + H^+
537.3	n.a.
544.3	LPC 18:1 + Na^+
546.3	LPC 18:0 + Na^+
553.3	n.a.
606.3	n.a.
659.3	n.a.
675.3	n.a.
703.6	SM 16:0 + H^+
725.6	SM 16:0 + Na^+
740.5	PE 16:0, 20:4 (acyl-acyl) + H^+
746.6	PC 16:0, 18:1 (alkyl-acyl) + H^+
760.6	PC 16:0, 18:1 (acyl-acyl) + H^+
766.5	PE 18:0, 18:2 (acyl-acyl) + Na^+
768.5	PE 18:0, 20:4 (acyl-acyl) + H^+ or PE 18:0, 18:1 + Na^+
768.6	PC 16:0, 18:1 (alkyl-acyl) + Na^+
790.5	PE 18:0, 20:4 (acyl-acyl) + Na^+
808.6	PC 18:0, 18:2 (acyl-acyl) + Na^+ and PC 16:0, 22:5 (acyl-acyl) + H^+
835.6	SM 24:1 + Na^+
881.8	TAG 52:2 (2 × 18:1, 16:0) + Na^+
907.8	TAG 54:3 (3 × 18:1) + Na^+

LPC, Lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; TAG, triacylglycerol; n.a., not assigned.

lished data (Cabot *et al.*, 1980). The contribution of ether-linked lipids in HL-60 cells increases, however, after their differentiation (Naito *et al.*, 1987).

In order to verify the presence of ether-linked PLs – as well as to confirm the peak identity – the lipid extracts of HL-60 cells were subjected to digestion with pancreatic PLA_2. This approach was chosen, because residual LPLs can be easily detected and analyzed by MALDI-TOF MS and possess lower variability of fatty acid residues. Moreover, it is possible to confirm the identity of the fatty acid residue at the *sn*-1 position since PLA_2 catalyzes the cleavage of the fatty acid residue at the *sn*-2 position of the glycerol backbone (Murakami *et al.*, 1997). By this approach, it was possible to detect alkyl LPC 16:0 and alkyl LPC 18:0 in the mass spectra of the lipid extracts of the HL-60 cell line (data not shown).

Some additional peaks are detectable in Figs. 2c and 2d in the mass range between *m/z* ~ 550 and *m/z* ~ 700. The origin of these peaks could not be identified so far, but they might represent fragmentation products of PLs of still unidentified nature. They were, however, still present in the spectra of the PLA_2-digested lipid extracts (data not shown), indicating that they do not possess the fatty acid residue at the *sn*-2 position that is susceptible to the PLA_2-catalyzed scission.

Sphingomyelin (SM) (16:0) was detectable at *m/z* = 703.6 (the proton adduct) and 725.6 (the sodium adduct) in the spectra. Besides that, the SM species containing a stearic acid residue is also detectable (cf. Table II, not labelled in the spectra).

The mass region between *m/z* ~ 750 and *m/z* ~ 830 is overcrowded by peaks and, thus, difficult to analyze. However, the main peaks were successfully previously assigned in the MALDI-TOF mass spectra of organic extracts of human neutrophils (Schiller *et al.*, 1999). Many peaks detectable in this mass range cannot be unequivocally assigned because they are caused by overlapping species. In the spectra given in Fig. 2, some of the most intense peaks are already labelled and could be easily assigned. The most abundant diacyl PC species is identified at *m/z* = 786.6 as the proton adduct of PC 18:0, 18:2. According to the molecular weight, these peaks could also arise from PC 18:1, 18:1. However, after incubation with PLA_2 no peak corresponding to LPC 18:1 could be detected (data not shown). That confirmed the peak identity. Small amounts of LPC 18:1 were detectable in the spectra of the lipid extracts, but they were not present in the spectra of the digested extracts. This might indicate that these species were present in rather low amounts in the cells and corresponding peaks are suppressed by other LPC species generated after treatment with PLA_2.

The sodium adduct of PC 18:0, 18:2 is detectable at *m/z* = 808.6. The alkyl-analogue of PC 18:0, 18:2 is also detectable in the spectra at *m/z* = 772.6 (proton adduct) and at *m/z* = 794.6 (the corresponding sodium adduct; not labelled).

Although the peaks arising from PE and PS could be suppressed in the presence of high PC amounts (Petković *et al.*, 2001a), some PE peaks

could be distinguished in the spectra. Peaks at m/z = 740.5 and 762.5 correspond to the proton and the sodium adduct of PE 16:0, 20:4, respectively, whereas the analogous PE with stearic instead of palmitic acid could be detected at m/z = 768.5 and 790.5. PS is more difficult to detect in comparison to PC, due to its acidic nature and higher molecular weight. In addition, high amounts of PC might suppress the corresponding peaks. Therefore, analysis of this PL species by MALDI-TOF MS in samples of biological origin (containing high PC amounts) would not be possible. In the case of a lower number of species this problem could be overcome by recording the negative ion mode, since it was shown that under these conditions some PS peaks could be detected (Petković *et al.*, 2001a). But, the presence of more lipid species in the cellular extracts additionally decreases the detectability of the negatively-charged PLs, such as PS. Therefore, only the positive ion MALDI-TOF mass spectra are presented in this work.

Some additional peaks at higher m/z ratios were observed in traces (Fig. 2a–c). Peaks at m/z = 881.8 and 907.8 correspond to the sodium adducts of TAG 52:2 and TAG 54:3 (cf. Table II), respectively. TAGs (as well as DAGs) give only sodium and potassium adducts whereas proton adducts are not detectable at all (Benard *et al.*, 1999). Also, peaks at m/z = 537.3 and 553.3 are detectable in Fig. 2d, but their identity is not known so far. Since they are present only in the spectra of isopropanol/hexane extracts, we can only assume that they arise from the solvent used (hexane). Other qualitative differences could not be observed in the summary spectra given in Fig. 2.

Acknowledgements

This work was supported by the Interdisziplinäres Zentrum für Klinische Forschung (IZKF) Leipzig at the Faculty of Medicine of the University of Leipzig (Projects A17 and A19). M. Petković has a Research Fellowship from the German Research Council (Deutsche Forschungsgemeinschaft, PE 895/1–1).

Asbury G. R., Al-Saad K., Siems W. F., Hannan R. M., and Hill H. H. (1999), Analysis of triacylglycerols and whole oils by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J. Am. Soc. Mass Spectrom. **10**, 983–991.

Benard S., Arnhold J., Lehnert M., Schiller J., and Arnold K. (1999), Experiments towards quantification of saturated and polyunsaturated diacylglycerols by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Chem. Phys. Lipids **100**, 115–120.

Bjerve K. S., Daae L. N. W., and Bremer J. (1974), The selective loss of lysophospholipids in some commonly used extraction procedures. Anal. Biochem. **58**, 238–245.

Bligh E. G. and Dyer W. J. (1959), A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**, 911–917.

Bremer J. (1963), Carnitine in intermediary metabolism. The biosynthesis of palmylcarnitine by cell subfractions. J. Biol. Chem. **238**, 2774–2779.

Cabot M. C. and Welsh C. J. (1981), Fatty acid metabolism in phorbol ester-differentiating human leukaemia cells. Canc. Res. **41**, 4910–4915.

Cabot M. C., Welsh C. J., Callaham M. F., and Huberman E. (1980), Alterations in lipid metabolism induced by 12-*O*-tetradecanoylphorbol-13-acetate in differentiation of human myeloid leukaemia cells. Canc. Res. **40**, 3674–3679.

Collins S. J., Ruscetti E. W., Gallagher R. E., and Gallo R. C. (1978), Terminal differentiation of human promyelocytic leukaemia cells induced by dimethylsulfoxide and other polar compounds. Proc. Natl. Acad. Sci. USA **75**, 2458–2462.

Folch J., Lees M., and Stanley G. H. S. (1957), A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**, 497–509.

Hara A. and Radin N. S. (1978), Lipid extraction of tissues with a low-toxicity solvent. Anal. Biochem. **90**, 420–426.

Hillenkamp F., Karas M., Beavis R. C., and Chait B. T. (1991), Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of biopolymers. Anal. Chem. **63**, 1193A–1203A.

Lennartz M. R. (1999), Phospholipases and phagocytosis: the role of phospholipid-derived second messengers in phagocytosis. Int. J. Cell Biol. **31**, 415–430.

Leßig J., Gey C., Süß R., Schiller J., Glander H.-J., and Arnhold J. (2004), Analysis of the lipid composition of human and boar spermatozoa by MALDI-TOF mass spectrometry, thin layer chromatography and ^{31}P NMR spectroscopy. Comp. Biochem. Phys. B **137**, 265–277.

Manning R., Fallani A., and Ruggieri S. (1995), Lipid changes in HL-60 cells on differentiation into macrophages by treatment with a phorbol ester. Lipids **30**, 811–815.

Marinetti G. V. and Cattieu K. (1982), Composition and metabolism of phospholipids of human leukocytes. Chem. Phys. Lipids **31**, 169–177.

Murakami M., Nakatani Y., Atsumi G., Inoue K., and Kudo I. (1997), Regulatory functions of phospholipase A_2. Crit. Rev. Immunol. **17**, 225–283.

Naito M., Kudo I., Nakagawa Y., Waku K., Nojiri H., Saito M., and Inoe K. (1987), Lipids of human promyelocytic leukaemia cell HL-60: increasing levels of ether-linked phospholipids during retinoic acid-induced differentiation. J. Biochem. **102**, 155–162.

Petković M., Schiller J., Müller M., Benard S., Reichl S., Arnold K., and Arnhold J. (2001a), Detection of individual phospholipids in lipid mixtures by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: Phosphatidylcholine prevents the detection of further species. Anal. Biochem. **289**, 202–216.

Petković M., Schiller J., Müller J., Müller M., Arnold K., and Arnhold J. (2001b), The signal-to-noise ratio as the measure for the quantification of lysophospholipids by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Analyst **126**, 1042–1050.

Petković M., Schiller J., Müller M., Arnold K., and Arnhold J. (2002a), Investigations of the lysophospholipid composition of human neutrophils under different stimulation conditions by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J. Serb. Chem. Soc. **67**, 149–163.

Petković M., Müller J., Müller M., Schiller J., Arnold K., and Arnhold J. (2002b), Application of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for monitoring the digestion of phosphatidylcholine by pancreatic phospholipase A_2. Anal. Biochem. **308**, 61–70.

Rose H. and Oklander M. (1965), Improved procedure for the extraction of lipids from human erythrocytes. J. Lipid Res. **6**, 428–431.

Schiller J., Arnhold J., Benard S., Müller M., Reichl S., and Arnold K. (1999), Lipid analysis by matrix-assisted laser desorption and ionization mass spectrometry: A methodological approach. Anal. Biochem. **267**, 45–56.

Schiller J., Zschörnig O., Petković M., Müller M., Arnhold J., and Arnold K. (2001a), Lipid analysis of human HDL and LDL by MALDI-TOF mass spectrometry and ^{31}P NMR spectroscopy. J. Lipid Res. **42**, 1501–1508.

Schiller J., Arnhold J., Benard S., Müller M., Petković M., Zschörnig O., and Arnold K. (2001b), MALDI-TOF mass spectrometry and ^{31}P NMR spectroscopy in lipid research. In: Recent Research Developments in Lipids (Pandalai S. G., ed.). Transworld Research Network, Trivandrum, India, pp. 179–200.

Singer W. D., Brown H. A., and Sternweis P. C. (1997), Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. Annu. Rev. Biochem. **66**, 475–509.

Stewart J. C. M. (1980), Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal. Biochem. **104**, 10–14.

Satz und Druck: AZ Druck und Datentechnik GmbH, Kempten