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Short communication

A simple method to generate oxidized phosphatidylcholines in amounts close to one milligram

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ABSTRACT

Oxidized (phospho)lipids are of paramount interest from different reasons: beside their significant *in vivo* relevance, these products are often needed in the laboratory to study the response of selected cells to oxidized lipids. Unfortunately, the commercial availability of oxidized lipids is limited and scientists interested in studying the physiological impact of oxidized lipids are normally forced to prepare the required compounds by themselves.

We will show here that chain-shortened products of oxidized phosphatidylcholines (PCs) such as aldehydes and carboxylic acids can be easily (and in nearly quantitative yields) generated by the Fenton reaction ($H_2O_2 + Fe^{2+}$) or the $KMnO_4$-induced oxidation of the PC. Using the Fenton reaction and physiological saline, chlorinated oxidation products such as chlorohydrins are also readily available. Additionally, it will be shown that preparative thin-layer chromatography (TLC) is a convenient but simple method to isolate the individual oxidation products in reasonable yields and high purities: all relevant products could be successfully identified by matrix-assisted laser desorption and ionization (MALDI) mass spectrometry and the amounts of the oxidized products determined by a simple colorimetric assay.

1. Introduction

Phospholipids (PLs) are (beside cholesterol and selected membrane proteins) important constituents of all cellular membranes. Because of their amphiphilic properties PLs enable the formation of stable bilayers that protect the interior of the cells but enable also selective transport processes. Additionally, selected PLs such as phosphatidic acids or poly-phosphoinositides are molecules with important regulatory functions serving as lipid second messengers (Martelli et al., 2004).

The oxidation of PLs by reactive oxygen species (ROS) is a very important reaction and many oxidized PLs are of considerable biological significance (Fuchs et al., 2011). This particularly applies at inflammatory conditions that are accompanied by increased ROS generation by immune cells such as neutrophilic granulocytes. Lipid oxidation is involved in the pathogenesis of many diseases such as asthma (Olopade et al., 1997), rheumatoid arthritis (Schiller et al., 2003; Fuchs et al., 2005), atherosclerosis (Victor et al., 2009) and Parkinson's disease (Mattson, 2009). Finally, selected oxidized PLs (for instance, oxidatively-modified phosphatidylcholines and lyso-phosphatidylcholines) are known to possess biological activities similar to platelet-activating factors (PAF) (Marathe et al., 2000) and are likely to destabilize the structures of biological membranes.

Phosphatidylcholines (PCs) are the most abundant PLs in the human body as well as in virtually all mammalian cells (Henry et al., 1998). This – in combination with their commercial availability – renders PCs interesting compounds to study their oxidation products. The related oxidation reactions are not only of interest regarding mechanistic considerations (e.g., to investigate the stabilities of the oxidation products) but as well to study the response of selected cells towards selected oxidation products. Unfortunately, oxidized (phospho)lipids are scarcely commercially available and, thus, scientists are often forced to synthesize the desired oxidation products by themselves.

The oxidation of unsaturated PCs such as PLPC (1-palmitoyl-2-linoleoyl-*sn*-phosphatidylcholine) leads to per-oxidized compounds (endo- and hydroperoxides) as primary products which decay subsequently under generation of aldehydes as well as carboxylic acids as secondary products, i.e., a scission of the double bonds occurs (Niki, 2009; Fuchs et al., 2011). Although oxidation reactions are commonly used to determine the positions of double

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bonds within an unsaturated fatty acyl chain, many details of lipid oxidation processes remain to be elucidated (Fuchs et al., 2011).

The present work has two different aims: on the one hand, different oxidizing agents (atmospheric oxygen, $KMnO_4$ and the Fenton reagent) are compared (a) regarding their ability to induce lipid oxidation and (b) the generated product patterns. On the other hand, preparative thin-layer chromatography (TLC) will be used to isolate the most abundant lipid oxidation products: although the separation quality achievable by TLC is rather poor in comparison to HPLC (high performance liquid chromatography), the required TLC equipment is inexpensive and simple and the method can be, thus, easily established in virtually all laboratories. PLPC will be exclusively used here because it represents in these author's opinion an excellent compromise: due to its two double bonds (within the linoleoyl residue) it is more readily oxidized in comparison to POPC (1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine) that contains only a single double bond. On the other hand, the variety of products is much smaller in comparison to higher unsaturated PLs (Reis et al., 2004).

The characterization of the generated oxidation products will be performed by matrix-assisted laser desorption and ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) (Fuchs et al., 2010), which is a simple, but powerful method to characterize lipids and their oxidation products. Finally, the yields of the different oxidation products will be determined by using a simple colorimetric assay.

2. Materials and methods

2.1. Chemicals

All chemicals and all solvents (methanol, ethanol, chloroform, isopropanol, acetonitrile, water, triethylamine and glacial acetic acid) were obtained in the highest commercially available purity from Sigma–Aldrich (Taufkirchen, Germany) and used as supplied.

PLPC was purchased from AVANTI Polar Lipids (Alabaster, AL, USA) as 10 mg/ml chloroform solution and used as supplied. Chemicals used for lipid oxidation were purchased either from Fluka ($KMnO_4$ and H_2O_2) or from Sigma–Aldrich ($FeCl_2$).

The dye primuline (for monitoring the presence of lipids on the TLC plate) was obtained from Sigma–Aldrich (Taufkirchen, Germany) and used as previously described (Fuchs et al., 2008; White et al., 1998). Phospholipase A_2 (PLA_2) from hog pancreas was also obtained from Sigma–Aldrich.

2.2. Oxidation of PLPC at room temperature (296 K)

An aliquot of about 66 nmol PLPC (50 μg, dissolved in 10 μl $CHCl_3$) was evaporated to dryness and the resulting lipid film subsequently exposed to atmospheric oxygen in a small glass vessel (the area of the lipid film exposed to oxygen was estimated to be about 19.6 mm^2). The oxidation was performed for "0" (control), 2 and 7 days at 296 K. The incubation was stopped by the addition of the MALDI matrix and the resulting solution immediately analyzed by MALDI–TOF MS (vide infra).

2.3. Oxidation of PLPC by $KMnO_4$

An aliquot of 33 nmol PLPC (25 μg, dissolved in 10 μl $CHCl_3$) was evaporated to dryness. Oxidation was induced by the addition of 100 μl of a 1.58 mol/l $KMnO_4$ (250 mg/ml dissolved in H_2O) solution and incubated for 10 min at 37 °C. The oxidation was stopped by the addition of organic solvents (chloroform/methanol (1:1 (v/v); 200 μl)) to extract the apolar lipids from the aqueous phase, whereby the $KMnO_4$ remains nearly quantitatively in the aqueous phase (the organic phase was nearly colorless, while the aqueous phase was violet).

2.4. Oxidation of phospholipids by the Fenton reagent (H_2O_2 and $FeCl_2$)

An aliquot of 33 nmol PLPC (25 μg dissolved in 10 μl $CHCl_3$) was evaporated to dryness. The resulting lipid film was incubated with a fixed amount of 50 μl of a 500 mM aqueous solution of H_2O_2 (spectrophotometrically determined ($\varepsilon_{230} = 74\ M^{-1}\ cm^{-1}$)) (Beers and Sizer, 1952) and 50 μl of a 50 mM aqueous $FeCl_2$ solution. These conditions resulted in our hands in a maximum yield of oxidation products. Incubations were performed for 1 h, 2 h or over night (about 16 h). The incubation was stopped by adding chloroform/methanol (1:1 (v/v)) to extract the lipids (Bligh and Dyer, 1959) and to reduce the amounts of inorganic salts.

2.5. Large scale oxidation of PLPC with H_2O_2 and $FeCl_2$

An aliquot of 1.32 μmol PLPC (1 mg dissolved in 100 μl $CHCl_3$) was evaporated to dryness. The resulting lipid film was incubated with a fixed amount of 50 μl H_2O_2 (500 mM dissolved in H_2O) and 50 μl $FeCl_2$ (50 mM dissolved in H_2O). Incubations were performed over night (about 12 h). The incubation was stopped by adding chloroform/methanol (1:1 (v/v)) to extract the lipids (Bligh and Dyer, 1959). Six different reaction batches were combined and used for preparative TLC. The total amount of lipid was, thus, 6 mg corresponding to about 7.9 μmol.

2.6. Lipid extraction

Lipid extraction was performed in all cases according to Bligh and Dyer (1959). After addition of the organic solvent mixtures (aqueous phase/chloroform/methanol = 1:1:1 (v/v/v)) the sample was vigorously vortexed and the mixture centrifuged at 1000 × *g* for 5 min (296 K) to expedite the separation of the organic and the aqueous phase. The lower (chloroform) phase was carefully isolated by using a Hamilton syringe whereas the upper phase (aqueous methanol) was discarded. Samples were directly used for subsequent MALDI–TOF MS characterization or TLC separation.

2.7. Thin-layer chromatography (TLC)

Oxidized lipid extracts were applied onto HPTLC silica gel 60 plates (10 × 10 cm in size with aluminum backs (Merck, Darmstadt, Germany)), using a Linomat 5 device (CAMAG; Berlin, Germany), and developed in a vertical TLC chamber with $CHCl_3$, methanol, water (60:30:5 (v/v/v)) as the mobile phase (Kupke and Zeugner, 1978). Lipids were visualized by spraying the plate with primuline (Direct Yellow 59), which is known to bind non-covalently to the apolar fatty acyl residues of PLs without affecting the molecular weights (White et al., 1998). Upon illumination with UV light (366 nm), individual lipid classes are detectable as colored spots. These spots were assessed using a digital image system in combination with the program Argus X1 (BioStep, Jahnsdorf, Germany).

In one selected case, two-dimensional TLC (using commercially available diol-modified (Merck, Darmstadt, Germany) HPTLC silica plates) was also applied in an attempt to improve the separation quality. Chloroform, methanol, water, ethanol, triethylamine (13/4/2/7/7, v/v/v/v/v) was used in the first dimension and (after careful drying of the TLC plate) chloroform, methanol, acetic acid, water (45/20/6/1, v/v/v/v) in the second dimension.

2.8. Phospholipase A_2 digestion

Selected PL oxidation mixtures were digested by the enzyme PLA_2 to obtain the corresponding lysolipids and to confirm peak

assignments (Fuchs et al., 2007a). Briefly, organic aliquots were evaporated to dryness. Lipid vesicles were prepared by suspending the resulting PL film in water and vortexing vigorously for 30 s. Vesicles were treated with 0.5 mg/ml PLA_2 (about 100 U/ml) for 2 h at 37 °C. Afterwards, lipids were extracted as described (vide supra) and the organic solution directly used for MS characterization.

2.9. Lipid re-extraction from silica gel

The marked lipid spots (subsequent to primuline staining) were scratched off from the TLC plate with a steel spatula. Subsequently, the silica gel was eluted with a 20-fold excess of 0.9% aqueous NaCl/methanol/chloroform (1:1:1 (v/v/v)). The amount of the used solvent is related to the dry weight of the silica gel. The silica gel was washed three times with the indicated excess of the solvent system to minimize lipid losses (Teuber et al., 2010). The obtained extracts were combined and dried under reduced pressure. According to our experience there is no major difference and the use of an inert gas is, thus, not an absolute must.

2.10. Determination of PL amounts (Stewart-assay)

This assay developed by Stewart (1980) is a fast and simple colorimetric method to determine absolute amounts of PLs. It is based on the complex formation between PLs and an inorganic, colored iron salt (ammonium ferrothiocyanate) which gets soluble in organic solvents after binding to the PL.

A solution of ferrothiocyanate was prepared by dissolving 27.03 g $FeCl_3 \times 6H_2O$ and 30.4 g NH_4SCN in one liter of water. The lipid sample of interest (dissolved in 2 ml $CHCl_3$) was added to 2 ml ferrothiocyanate solution. After careful mixing, the organic phase was isolated by centrifugation. The optical density of the chloroform phase was determined at $\lambda = 488$ nm in a quartz cuvette on a Hitachi U-2000 photometer. Pure $CHCl_3$ served as blank control.

2.11. MALDI–TOF mass spectrometry

Two different matrices were applied: 2,5-dihydroxybenzoic acid (DHB) (from Fluka) was used as 0.5 M solution in methanol (Schiller et al., 1999) and 9-aminoacridine (9-AA) hemihydrate (from Acros Organics, distributed by Fisher Scientific GmbH, Niederau, Germany) as 10 mg/ml solution in 60/40 (v/v) isopropanol/acetonitrile (Sun et al., 2008). These both matrices were used due to their different pK values (Fuchs et al., 2009). All organic extracts were pre-mixed (1:1 (v/v)) with either 9-AA or DHB and 1 μl of this mixture was transferred onto the MALDI target (gold-coated aluminum). All mass spectra were acquired on a Bruker Autoflex mass spectrometer (Bruker Daltonics, Bremen, Germany). This system utilizes a pulsed nitrogen laser emitting at 337 nm. The extraction voltage was 20 kV and gated matrix suppression was applied to prevent the saturation of the detector by matrix ions. One hundred single lasers shots were averaged for each mass spectrum. In order to enhance the resolution, all spectra were acquired in the reflector mode using "delayed extraction" conditions. A more detailed methodological description of MALDI–TOF MS with the focus on lipid analysis is available in a recent review by Fuchs et al. (2010) as well as Bresler et al. (2011). Raw data were processed with the software "Flex Analysis" version 2.2 (Bruker Daltonics).

3. Results and discussion

The first aim of this study was to compare the effects of different oxidizing agents on PLPC as one selected PL. The obtained oxidation products were identified by MALDI–TOF MS which is a simple and convenient method to characterize lipids and the related oxidation products (Fuchs et al., 2010).

Fig. 1 compares the positive ion MALDI–TOF mass spectra of PLPC subjected to different oxidizing conditions. Trace (1A) reflects pure PLPC as the control. PLPC results in two peaks at $m/z = 758.6$ and 780.6 corresponding to the H^+ and the Na^+ adduct (Schiller et al., 1999). If the oxidation is performed by exposing the PLPC to atmospheric oxygen at RT (296 K) for 1 day (trace (1B)), there is no more original PLPC detectable. However, there are several signals that can be easily assigned to characteristic oxidation products: the peak at $m/z = 496.3$ corresponds to lysophosphatidylcholine (LPC) 16:0 (H^+ adduct) that is generated as a minor product upon PLPC oxidation by the complete cleavage of the unsaturated (or oxidatively modified) fatty acyl residue. Although it is commonly accepted that LPC is primarily generated under the influence of the enzyme phospholipase A_2, oxidation processes are also accompanied by LPC generation: Arnhold et al. (2002) were able to show that the introduction of electronegative

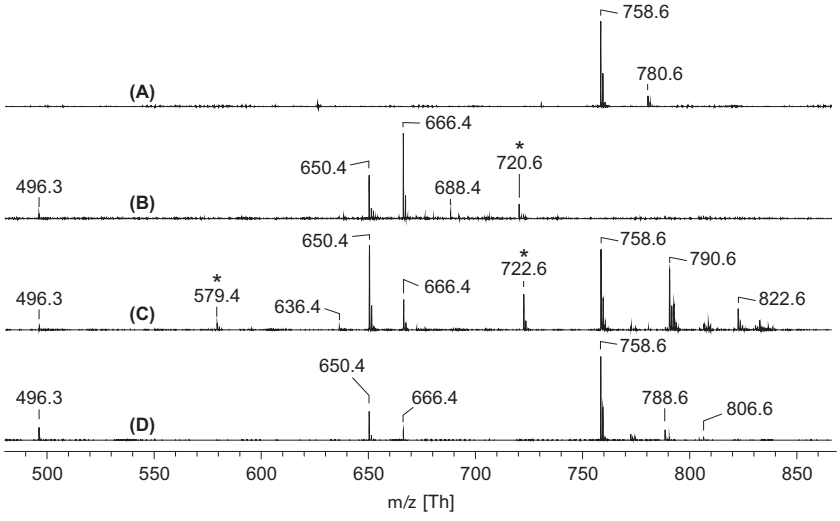

Fig. 1. Selected positive ion MALDI–TOF mass spectra of oxidized PLPC. All spectra were recorded with 9-AA as matrix and the most prominent peaks are marked by the corresponding m/z values. (A) represents the spectrum of native PLPC (control) while the PLPC was oxidized by exposition to atmospheric oxygen (B), $KMnO_4$ (C) and $FeCl_2$ and H_2O_2 (Fenton conditions) (D). So far unidentified peaks are marked by asterisks. Additional experimental details are available in Section 2.

Table 1
Assignment of the most intense peaks observed in the positive ion MALDI–TOF mass spectra of PLPC oxidized by different oxidizing reagents.

m/z	Assignment
496.3	LPC 16:0 ($+H^+$)
518.3	LPC 16:0 ($+Na^+$)
579.4	Unidentified
636.4	1-palmitoyl-2-(8-oxo-octanoic acid)-*sn*-phosphatidylcholine ($+H^+$)
638.4	1-palmitoyl-2-(8-hydroxyoctanoic acid)-*sn*-phosphatidylcholine ($+H^+$)
650.4	1-palmitoyl-2-(9-oxo-nonaoic acid)-*sn*-phosphatidylcholine ($+H^+$)
660.4	1-palmitoyl-2-(8-hydroxyoctanoic acid)-*sn*-phosphatidylcholine ($+Na^+$)
666.4	1-palmitoyl-2-(nonadioic acid)-*sn*-phosphatidylcholine ($+H^+$)
672.4	1-palmitoyl-2-(9-oxo-nonaoic acid)-*sn*-phosphatidylcholine ($+Na^+$)
688.4	1-palmitoyl-2-(nonadioic acid)-*sn*-phosphatidylcholine ($+Na^+$)
720.6	Unidentified
722.6	Loss of headgroup from *m/z* 780.6
758.6	PC 16:0 18:2 ($+H^+$) (PLPC)
780.6	PC 16:0 18:2 ($+Na^+$) (PLPC)
788.6	PLPC + O_2 − 2H ($+H^+$)
790.6	PLPC + O_2 ($+H^+$)
804.6	PLPC + 2 O_2 − H_2O ($+H^+$)
806.6	PLPC + 3/2 O_2 ($+H^+$)
810.6	PLPC + HOCl ($+H^+$)
812.6	PLPC + O_2 ($+Na^+$)
814.6	Unidentified
822.6	PLPC + 2 O_2 ($+H^+$)
826.6	PLPC + 2HOCl − HCl ($+H^+$)
862.6	PLPC + 2HOCl ($+H^+$)
884.6	PLPC + 2HOCl ($+Na^+$)

elements into an unsaturated fatty acyl residue upon oxidation leads to a reduction of the strength of the ester linkage resulting in an increased hydrolysis rate.

Additionally, the cleavage at one double bond position is obvious by the generation of the characteristic aldehyde (1-palmitoyl-2-(9′-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine) at *m/z* = 650.4 (Itabe et al., 1996) and the corresponding carboxylic acid (1-palmitoyl-2-(1,9-nonanedicarboxylic acid)-*sn*-glycero-3-phosphocholine) at *m/z* = 666.4 (and to a much smaller extent at *m/z* = 688.4 because the carboxylic acid undergoes exchange reactions with alkali metal ions) (Podrez et al., 2002). Products with higher masses in comparison to the original PLPC are not detectable under these reaction conditions. Therefore, air-oxidation is the method of choice when only chain-shortened products are of interest. In contrast, such products are detectable (in addition to the products with shortened fatty acyl residues) when the oxidation is induced by $KMnO_4$ (trace (1C)) or the Fenton reagent (trace (1D)). Particularly the $KMnO_4$-induced oxidation leads to the addition of one (*m/z* = 790.6) or two (*m/z* = 822.6) O_2 molecules. Addition of H_2O_2 (*m/z* = 792.6) to the PLPC molecule may also occur. Unfortunately, some so far unknown products (*m/z* = 579.4, 720.6 and 722.6; marked by asterisks in Fig. 1) are also generated under these experimental conditions. A more detailed assignment of all observed peaks is available in Table 1.

In a nutshell, all three oxidation methods lead to similar product patterns although the yields of the individual compounds are obviously different. It is surprising that the oxidation by the Fenton reagent resulted in the smallest yield of reaction products because the generated hydroxyl radicals ($HO^•$) would be expected to react in a diffusion-controlled manner. Therefore, our next step was to evaluate the time-dependence of PLPC oxidation under Fenton conditions: the ratios between PLPC, H_2O_2 and $FeCl_2$ were kept constant in all cases and the only variable was the incubation time. The corresponding positive ion MALDI mass spectra are shown in Fig. 2.

It is evident that the incubation time has a considerable impact on the product pattern: while no oxidation products are detectable in the spectrum of the pure PLPC (2A), peaks of LPC 16:0, the aldehyde, the carboxylic acid and an oxidized product with *m/z* = 788.6 (Davis et al., 2008; cf. Table 1) are clearly detectable after incubation for 1 h with the Fenton reagent (2B) although the peaks of the educt (PLPC) are still dominating. Using a prolonged incubation time of 2 h (2C) the yield of the carboxylic acid (*m/z* = 666.4 and 688.4) is significantly increased while that of the PLPC as the educt is reduced. In addition, the peroxidized product at *m/z* = 790.6 and the bis-chlorohydrin at *m/z* = 862.6 are detectable, whereas *m/z* = 826.6 corresponds to the loss of HCl from *m/z* = 862.6. The generation of the chlorohydrins under conditions of the Fenton reaction may be surprising but this fact was recently established by Wu et al. (2011): these authors suggested that chloride reacts with $HO^•$ in a multiple step reaction to give HOCl which readily adds to the unsaturated fatty acyl residues of PLs.

After 16 h (2D), the peak of the PLPC is not detectable anymore and the carboxylic acid (*m/z* = 666.4 and 688.4) is the most abundant product. From these data it is obvious, that the incubation time has a considerable effect: using prolonged incubation times, only LPC 16:0, the carboxylic acid and the

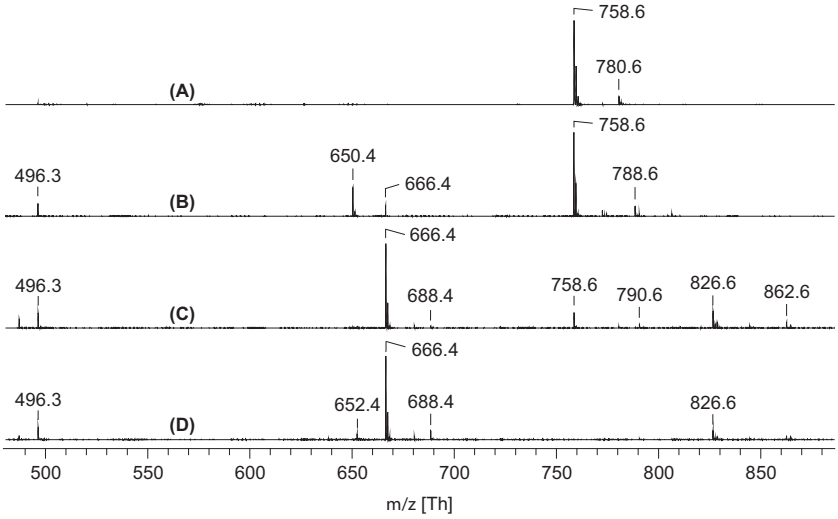

Fig. 2. Selected positive ion MALDI–TOF mass spectra of PLPC oxidized by the Fenton reagent ($FeCl_2$ and H_2O_2) in dependence on the incubation time. All spectra were recorded with 9-AA as matrix and all peaks are marked by the corresponding *m/z* values. In (A) the spectrum of non-oxidized PLPC (control) is shown. PLPC was oxidized with $FeCl_2$ and H_2O_2 for 1 h (B), 2 h (C) and 16 h (D). All spectra are scaled in the way that the most intense peak possesses the same intensity. For details see text.

bis-chlorohydrin subsequent to HCl elimination (m/z = 826.6) are detectable. In contrast, shorter incubation times favor the generation of the corresponding aldehyde (m/z = 650.4). This is not surprising because the carboxylic acid results from the oxidation of the aldehyde.

The so far performed experiments were done on an analytical scale to optimize the experimental conditions to generate the desired products in maximum yields. The oxidation of PLPC by $FeCl_2$ and H_2O_2 for 16 h was consequently chosen to isolate the individual oxidation products on a larger scale by means of preparative TLC. TLC is a highly established method for lipid separation of even complicated mixtures (Fuchs et al., 2007b) and has the most significant advantage that it can be easily established in all laboratories because no sophisticated and/or expensive equipment is necessary. It is also obvious from the chromatogram (Fig. 3, left) that a one-dimensional TLC run is already sufficient to separate the majority of the different oxidation products of PLPC: even very similar compounds (e.g., the aldehyde and the carboxylic acid) can be separated and subsequently isolated at a preparative scale. Of course, normal phase TLC is normally more useful to separate different PL classes, while reversed phase (RP) TLC (for instance C18 modified silica) is the method of choice to separate lipids with different fatty acyl residues. Unfortunately, RP TLC cannot be used at the so far established conditions: using the dye primuline the entire RP TLC plate is stained since the primuline binds to the apolar (C18) residues (data not shown). This is the reason why we have used exclusively non-modified silica.

The positive ion MALDI–TOF mass spectra of the identified six TLC spots (subsequent to re-elution of the oxidized PLs from the silica gel) are shown at the right hand of Fig. 3. Since the intensity of the LPC on the TLC is very low, this part of the TLC plate is not shown. The most apolar compound in spot (3A) possesses only low intensity and can be unequivocally assigned to non-oxidized PLPC with m/z = 758.6 and 780.6. The second spot (2B) is much more intense and corresponds to a mixture of different compounds that could not be resolved by 1D TLC: beside the peroxide (m/z = 790.6) there is also a significant contribution of the bis-chlorohydrin (m/z = 862.6) and an additional product corresponding to the loss of HCl (m/z = 826.6) from the chlorohydrin. The last detectable product at m/z = 804.6 is tentatively assigned to the addition of two O_2 molecules to PLPC followed by the loss of water. Due to the very low concentration of this compound, we unfortunately failed to record reasonable MS/MS spectra (even if an electrospray ionization (ESI) MS with an ion trap mass analyzer is used) which would make peak assignments unambiguous. However, we did not pay very much attention to this problem since fraction (3B) represents a mixture and our aim was to obtain chromatographically pure compounds.

Due to the identical lengths of their acyl residues it is not surprising that these oxidation products (3B) cannot be differentiated by one-dimensional (normal phase) TLC. In contrast, chain-shortened oxidation products can be easily differentiated: in spot (3C) the aldehyde and in (3E) the corresponding carboxylic acid can be easily identified although the molecular structure of the compound with m/z = 814.6 (3C) remains to be elucidated. We do also not yet have a convincing explanation of the peaks at m/z = 638.4 and 660.4 detected in spot (3D) although it seems likely that this compound has a very similar chemical structure as the compound observed in trace (3C). It is well known that the oxidation of polyunsaturated lipids may lead to the shift of a double bond which would lead to a mass difference of 14 u in comparison to the so far discussed products. For instance, Reis et al. (2005) detected upon oxidation of PLPC in addition to the aldehyde at m/z = 650.4 an additional product at m/z = 636.4 which was assigned to the chain-shortened aldehyde 1-palmitoyl-2-(8-oxo-octanoic acid)-*sn*-phosphatidylcholine (one CH_2 group results in a mass difference of 14 u). However, the mass difference observed here is only 12 u, not 14 u. A mass difference of 2 u might be explained by the reduction of the aldehyde to the corresponding alcohol. Of course, a reduction is not very likely because the reaction was performed under strongly oxidizing conditions. Nevertheless, a disproportionation of the aldehyde into the carboxylic acid and the alcohol cannot be completely ruled out. As the quality of the achieved MS/MS spectra was rather poor and did not allow a clear assignment of the residue in *sn*-2 position (data not shown), we have chosen an alternative way to confirm the molecular structure of the product detected at m/z 638.4 and 660.4.

The enzyme phospholipase A_2 is known to cleave exclusively the fatty acyl residue in the *sn*-2 position of a given PL under generation of the corresponding LPC and the related free fatty acid. If the alcohol (8-hydroxyoctanoic acid) instead of linoleic acid would be released, this should result in an altered polarity. Since 8-hydroxyoctanoic acid is commercially available from Aldrich, this compound can be used to compare the related TLC migration properties and, thus, to verify the potential presence of 8-hydroxyoctanoic acid. The corresponding developed TLC plate is shown in Fig. 4. In lane (4C) the result of a partial digestion of PLPC is shown as control. It is obvious that there are – in addition to PLPC – only two products: LPC 16:0 and free linoleic acid. Unfortunately, the digestion of the compounds present in the fraction which results in the MS peaks at m/z 638.4 and

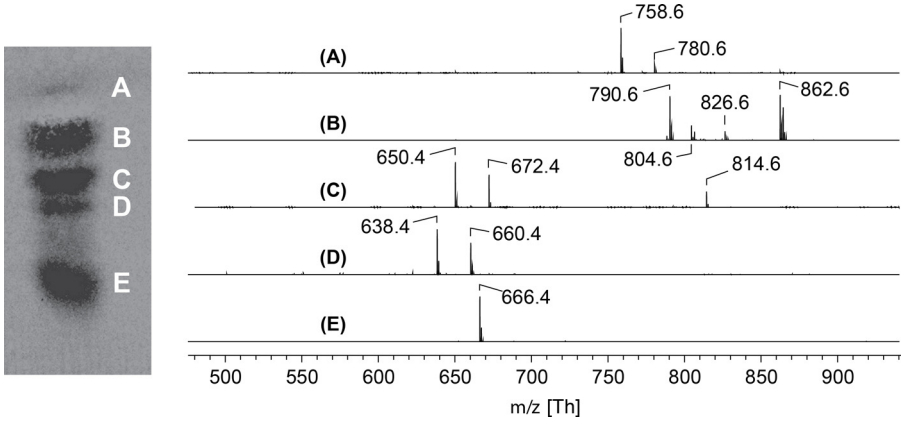

Fig. 3. Left: image of a typical HPTLC plate used to separate the oxidation products of PLPC after incubation with the Fenton reagents (cf. Section 2) for 16 h. Staining was performed by using the non-destructive dye primuline. Right: positive ion MALDI–TOF mass spectra recorded to confirm the identities of the lipids detectable in the individual TLC fractions. Peak assignments are available in Table 1.

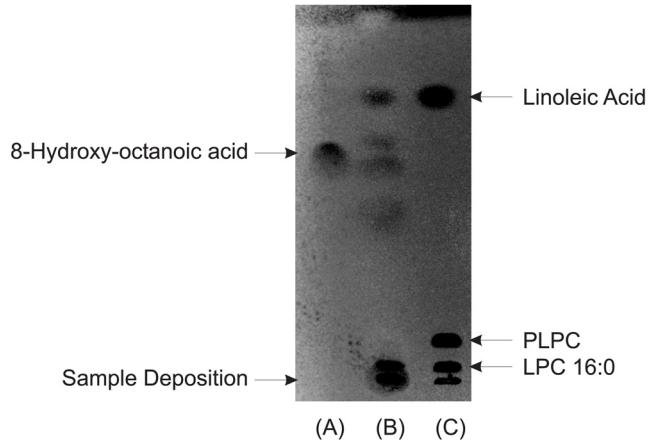

Fig. 4. TLC chromatogram to verify the generation of 1-palmitoyl-2-(8-hydroxyoctanoic acid)-*sn*-phosphatidylcholine as oxidation product of PLPC. In lane (C) the products obtained by PLA_2 digestion of PLPC (LPC and free linoleic acid) are shown as control, while the digestion products of putative 1-palmitoyl-2-(8-hydroxyoctanoic acid)-*sn*-phosphatidylcholine are shown in (B). The aqueous reaction mixture was directly used in this case to avoid potential lipid losses upon the extraction. This might be the reason why there are some pale spots that could not be identified; these are presumably polar compounds that would be lost upon extraction. Finally, isolated (commercially available) 8-hydroxyoctanoic acid is shown in lane (A).

660.4 provides less convincing results (lane (4B)). This is caused by two reasons: on the one hand, the concentration of this compound is much smaller and, thus, the obtained bands of the chromatogram are comparably pale. On the other hand, an aqueous sample without previous extraction was used in lane (4B). This was done to minimize the potential loss of 8-hydroxyoctanoic acid by the extraction with organic solvents. The use of the aqueous layer may be one reason why there are several spots (presumably corresponding to water-soluble, i.e., polar compounds) detectable which are normally not visible if the organic extracts are investigated. Nevertheless, there is a weak spot which nicely corresponds to the migration properties of the pure 8-hydroxyoctanoic acid that was applied as reference in lane (4A). These results are in agreement with the generation of 1-palmitoyl-2-(8-hydroxyoctanoic acid)-*sn*-phosphatidylcholine upon the oxidation of PLPC.

It is remarkable that 1-palmitoyl-2-(8-hydroxyoctanoic acid)-*sn*-phosphatidylcholine is only detectable when the PLPC oxidation is scaled up but is not detectable if smaller amounts of PLPC oxidized. It should also be noted that the tendencies of Na^+ adduct generation are significantly different: while the products detected in traces (3C) and (3D) have similar affinities to H^+ and Na^+, all other fractions are dominated by the H^+ adducts. Although this is an interesting aspect from the mass spectrometric viewpoint (all fractions should have similar alkali metal ion concentrations since they were re-extracted from the same TLC plate), a more detailed evaluation of the related mechanisms was beyond the scope of this paper. It should be noted that this oxidation is also accompanied by LPC generation. However, the LPC spot (as well as the corresponding mass spectrum) is not shown in order to emphasize the other spots. Thus, the compositions of the obtained fractions agree with the data illustrated in Figs. 1 and 2.

Although MALDI MS is a convenient method to qualitatively detect small amounts of lipids, it is not the method of choice to obtain quantitative data. This particularly applies because the different oxidation products may result in different ion yields making the use of internal standards (which are not readily available so far) mandatory. Therefore, the photometric approach according to Stewart (that has also been successfully applied by Teuber et al. (2010)) was used to determine the quantitative amounts of the individual oxidation products. As the outcome of this assay is primarily determined by the headgroup of the PL of interest (Stewart, 1980) and the phosphorylcholine headgroup is not altered upon the used oxidation conditions, quantitative data can be easily obtained. These data (which were obtained subsequent to re-elution of the identified lipid spots from the TLC plate (cf. Fig. 3)) are summarized in Table 2.

Table 2
Quantitative determination of the oxidation products of PLPC by means of the Stewart assay. Because of the very low concentration of LPC, this compound cannot be determined by the Stewart assay and is, thus, not listed in this table. Please note that it was not possible to absolutely differentiate spots (c) and (d). Therefore, both fractions were combined. For details see text.

Observed spot	Absorption	Amount (μg)	Yield (%)
(A)	0.27	265	4.2
(B)	0.93	916	15.2
(C) and (D)	0.57	560	9.3
(E)	0.99	973	16.2
Total		2714	44.9

Although clear yield differences of the individual oxidation products are obvious, it is also evident that there are significant lipid losses, i.e., a certain portion of the lipid sticks tightly to the silica gel and can be hardly extracted – even if a considerable excess of solvents is used. However, this lipid loss only slightly depends on the lipid structure (Teuber et al. (2010)): while there is a significant loss of the absolute lipid amount, the relative moieties of the individual PLPC oxidations products are presumably not significantly altered. Using preparative TLC about 45% of the originally applied PL can be recovered. A more detailed evaluation whether the most pronounced lipid loss occurs during the extraction from the aqueous phase (i.e., prior to the TLC) or as the result of the TLC separation and the subsequent re-elution was beyond the scope of this paper. Nevertheless, according to the data shown in Table 2, the carboxylic acid and the aldehyde are the by far most abundant products and can be isolated in amounts close to 1 mg. Therefore, it is concluded that the suggested method is simple and convenient and allows even non-experts to prepare oxidized, chain-shortened lipids when they are required in the laboratory. The simple approach described in this work might be particularly useful for cell biologists who would like to treat dedicated cell lines with these lipid oxidation products.

However, it must be admitted that the lipid fractions (with the exception of the aldehyde and the corresponding carboxylic acid) isolated by our TLC approach are not pure. There are basically three different approaches how this problem could be overcome: (a) by using HPLC (b) applying reversed phase TLC and (c) by performing two-dimensional TLC. We did not want to use HPLC because the method should be as simple as possible and suitable HPLC protocols are presumably not available in all laboratories. As already discussed (vide supra) reversed phase TLC cannot be combined with primuline staining and, thus, major modifications of the workflow would be necessary. Therefore, we relied on (c) and used 2D TLC whereby the very best results were obtained when (commercially available) diol-modified silica was used – although even these data are not very convincing. The separation of Fenton-oxidized PLPC by 2D TLC is shown at the left of Fig. 5 and the corresponding positive ion MALDI–TOF mass spectra are shown at the right. It is obvious that exclusively spots (B) and (C) represent chromatographically pure compounds while all other spots (particularly (A)) represent still mixtures (cf. Table 1 for detailed peak assignments). This is of course disappointing although we tried different stationary phases as well as solvent systems. Therefore, it must be concluded that the so far performed

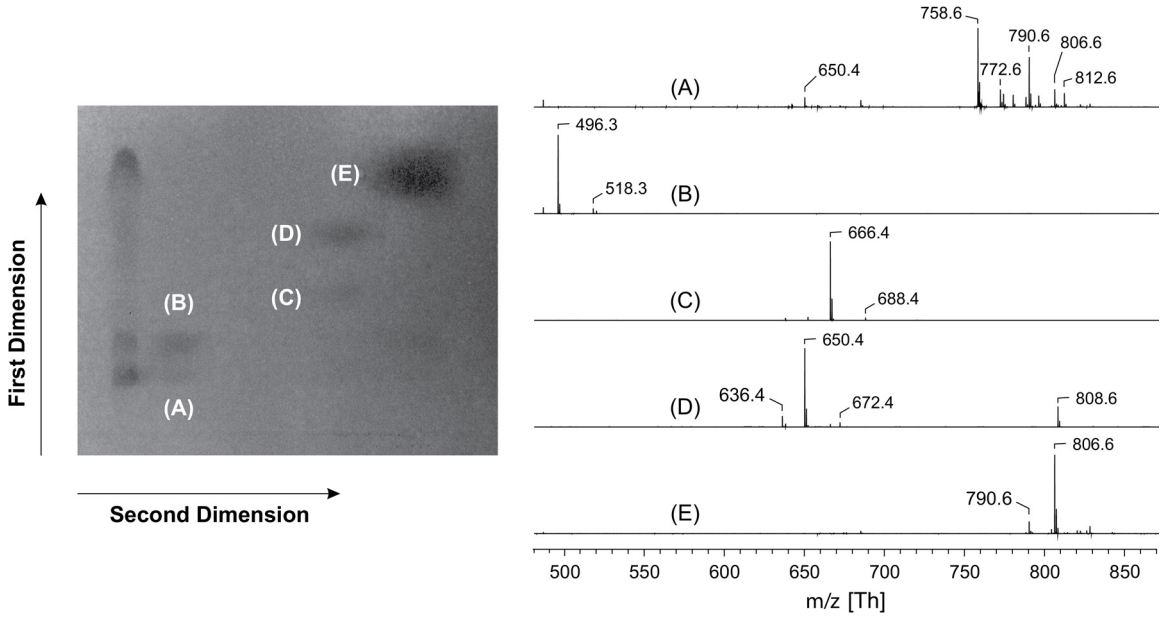

Fig. 5. Left: two-dimensional HPTLC separation of the oxidation products of PLPC after incubation with the Fenton reagents (cf. Section 2) for 16 h. Diol-modified silica was used as stationary phase. Chloroform, methanol, water, ethanol, triethylamine (6.5/2/1/3.5/3.5, v/v/v/v/v) was used in the first dimension and chloroform, methanol, acetic acid, water (90/40/12/2, v/v/v/v) in the second dimension. Right: positive ion MALDI–TOF mass spectra recorded from the positions indicated in the TLC image. Peak assignments are available in Table 1.

TLC separations are only useful to isolate chain-shortened products. Nevertheless, we are confident that even this problem can be overcome in the future.

Conflict of interest

The authors declare that there is no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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