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Analysis of oxidized phosphatidylcholines as markers for oxidative stress, using multiple reaction monitoring with theoretically expanded data sets with reversed-phase liquid chromatography/tandem mass spectrometry☆

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ABSTRACT

Recently, it was reported that oxidized phosphatidylcholine shows biological activities via scavenger receptor CD36 or Toll-like receptor 4 (TLR4)-TRIF. Thus, the analysis of oxidized phospholipids is essential in understanding these biological roles. Here, we report an analytical method for oxidized phosphatidylcholines using multiple reaction monitoring (MRM) with theoretically expanded data sets. This analytical method was performed by a quadrupole linear ion trap mass spectrometer with ultra performance LC (UPLC). To investigate whether this established analytical method was applicable to biological samples, we performed variation analysis of oxidized PCs using a myocardial ischemia-reperfusion model. Most oxidized PCs were detected in higher amounts in the ischemic myocardium than in the non-ischemic myocardium. From these application results, this established method is a valuable tool for the global analysis of oxidized PCs. In the future, our study can provide further understanding of how oxidized phospholipids are produced and are correlated to various diseases.

1. Introduction

Polyunsaturated fatty acids (PUFAs) such as linoleic acid (LA, 18:2), arachidonic acid (AA, 20:4) and docosahexaenoic acid (DHA, 22:6) are easily oxidized under oxidative stress. Generally, PUFAs are esterified to glycerophospholipids that make up biological membranes. Oxidized phospholipids (PLs) are produced by enzymatic and non-enzymatic reactions, which are initiated by peroxidation of PUFAs by oxidizing enzymes (e.g., lipoxygenase) and free radicals, respectively [1,2]. In addition, oxidized PLs can exist as many different derivatives and isomers *in vivo*. It is known that oxidized PLs and their decomposition products are closely correlated to atherosclerosis, cancer, and various neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [3,4]. Conventionally, analyses of oxidized PLs have been performed using various methods, such as high performance liquid chromatography (HPLC) [5,6], the analysis of thiobarbituric acid-reactive substance (TBARS) [7–9] and fluorescence analysis [10,11]. However, the precise molecular species of oxidized PLs cannot be determined by these methods. Therefore, it is possible that important biological implications have been overlooked by these methods.

Recently, mass spectrometry has been applied widely to the analysis of PLs [12–16] and oxidized PLs [17–22]. Liquid chromatography electrospray ionization mass spectrometry (LC–ESIMS) is an effective technique for lipidomics. We previously described analytical methods using normal-phase (NP) and reversed-phase (RP) LC–MS for PLs [23–25]. Analytical methods using precursor ion scanning or neutral loss scanning have also been reportedly very useful [26,27]. Data based on detection of specific fragment ions or of neutral losses, such as fatty acyl moieties and the phosphoryl base obtained from glycerophospholipids, can be very effective in identifying individual molecular species [28–34]. In this study, we report analytical methods for oxidized phosphatidylcholines (PCs) using RPLC–ESIMS/MS.

Phosphatidylcholine is a major component of cellular membranes, and an important constituent of pulmonary surfactant and serum lipoproteins *in vivo*. Low-density lipoprotein (LDL) is known as a transporter of cholesterol in plasma. Oxidized LDL is thought to play an important role in atherogenesis and cardiovascular disease in humans [35–37]. Recently, a novel family of oxidized PCs was

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identified as highly specific ligands for scavenger receptor CD36 [38–40]. It was reported that oxidized PC_{CD36} accumulate *in vivo* and mediate macrophage foam cell formation as well as promote platelet hyper-reactivity in hyperlipidemia via CD36. Furthermore, oxidized PCs were identified as induce lung injury and cytokine production by lung macrophages via Toll-like receptor 4 (TLR4)-TRIF [41]. Thus, the analysis of oxidized PCs is very important to not only understand these physiological and pathological phenomena but also exploration of novel bioactive substances for candidate biomarkers of various inflammatory diseases and common diseases.

In the current study, we present an analytical method for oxidized PC using multiple reaction monitoring (MRM) with RPLC/tandem mass spectrometry. MRM analysis is usually used for quantification of already-known molecules. However, theoretical values for the characteristic fragment ions from fragmentation patterns of structural analogs can be used in the absence of standard compounds. In this experiment, we applied MRM with theoretically expanded data sets (MRM with expansion), which is used for analysis of many unknown molecules predicted from a theoretically constructed database, to the specific and global analyses of oxidized PCs. In addition, we used ultra performance LC (UPLC) as the separation system, which is essentially a high resolution HPLC system using a high-pressure pump and very small particle silica gel. Highly selective and sensitive analysis can be achieved by this measurement system for oxidized PCs. Our study provides further understanding of how oxidized phospholipids are produced and are correlated to various diseases.

2. Experimental

2.1. Materials

1,2-diacyl-sn-glycero-3-phosphocholine (PC) standards such as 16:0/18:2 PC (PLPC), 16:0/20:4 PC (PAPC) and 16:0/22:6 PC (PDPC) and oxidized PC standards such as 16:0/9COOH PC (PAzPC), 16:0/5CHO PC (POVPC) and 16:0/5COOH PC (PGPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The 16:0/9CHO PC (PONPC) was obtained from Cayman Chemical (Arbor, MI, USA). The 16:0/4CHO PC standard was kindly provided by Mr. S. Souda (Alfresa Pharma Corp., Osaka, Japan). Soybean 15-lipoxygenase, sodium borohydride and deoxycholic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). All solvents were HPLC or LC–MS grade and the other chemical reagents were analytical grade (Wako Pure Chemicals, Osaka, Japan). Ultrapure water was obtained from a Milli-Q water system (Millipore, Milford, MA, USA).

2.2. Preparation of oxidized phosphatidylcholine by 15-lipoxygenase

PCs such as PLPC, PAPC and PDPC were oxidized by soybean 15-lipoxygenase. PCs were solubilized in 150 μL of 0.2 M borate buffer (pH 9.0) containing 10 mM deoxycholate and reacted with 7.5 μg (131000 units/mg) soybean 15-lipoxygenase at room temperature for 30 min. The oxidation reaction was monitored by its absorbance at 234 nm. PC hydroxide was prepared by the reduction reaction of PC hydroperoxide using $NaBH_4$ for 10 min on ice. The phosphatidylcholine hydroxide was extracted using solid-phase extraction (SPE) [42,43] (see Section 2.4).

2.3. Myocardial ischemia–reperfusion model

We made up the myocardial ischemia-reperfusion mouse as an oxidative stress model. Three-month-old male C57BL/6J mice were obtained from CLEA Japan, Inc. (Fuji, Shizuoka, Japan). Regional myocardial ischemia was induced by transient occlusion of the left anterior descending coronary artery. After 30 min of occlusion and a subsequent 6-h period of reperfusion, the left ventricle of the heart was removed and quickly frozen in liquid nitrogen. The oxidized PC was extracted using SPE (see Section 2.4).

2.4. Sample preparation for LC–MS

The oxidized phospholipids were extracted using SPE. Briefly, the hearts of mice were homogenized with 1 mL methanol including an internal standard (17:0-lysoPC) using a glass homogenizer and extracted after stand for 1 h at on ice. The samples were centrifuged (7000 rpm, 4 °C, 5 min) to remove cellular and protein materials then the supernatant was diluted with 10 volumes of water then adjusted to pH 3.0 with 0.1 N HCl. The samples were applied to preconditioned (20 mL of methanol and 20 mL of water) C18 Sep-Pak cartridges (500 mg, Waters, Millford, MA, USA), and washed with 20 mL of water to exclude nonvolatile ions followed by 10 mL of hexane to exclude cholesterols and neutral lipids. The samples were eluted with 10 mL of methanol to obtain oxidized PLs. The lipid extracts were dried under a gentle stream of nitrogen, dissolved in 1 mL of methanol, and stored at −80 °C until use.

2.5. LC–ESIMS/MS system

The LC–ESIMS/MS analysis was performed by using 4000 Q-TRAP® quadrupole linear ion trap hybrid mass spectrometer (Applied Biosystem/MDS Sciex, Concord, ON, Canada) with an ACQUITY Ultra Performance LC® (Waters). The sample was subjected to LC–ESIMS/MS analysis by using the ACQUITY UPLC™ BEH C18 (0.17 μm, 150 mm × 1.0 mm) column. Sample (10 μL) was simply injected by the autosampler, and the oxidized PL fractions were separated by a step gradient with mobile phase A (acetonitrile:methanol:water = 2:2:1 v/v/v containing 0.1% formic acid and 0.028% ammonia):mobile phase B (isopropanol containing 0.1% formic acid and 0.028% ammonia) ratios of 100:0 (0–5 min), 50:50 (5–25 min), 50:50 (25–59 min), 100:0 (59–60 min), and 100:0 (60–75 min) at a flow rate of 70 μL/min and a column temperature of 30 °C. The specific detection was performed by MRM. Precursor

Table 1
MRM mode conditions.

Standard PCs	Q1[a] (*m*/*z*)	Q3[b] (*m*/*z*)	CE (eV)
16:0/4CHO	624	255	−50
		101	−50
16:0/5CHO	638	255	−50
		115	−40
16:0/5COOH	608	255	−45
		145	−30
16:0/9CHO	694	255	−50
		171	−50
16:0/9COOH	664	255	−45
		201	−40
16:0/18:2 +O	818	255	−60
		295	−55
		195	−70
16:0/20:4 +O	842	255	−60
		319	−50
		219	−55
16:0/22:6 +O	866	255	−60
		343	−50
		245	−50

[a] First quadrupole.
[b] Third quadrupole.

ion scanning or neutral loss scanning is useful to detect or find out expected metabolites within focused metabolites. We think that often the existence of specified molecules was assumed or detected by MRM more sensitively and accurately than other MS detection method. Oxidized PC is relatively low amounts in whole PC, thus we selected the method of MRM with expansion.

2.6. Flow injection experiment

The ESIMS analysis was performed using a 4000 Q-TRAP® with an UltiMate 3000 HPLC system (Dionex, Sunnyvale, CA, USA) combined with an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland). The sample (10 μL) was subjected to ESIMS analysis without LC separation by flow injection. The mobile phase composition was A:B = 1:1 v/v (as described in Section 2.5) at a flow rate of 10 μL/min.

2.7. MS conditions

MS/MS analysis was performed in negative electrospray ionization (ESI) mode with the following settings, ion spray voltage, −4500 V; curtain (nitrogen), 10 arbitrary units; and collision gas (nitrogen), "high". Specific detection was performed by MRM with the following settings, dwell times, 50 ms; declustering potential, −80 V, and resolutions of *Q*1 and *Q*3, "unit". Conditions for the detection in MRM mode are shown in Table 1.

The characteristic fragmentation patterns of individual oxidized PCs were determined by enhanced product ion scanning (EPI). These EPI experiments were performed as continuous, not data-dependent, measurements for obtaining mass chromatograms of characteristic product ions with the following settings, collision energies (CEs), 20–65 eV; scan range of the instrument, *m*/*z* 50–950; scan speed, 1000 Th/s; *Q*0 trapping, "on"; linear ion trap fill-time, 10 ms; declustering potential, −105 V; and resolution of *Q*1, "unit".

3. Results and discussion

3.1. MS/MS analysis of standard oxidized PCs

First, we investigated collision induced dissociation (CID) patterns of standard oxidized PCs, to determine the optimal fragmentation reactions for MRM analysis in terms of higher abundance

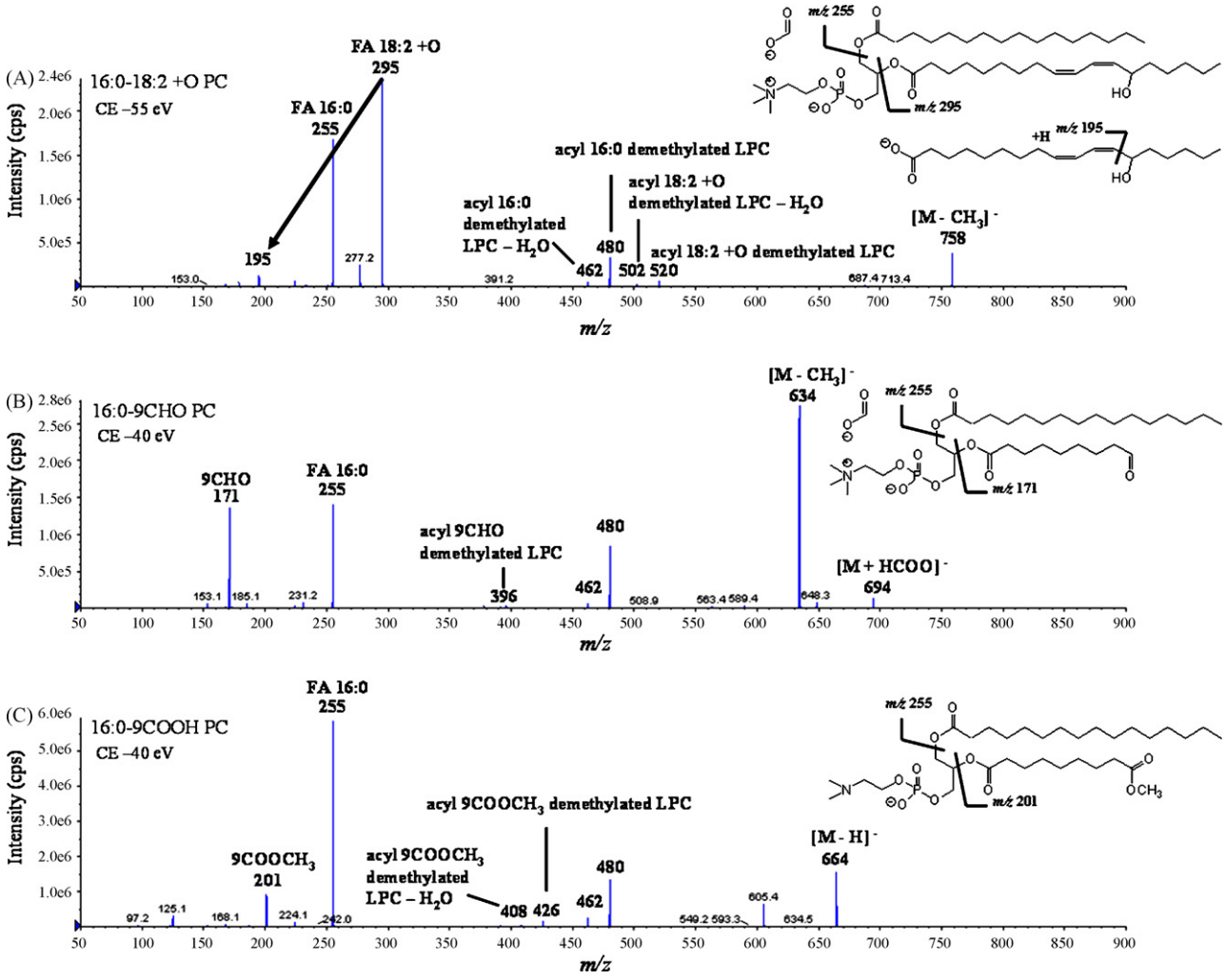

Fig. 1. CID spectra of the 16:0/18:2 oxidized PC series. 16:0/18:2 +O PC (*m*/*z* 818) and 16:0/9CHO PC (*m*/*z* 694) were detected as the formate adduct ions at $[M+HCOO]^-$ and 16:0/9COOH PC (*m*/*z* 664) was detected as a proton adduct ion at $[M-H]^-$. The formate adduct ions were easily dissociated and detectable as the $[M-CH_3]^-$ ions by MS/MS. The demethylated LPC-related ions were then detected as the fragment ions in two different forms, as the $[M-CH_3-RCH_2COOH]^-$ resulting from neutral losses of free FAs, and as the $[M-CH_3-RCH{=}C{=}O]^-$ resulting from losses of ketenes. 16:0/18:2 +O PC (A), 16:0/9CHO PC (B), and 16:0/9COOH PC (C) were detected as the *m*/*z* 255 (FA 16:0) at the *sn*-1 position as a common FA fragment ion and *m*/*z* 295 (FA 18:2 +O), 171 (9CHO), and 201 ($9COOCH_3$) at the *sn*-2 position as the specific FA fragment ions, respectively. The ion at *m*/*z* 295 yielded the product ion of *m*/*z* 195, which originates from the characteristic fragmentations of 13-HODE. cps, count per second. FA, fatty acid.

and stability of at least two diagnostic product ions. In negative ESI mode with a triple quadrupole instrument, the oxidized PCs were mainly detected as the $[M+HCOO]^-$ ions by using ammonium formate as an elution buffer. These formate adduct ions were easily dissociated and detectable as the $[M-CH_3]^-$ ions by MS/MS. In addition, the fatty acid (FA) ions produced from two fatty acyl groups by CID were detected as deprotonated $[FA-H]^-$ ions. However, the oxidized FAs of short-chain carbonic acid methyl derivatives were detected 14 Da larger than those of proton elimination. This result indicates that a methyl group of choline was transferred to a carboxyl group. Consequently, short-chain carbonic acid derivative PCs were detected as $[M-H]^-$ ions [19,44].

3.1.1. 16:0/18:2 oxidized phosphatidylcholine series

Fig. 1 shows the CID spectra of 16:0/18:2 +O PC (A) and 16:0/9CHO PC (B) obtained by collision of the corresponding $[M+HCOO]^-$ at *m/z* 818 and 694 and 16:0/9COOH PC (C) obtained by collision of the corresponding $[M-H]^-$ at *m/z* 664 (flow injection experiments). The demethylated-PC $[M-CH_3]^-$ ion was obtained by loss of (HCO_2+CH_3) from the precursor ion (16:0/18:2 +O PC $[M+HCOO]^-$) by product ion scanning. The demethylated LPC-related ions were detected as the fragment ions in two different forms, as the $[M-CH_3-RCH_2COOH]^-$ at *m/z* 462 (acyl 16:0 demethylated LPC–H_2O) and *m/z* 502 (acyl 18:2 +O demethylated LPC–H_2O) resulting from neutral losses of free FAs, and as the $[M-CH_3-RCH{=}C{=}O]^-$ at *m/z* 480 (acyl 16:0 demethylated LPC) and *m/z* 520 (acyl 18:2 +O demethylated LPC) resulting from losses of ketenes. The fatty acyl anions of 16:0 at the *sn*-1 and 18:2 +O at the *sn*-2 position were detected as $[FA-H]^-$ at *m/z* 255 and 295, respectively. Furthermore, *m/z* 195 was produced from the fragment ion of 18:2 +O (*m/z* 295) by increasing the CEs. The ion at *m/z* 195 was derived from the fragmentation of 13-hydroxyoctadecadienoic acid (13-HODE). The MS/MS spectra of 16:0/9CHO PC $[M+HCOO]^-$ were also detected as the demethylated-PC $[M-CH_3]^-$ ions at *m/z* 634 and demethylated LPC-related ions, which are *m/z* 480 and 462 at the *sn*-1 position, and *m/z* 396 (acyl 9CHO demethylated LPC) at the *sn*-2 position. In addition, each FA fragment ion was detected as the *m/z* 255 at *sn*-1 (16:0 FA) and *m/z* 171 at *sn*-2 (9CHO), which is the fragment ion from 9-HODE. The 16:0/9COOH PC (*m/z* 664) was detected as a proton adduct ion at $[M-H]^-$ because the methyl group of choline

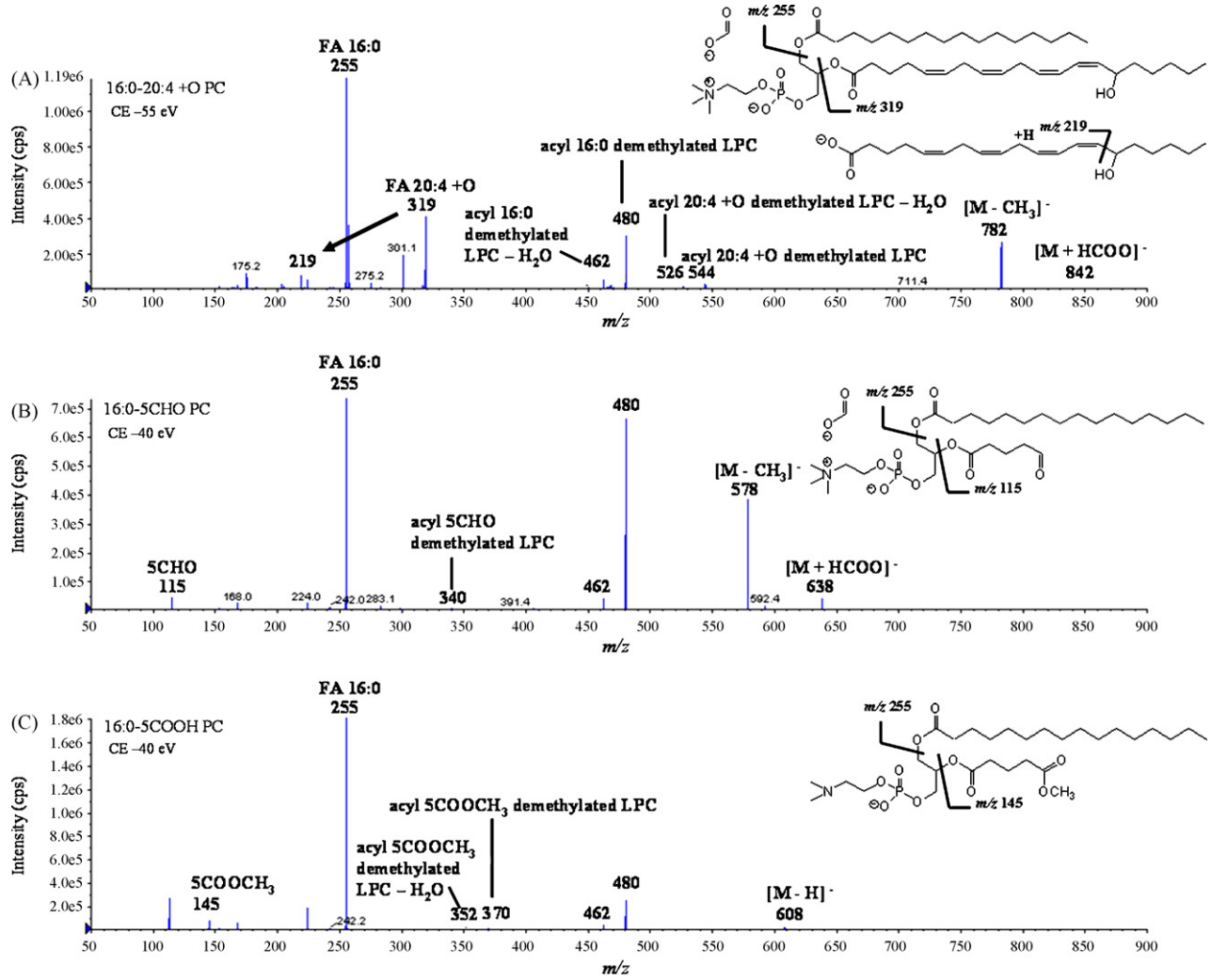

Fig. 2. CID spectra of the 16:0/20:4 oxidized PC series. MS/MS fragments of 16:0/20:4 +O PC (*m/z* 842), 16:0/5CHO PC (*m/z* 638) and 16:0/5COOH PC (*m/z* 608) are shown in A, B and C, respectively. 16:0/20:4 +O PC (A), 16:0/5CHO PC (B), and 16:0/5COOH PC (C) were detected as the *m/z* 255 (FA 16:0) at the *sn*-1 position as a common FA fragment ion and *m/z* 319 (FA 20:4 +O), 115 (5CHO) and 145 ($5COOCH_3$) at the *sn*-2 position as the specific FA fragment ions, respectively. The ion at *m/z* 319 yielded the product ion of *m/z* 219, which originates from the characteristic fragmentations of 15-HETE. cps, count per second. FA, fatty acid.

was transferred to a carboxyl group. As a result, precursor ions formed the analogs of demethylated-PC $[M-CH_3]^-$ ions. Therefore, their fragment ions obtained by MS/MS analysis were detected as demethylated LPC-related ions, which are *m/z* 480 and 462 at the *sn*-1 position, and *m/z* 426 (acyl 9COOCH$_3$ demethylated LPC) and *m/z* 408 (acyl 9COOCH$_3$ demethylated LPC–H$_2$O) at the *sn*-2 position, *m/z* 255 at *sn*-1 (16:0 FA) and *m/z* 201 at *sn*-2 (9COOCH$_3$).

3.1.2. 16:0/20:4 oxidized phosphatidylcholine series

Next, we performed analyses of the 16:0/20:4 oxidized PC series. Similar to the 16:0/18:2 oxidized PC series, 16:0/20:4 +O PC (*m/z* 842) and 16:0/5CHO PC (*m/z* 638) were detected as the formate adduct ions at $[M+HCOO]^-$ and 16:0/5COOH PC (*m/z* 608) was detected as a proton adduct ion at $[M-H]^-$. Typical MS/MS fragmentation patterns of 16:0/20:4 oxidized PCs series are shown in Fig. 2. Molecular species such as 16:0/20:4 +O PC (A), 16:0/5CHO PC (B), and 16:0/5COOH PC (C) were detected as *m/z* 255 (16:0 FA) at the *sn*-1 position as a common FA fragment ion and *m/z* 319 (20:4 +O), 115 (5CHO), and 145 (5COOCH$_3$) at the *sn*-2 position as the specific FA fragment ions, respectively. The ion at *m/z* 319 (20:4 +O) yielded the product ion of *m/z* 219, which originates from the specific fragmentations of 15-hydroxyeicosatetraenoic acid (15-HETE), by different CEs.

3.1.3. 16:0/22:6 oxidized phosphatidylcholine series

Finally, we performed analyses of the 16:0/22:6 oxidized PC series. Molecular species such as 16:0/22:6 +O PC (*m/z* 866) and 16:0/4CHO PC (*m/z* 624) were detected as the formate adduct ions at $[M+HCOO]^-$ (Fig. 3). In product ion scanning, they were easily dissociated as the *m/z* 806 and 564 $[M-CH_3]^-$ ions, respectively. The demethylated LPC-related ions were also detected in the 16:0/22:6 oxidized PC series as well as other oxidized PC series. In addition, *m/z* 343 (22:6 +O) and 101 (4CHO) were obtained from 16:0/22:6 +O PC and 16:0/4CHO PC, respectively. These fragments were the characteristic FA fragment ions of the *sn*-2 position. Furthermore, *m/z* 343 yielded the product ion of *m/z* 245 by increasing the CEs. The ion at *m/z* 245 is derived from the fragmentation of 17-hydroxydocosahexaenoic acid (17-HDoHE) (Fig. 3B).

In this experiment, the 16:0/PUFA +O PCs were prepared by soybean 15-lipoxygenase. Hence, each FA fragment ion of 18:2 +O, 20:4 +O, and 22:6 +O was detected as its characteristic fragment ion at *m/z* 195, 219, and 245, respectively, derived from 13-HODE (18:2 +O), 15-HETE (20:4 +O), and 17-HDoHE (22:6 +O). In other words,

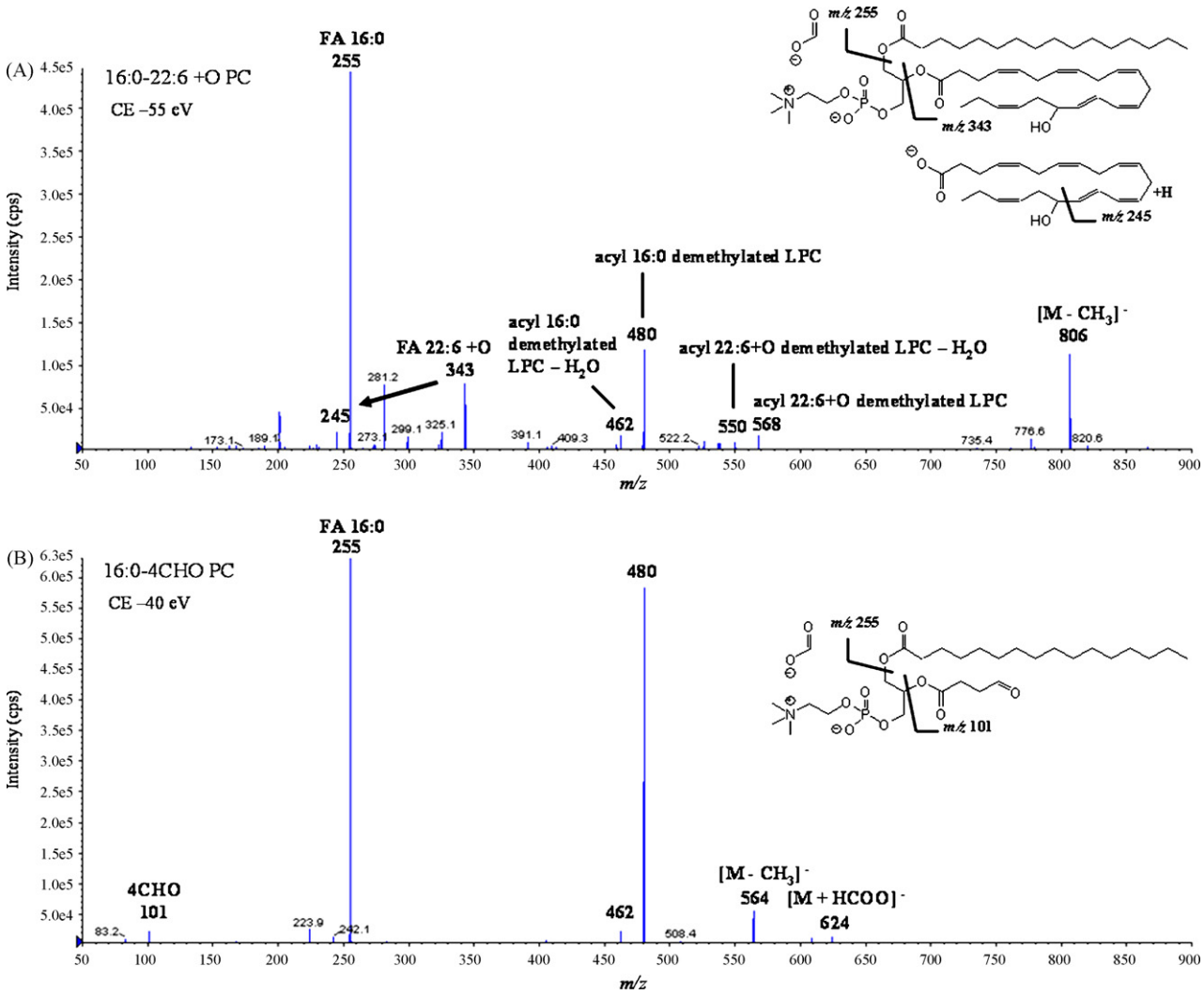

Fig. 3. CID spectra of the 16:0/22:6 oxidized PC series. MS/MS fragments of 16:0/22:6 +O PC (*m/z* 866) and 16:0/4CHO PC (*m/z* 624) are shown in (A) and (B), respectively. 16:0/22:6 +O PC (A) and 16:0/4CHO PC (B) were detected as the *m/z* 255 (FA 16:0) at the *sn*-1 position as a common FA fragment ion and *m/z* 343 (FA 22:6 +O) and 101 (4CHO) at the *sn*-2 position as the specific FA fragment ions, respectively. The ion at *m/z* 343 yielded the product ion of *m/z* 245, which originates from the characteristic fragmentations of 17-HDoHE. cps, count per second. FA, fatty acid.

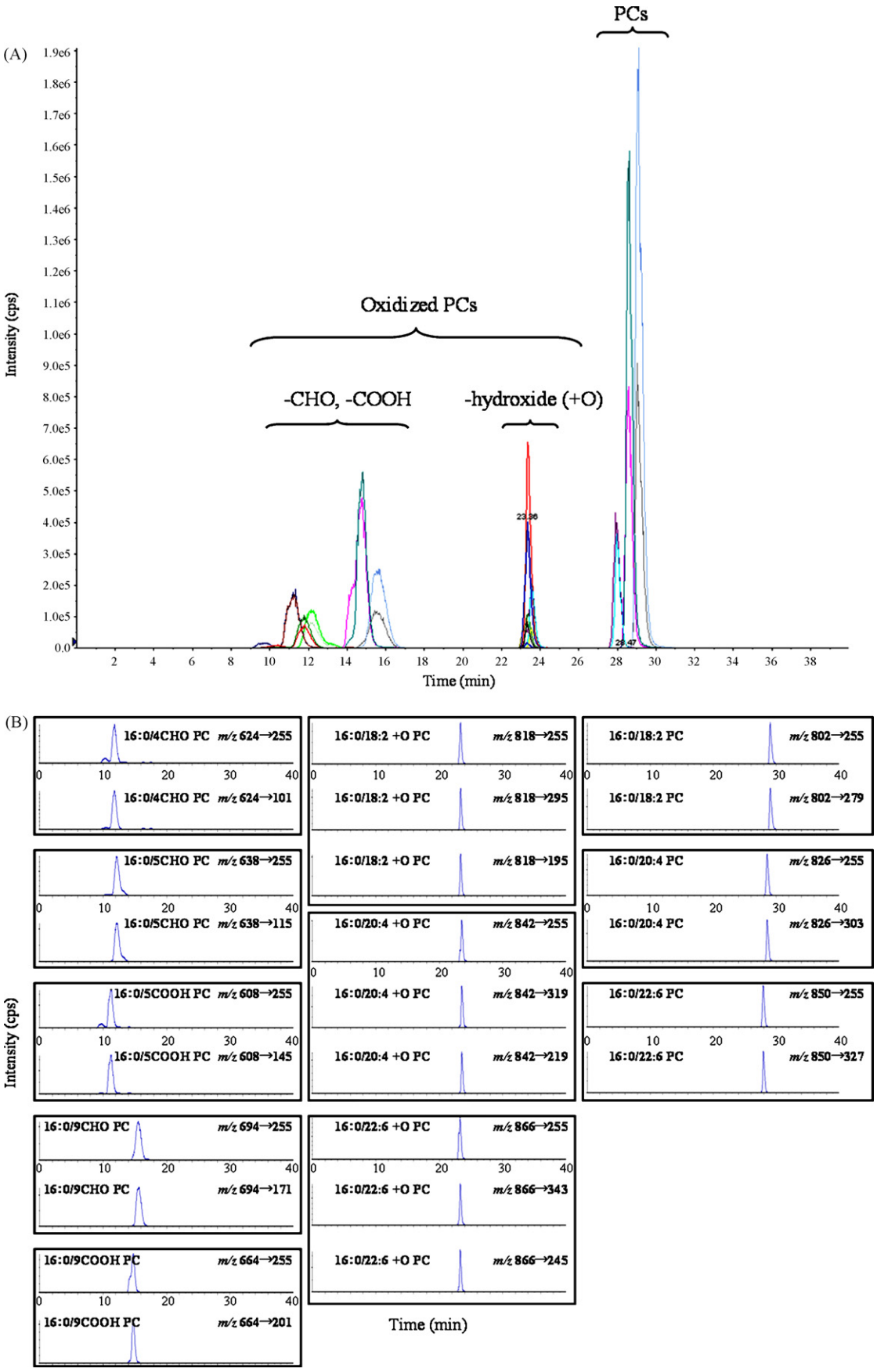
(A)
PCs
Oxidized PCs
-CHO, -COOH
-hydroxide (+O)
Intensity (cps)
Time (min)
(B)
16:0/4CHO PC m/z 624→255
16:0/4CHO PC m/z 624→101
16:0/5CHO PC m/z 638→255
16:0/5CHO PC m/z 638→115
16:0/5COOH PC m/z 608→255
16:0/5COOH PC m/z 608→145
16:0/9CHO PC m/z 694→255
16:0/9CHO PC m/z 694→171
16:0/9COOH PC m/z 664→255
16:0/9COOH PC m/z 664→201
16:0/18:2 +O PC m/z 818→255
16:0/18:2 +O PC m/z 818→295
16:0/18:2 +O PC m/z 818→195
16:0/20:4 +O PC m/z 842→255
16:0/20:4 +O PC m/z 842→319
16:0/20:4 +O PC m/z 842→219
16:0/22:6 +O PC m/z 866→255
16:0/22:6 +O PC m/z 866→343
16:0/22:6 +O PC m/z 866→245
16:0/18:2 PC m/z 802→255
16:0/18:2 PC m/z 802→279
16:0/20:4 PC m/z 826→255
16:0/20:4 PC m/z 826→303
16:0/22:6 PC m/z 850→255
16:0/22:6 PC m/z 850→327
Intensity (cps)
Time (min)

Table 2
MRM with expansion mode conditions.

sn-1 16:0				*sn*-1 18:0			
PCs	Q1[a] (*m*/*z*)	Q3[b] (*m*/*z*)	CE (eV)	PCs	Q1[a] (*m*/*z*)	Q3[b] (*m*/*z*)	CE (eV)
sn-2 18:2 series							
9CHO	694	255	−50	9CHO	722	283	−50
		171	−50			171	−50
9COOH	664	255	−45	9COOH	692	283	−45
		201	−40			201	−40
+O	818	255	−65	+O	846	283	−65
		295	−55			295	−55
(9OH)		171	−70	(9OH)		171	−70
(13OH)		195	−70	(13OH)		195	−70
+OO	834	255	−65	+OO	862	283	−65
		311	−45			311	−45
(9OOH)		185	−55	(9OOH)		185	−55
(13OOH)		195	−55	(13OOH)		195	−55
sn-2 20:4 series							
5CHO	638	255	−50	5CHO	666	283	−50
		115	−40			115	−40
5COOH	608	255	−45	5COOH	636	283	−45
		145	−30			145	−30
+O	842	255	−60	+O	870	283	−60
		319	−50			319	−50
(5OH)		115	−55	(5OH)		115	−55
(8OH)		155	−55	(8OH)		155	−55
(12OH)		179	−55	(12OH)		179	−55
(15OH)		219	−55	(15OH)		219	−55
+OO	858	255	−60	+OO	886	283	−60
		335	−45			335	−45
sn-2 22:6 series							
4CHO	624	255	−50	4CHO	652	283	−50
		101	−50			101	−50
4COOH	594	255	−45	4COOH	622	283	−45
		131	−40			131	−40
+O	866	255	−60	+O	894	283	−60
		343	−50			343	−50
(4OH)		101	−50	(4OH)		101	−50
(7OH)		141	−50	(7OH)		141	−50
(10OH)		153	−50	(10OH)		153	−50
(13OH)		193	−50	(13OH)		193	−50
(17OH)		245	−50	(17OH)		245	−50
(20OH)		187	−50	(20OH)		187	−50
+OO	882	255	−60	+OO	910	283	−60
		359	−40			359	−40

[a] First quadrupole.
[b] Third quadrupole.

the specific fragment ion is able to determine if the oxygenation position of carbon–carbon double bonds was predictable. Thus, the theoretical value of the characteristic fragment ion is applicable to MRM analysis.

3.2. Separation of normal and oxidized PCs by RPLC

3.2.1. Selectivity method

As oxidized PCs can exist as many different derivatives and isomers *in vivo*, identification of them is very difficult because of the detection of multiple peaks by RPLC. Moreover, accurate retention times of oxidized PCs are not known because there are few standard compounds. We used two or three different MRM pairs for identification of structural isomers of oxidized PCs. Each oxidized PC isomer was confirmed by the coincidence of the individual peak of the MRM to the detected retention time. In this experiment, normal and oxidized PCs were detected as several different pairs of precursor and product ions. Conditions for the detection of oxidized PCs by MS in MRM mode are summarized in Table 1. The normal and oxidized PCs were separated as –CHO, –COOH, –hydroxide

Fig. 4. MRM chromatograms of normal and oxidized PCs. The normal and oxidized PCs were separated into –CHO, –COOH types, –hydroxide (+O) type, and unoxidized type by RPLC (A). Typical chromatograms of normal and oxidized PCs are shown in (B). Identification of structural isomers of normal and oxidized PCs used two or three different MRM pairs. Each oxidized PC isomer was confirmed by the coincidence of the individual peak of the MRM to the detected retention time. The total chromatographic rum time was 40 min. cps, count per second.

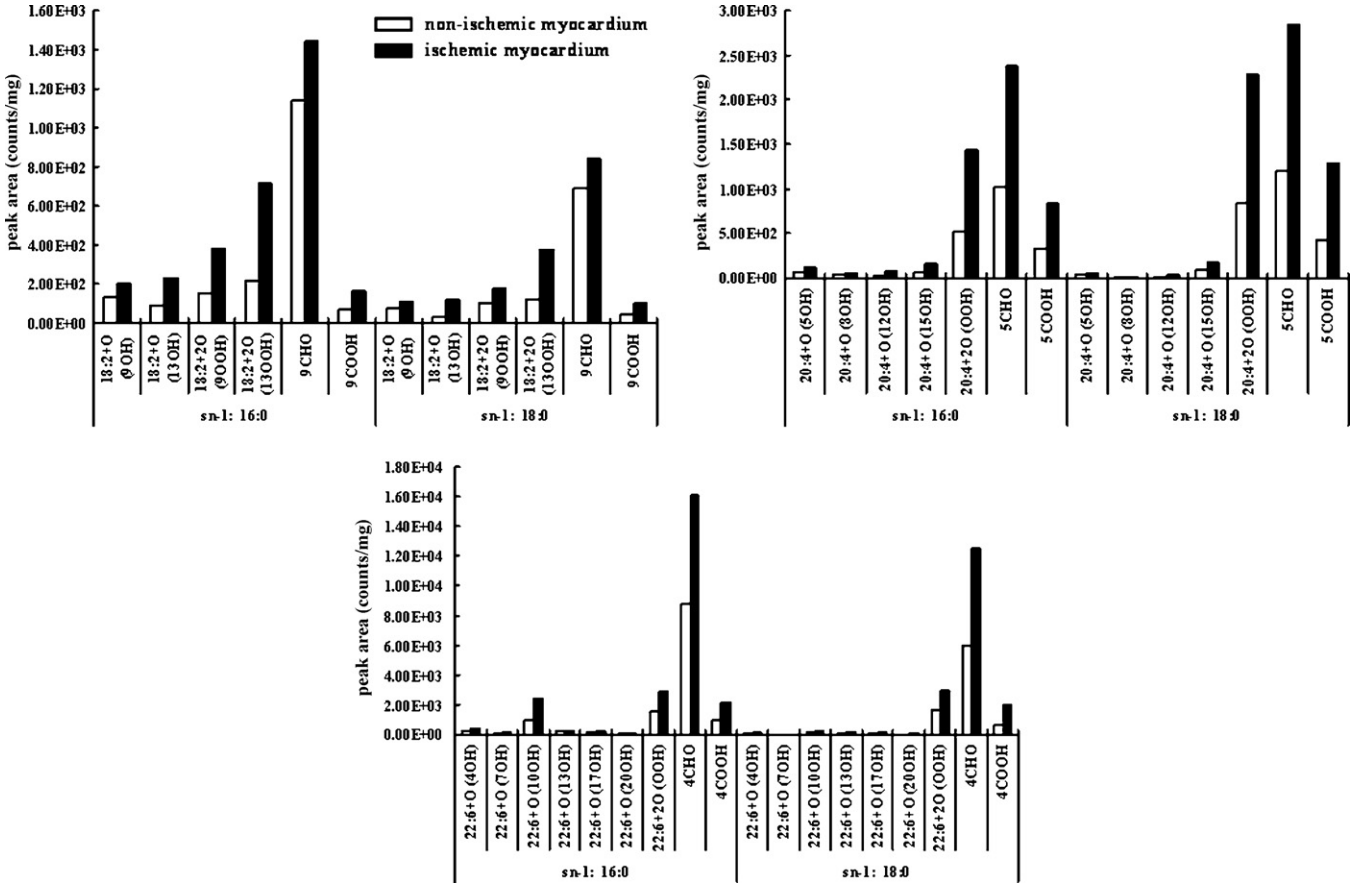

Fig. 5. Variation analysis of oxidized PCs using myocardial ischemia-reperfusion model mouse. The peak area is shown along the longitudinal axis, which was corrected using an internal standard (17:0-LPC) and the tissue weight. Most of the oxidized PCs tended to increase in the ischemic myocardium (*closed bars*) versus non-ischemic myocardium (*open bars*). A similar result was reproducibly obtained.

(+O), and unoxidized types by RPLC (Fig. 4A). Fig. 4B shows typical chromatograms of normal and oxidized PCs.

3.2.2. Sensitivity and linearity

The sensitivity and linearity were examined using the optimized conditions as described above. Quantitative calculation was possible in the linear range of 50–100000 fmol/10 μL. Typical regression lines were: 16:0/9CHO PC, $y = 204.34x + 54700$ ($r^2 = 0.9998$); 16:0/9COOH PC, $y = 278.90x + 82768$ ($r^2 = 0.9997$); 16:0/5CHO PC, $y = 96.02x + 39727$ ($r^2 = 0.9997$); 16:0/5COOH PC, $y = 127.56x + 8585$ ($r^2 = 0.9998$) (data not shown).

3.3. Application of biological samples

3.3.1. Structure of MRM with expansion method

We applied MRM with theoretically expanded data sets (MRM with expansion), which is used for analysis of many unknown molecules predicted from a theoretically constructed database, to the specific and global analyses of oxidized PCs *in vivo*. Effective characteristic fragment ions are predicted from the fragmentation patterns of standard oxidized PCs (Figs. 1–3). Here, FAs that exist in the *sn*-1/2 positions of oxidized PC were confined to 16:0 and 18:0 at *sn*-1 and 18:2, 20:4 and 22:6 at *sn*-2. Furthermore, the oxidized formations at *sn*-2 PUFA, e.g., 18:2, 20:4 and 22:6, were confined to hydroxides (+O), hydroperoxides (+OO), aldehydes (–CHO), and carboxylic acids (–COOH). Conditions for the MRM with expansion mode are summarized in Table 2.

3.3.2. Variation analysis of oxidized PCs using myocardial ischemia–reperfusion model

To investigate whether this established analytical method was applicable to biological samples, we performed variation analysis of oxidized PCs using a myocardial ischemia-reperfusion model. The mouse heart was subjected to acute oxidative stress induced by ischemia-reperfusion injury. The analysis data is shown in Fig. 5. The calibration of data was performed using an internal standard (17:0-LPC) and the tissue weight. As indicated in our experiment results, oxidized PCs were already produced in non-ischemic myocardium because the heart is always subjected to oxidative stress. As a result, most oxidized PCs were detected in higher amounts in the ischemic myocardium than in the non-ischemic myocardium. In brief, in the 18:2- or 20:4-containing oxidized PC series, hydroxide, hydroperoxide and aldehyde forms were detected at significant levels. On the other hand, 22:6-containing oxidized PC series consisted mainly of aldehyde forms than hydroxide or hydroperoxide forms. The aldehyde forms seemed to be significantly higher in 22:6-derived oxidized PCs than for those in 18:2- or 20:4-derived oxidized PCs. DHA (22:6)-containing PLs are abundant in heart tissue. It is believed that DHA plays a critical role as the major target of radical reactions by reactive oxygen species (ROS), and their turnover might be involved in oxidative stress within the cell. These results suggest that DHA and their oxidized metabolites provide the protective barrier of the myocardium to oxidative stress [45,46].

From these application results on biological samples, it was established that MRM with expansion with LC–ESIMS/MS method

is a valuable tool for the global analysis of oxidized PCs. In the future, analyses of quantitative and qualitative variation of PUFA-oxidized PLs will be applied to the biomarkers for myocardial disease.

4. Conclusion

We established a global analysis method for oxidized PCs using MRM with theoretically expanded data sets with LC–ESIMS/MS. This analytical method was determined to be applicable to biological samples. It was found that oxidized PCs in the ischemic myocardium increased more than those in the non-ischemic myocardium. Among these oxidized PCs, the proportion of 22:6-derived aldehyde PCs was larger than those of other oxidized PCs. This suggests that 22:6-containing PLs provide the protective barrier of the myocardium to oxidative stress. Research on these biological mechanisms and roles are currently underway. In the future, it may be possible to discover novel bioactive substances or candidate biomarkers. Furthermore, our technique will be helpful in clarifying the physiological and pathological phenomena of various inflammatory diseases and common diseases.

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