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Improvements in the High-Performance Liquid Chromatography and Extraction Conditions for the Analysis of Oxidized Fatty Acids Using a Mixed-Mode Spin Column

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Abstract

Lipidomics by liquid chromatography/mass spectrometry has been used for a better understanding of the roles of oxidized fatty acids in the development of various diseases. However, further work is required to improve the sample preparation process and the peak tailing of cysteinyl-leukotrienes. In this study, we evaluated various mobile phases and extraction conditions. The addition of phosphoric acid to the mobile phase improved the peak tailing of cysteinyl-leukotrienes. The extraction conditions were also optimized by spin-column possessing an anion-exchange and reversed-phase properties. The extraction efficiency of the modified extraction system was examined using 62 lipids, and 13 deuterated lipids were investigated to evaluate matrix effects and recovery from mouse lung homogenate samples. Extraction efficiencies of ≥70% were obtained for almost all of the lipids. Good results with standard deviations of <15% were obtained for the matrix effects and recovery. Finally, the efficiency of our extraction method was compared with those of several conventional methods, and those of leukotriene C4 was improved significantly using our method. Moreover, the proportion of variance between our method and the conventional methods was >0.99 for all the lipids tested. This newly developed method therefore represents a powerful tool to analyze lipids.

Keywords: Lipid; LC–MS/MS; Spin column; Extraction; Matrix effect; Reversed-phase solid-phase extraction

Introduction

The beneficial effects of polyunsaturated fatty acids for the prevention of cardiovascular disease were first recognized in the late 1960s following a series of epidemiological studies. At that time, thin-layer chromatography was widely used for the measurement of lipids in biological samples [1,2]. Although this method is suitable for distinguishing between the different classes of lipid, it is not suitable for gathering data pertaining to the individual lipid species. Since the 1960s, specific radioimmunoassay [3,4] and gas chromatography/mass spectrometry [5-7] techniques have been developed for the detection of trace quantities of the metabolites of polyunsaturated fatty acids. Furthermore, data collected using these methods have provided researchers with a deeper understanding of the various biological responses elicited by the prostaglandins, thromboxanes and Leukotriene's (LTs) derived from numerous fatty acids, including Arachidonic Acid (AA), Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA). For example, the results of several studies have suggested that the resolvins produced by the reactions of the omega-3 fatty acids EPA and DHA can reduce cellular inflammation by inhibiting the production and transportation of inflammatory cells and chemicals at lesion sites [8-13]. Compared with eicosanoids, resolvins are stored in relatively small amounts in the human body, making them difficult to detect with conventional analytical techniques. Prior to the development of liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods for the study of lipids, it was therefore difficult to achieve the detection of resolvins in human samples. The results of our previous studies involving the LC-MS/MS analysis of resolvins and eicosanoids [10,14-17] revealed that the analysis of eicosanoids such as cysteinyl-leukotrienes (Cys-LTs) was severely complicated by carryover and peak tailing issues. These issues were attributed to the adsorption of the eicosanoids on to the metallic surfaces of the analytical system [18], and we believe this could be resolved by optimizing the LC conditions.

Reversed-Phase Solid-Phase Extraction (RP SPE) methods are widely used for the extraction, purification and enrichment of the oxidized fatty acids present in biological samples [18-26], and RP SPE cartridges were used in our previous studies for this purpose [10,14-17]. However, in terms of the analytical process, the combination of different separation modes, such as two-dimensional High-Performance Liquid Chromatography (HPLC), has been shown to be more effective for the purification of biological samples than the combination of the same separation modes. Therefore, we were concerned about the possibility of ion suppression effects resulting from the different types of lipid as we used the same separation modes for the lipid extraction and HPLC processes. Although anion-exchange SPE has been used for the preparation of a wide variety of analytical samples, it is not suitable for the extraction of polar lipids such as prostaglandins and thromboxanes [26]. With this in mind, reversed-phase/anion-exchange SPE (mixed-mode SPE) was evaluated in the current study for the extraction of polar lipids from biological samples. Although mixed-mode SPE has been reported as a novel extraction approach for fatty acid metabolites [27,28], this technique has only been applied to a limited number of lipids and a detailed method has not yet been established.

In this study, we evaluated the use of a mixed-mode SPE system for the extraction of lipids and compared the performance of this method with that of several RP SPE systems using mouse lung homogenate samples.

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Materials and Methods

Ethical approval of the study protocol

This study was conducted in accordance with the guidelines set by Shionogi Innovation Center (Sapporo, Japan).

Chemicals

HPLC-grade methanol (MeOH) and isopropanol (IPA), ammonium acetate, butylated hydroxytoluene, hexane, methyl formate, sodium chloride, dipotassium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hydrochloric acid (HCl) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). HPLC-grade acetonitrile and phosphoric acid were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Liquid chromatography-mass spectrometry grade Formic Acid (FA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Isoflurane was obtained from DS Pharma Animal Health Co., Ltd. (Osaka, Japan). Lipid standards were purchased from Cayman Chemical Company (Ann Arbor, MI, USA) (Table 1). Ultrapure water was obtained using a Milli-Q system (Millipore, Billerica, MA, USA).

Animals

Male C57BL/6 mice were purchased from Clea Japan Inc. (Tokyo, Japan). All mice were maintained in microisolator cages, where they were exposed to a 12-h light-dark cycle and provided with ad libitum access to standard food and water.

Preparation of standard solutions

Primary stock solutions of 62 analytes and 13 Internal Standards (IS) were prepared in MeOH. Five mixed working solutions (100 pg/μL; AA, EPA and DHA were at 3000 pg/μL) were prepared from the primary stock solutions according to the 5 analytical methods in Table 1. An IS working solution (300 pg/μL; AA-d8 and DHA-d5 at 1000 pg/μL) was prepared from the primary stock solutions by dilution with 10 mmol/L butylated hydroxytoluene in MeOH. Calibration standards were prepared freshly when required by diluting the five mixed working solutions with MeOH to 0.1, 0.3, 1, 3, 10, 30 and 100 pg/μL (final concentrations of AA, EPA and DHA were all 3, 10, 30, 100, 300, 1000 and 3000 pg/μL in these solutions). These calibration standards contained the IS at 30 pg/μL (final concentrations of AA-d8 and DHA-d5 were both 100 pg/μL).

Extraction efficiency of oxidized fatty acids

The lipid extraction process was performed on a MonoSpin™ C18-AX system (GL Sciences Inc., Tokyo, Japan) according to the manufacturer's instructions. Before extraction, the column was pre-activated with 300 μL of methanol and 300 μL of 20 mmol/L potassium phosphate solution (pH 7.0), followed by centrifugation at 9,000 × g for 1 min at 4°C. The calibration standards of each concentration in MeOH (100 μL) and 300 μL of 20 mmol/L potassium phosphate solution (pH 7.0) were mixed and placed directly on the pre-activated spin column. The column was centrifuged at 9,000 × g for 1 min at 4°C and then washed with 300 μL of a 5% NaCl solution by centrifugation. Finally, the column was placed into a new silicon-coated tube and the analytes adsorbed onto the column were eluted twice with 300 μL of 5% NaCl/MeOH (1/9, v/v). The eluant was dried under a gentle stream of nitrogen at 40°C to give a residue, which was reconstituted in 100 μL of MeOH. A 5 μL sample of this solution was then collected and injected into the LC-MS/MS system. The extraction efficiency was assessed in triplicate using the calibration standards at 3, 10 and 30 pg/μL (AA, EPA and DHA concentrations were all 30, 100 and 300 pg/μL in these solutions). The extraction efficiency was calculated using the following equation, with the peak areas of the IS with and without extraction denoted as *A* and *B*, respectively:

$$\text{Extraction efficiency (\%)} = \frac{A}{B} \times 100$$

Matrix effect (ME) and recovery (RE) from lung homogenates

The ME and RE properties were assessed by analyzing the IS solutions in triplicate according to a previously published method [29]. The three different types of IS solution used in this study were prepared as follows.

The Type 1 IS solution was prepared by diluting the neat IS working solution ten times with MeOH. After mixing, the solution was transferred into a vial and a 5 μL sample was injected directly into the LC-MS/MS system.

The Type 2 IS solution was prepared using an IS working solution that had been spiked after the extraction process. After being treated with isoflurane, 36-week-old mice were sacrificed and samples of their lung tissue were extirpated. Lung homogenates (25% mass fraction) were prepared in ultrapure water using Micro Smash™ MS-100R (Tomy Seiko Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions and pooled. Lung homogenate aliquots of 25, 50, 100 and 200 μL (containing 6.25, 12.5, 25 and 50 mg of lung tissue, respectively) were diluted with MeOH (MeOH volume = nine times the volume of the homogenate). The resulting mixtures were then vigorously vortexed for 10 min before being centrifuged at 9,000 × g for 5 min at 4°C. The upper layer was transferred to a silicon-coated tube, where it was dried under a gentle stream of nitrogen at 40°C to give a residue, which was reconstituted in 100 μL of MeOH. This MeOH solution was then extracted according to the protocol in the "Extraction efficiency of oxidized fatty acids" section. After drying the eluent, the resulting residue was reconstituted in 10 μL of IS working solution and 90 μL of MeOH. A 5 μL sample of the reconstituted solution was then injected into the LC-MS/MS system for analysis.

The Type 3 IS solution was prepared using an IS working solution that had been spiked prior to the extraction process. A 10 μL sample of the IS working solution was added to an aliquot of the lung homogenate (25, 50, 100 or 200 μL) along with MeOH (MeOH volume = nine times the volume of the lung homogenate), and the resulting mixture was vortexed prior to being subjected to the lipid extraction process described in the Type 2 protocol. After drying the eluent, the resulting residue was reconstituted in 100 μL of MeOH and 5 μL of the reconstituted solution was injected into the LC-MS/MS system for analysis.

The ME and RE values were calculated using the following equations, with the peak areas of the IS in the Type 1, 2 and 3 denoted as *C*, *D* and *E*, respectively:

$$\text{ME (\%)} = \frac{D}{C} \times 100,$$

$$\text{RE (\%)} = \frac{E}{D} \times 100$$

Lipid extraction using RP SPE

The lipid extraction process was performed using MonoSpin™ C18 columns (GL Sciences Inc.) and Sep-Pak tC18 cartridges (500 mg/6 cc; Waters Corp., Milford, MA, USA). For the MonoSpin™ C18 column, the column was equilibrated with 300 μL of MeOH and 300 μL of H_2O prior to the extraction process. A 10 μL sample of the IS working solution was then added to 50 μL of the lung homogenate together with 450 μL of MeOH, and the resulting mixture was vigorously vortexed

No.	Lipid	Precursor Ion	Product ion	Declustering Potential	Collision energy	Retention Time (min)	Method	Internal standard
01	AA	303	259	-80	-15	7.44	1	AA-d8
02	5-HETE	319	115	-100	-25	5.60	1	15-HETE-d8
03	8-HETE	319	155	-100	-25	5.30	1	15-HETE-d8
04	9-HETE	319	151	-100	-25	5.35	1	15-HETE-d8
05	11-HETE	319	167	-100	-25	5.12	1	15-HETE-d8
06	12-HETE	319	179	-100	-25	5.19	1	15-HETE-d8
07	15-HETE	319	219	-100	-15	4.91	1	15-HETE-d8
08	16-HETE	319	189	-80	-25	4.59	1	15-HETE-d8
09	17-HETE	319	247	-100	-25	4.53	1	15-HETE-d8
10	18-HETE	319	261	-80	-25	4.47	1	15-HETE-d8
11	5;6-EET	319	191	-100	-15	5.99	1	5;6-EET-d11
12	8;9-EET	319	155	-100	-15	5.84	1	15-HETE-d8
13	11;12-EET	319	167	-100	-25	5.72	1	15-HETE-d8
14	14;15-EET	319	219	-100	-15	5.48	1	15-HETE-d8
15	LTB_4	335	195	-100	-25	3.38	1	14;15-DHET-d11
16	LXA_4	351	115	-80	-25	2.22	1	LXA_4-d5
17	PGD_2	351	189	-80	-25	3.19	2	PGD_2-d4
18	PGE_2	351	189	-80	-25	3.10	2	PGE_2-d4
19	$PGF_{2\alpha}$	353	193	-80	-35	3.16	2	PGE_2-d4
20	6-keto-$PGF_{1\alpha}$	369	163	-80	-35	2.09	2	TXB_2-d4
21	TXB_2	369	195	-80	-25	2.86	2	TXB_2-d4
22	dhk-PGD_2	351	175	-80	-35	3.64	2	PGD_2-d4
23	dhk-PGE_2	351	235	-80	-35	3.34	2	PGE_2-d4
24	15-deoxy-PGJ_2	315	271	-80	-25	5.86	2	15-deoxy-PGJ_2-d4
25	EPA	301	257	-80	-15	6.74	3	AA-d8
26	5-HEPE	317	115	-80	-25	4.85	3	15-HETE-d8
27	8-HEPE	317	127	-80	-25	4.56	3	15-HETE-d8
28	9-HEPE	317	167	-80	-25	4.61	3	15-HETE-d8
29	11-HEPE	317	167	-80	-25	4.42	3	15-HETE-d8
30	12-HEPE	317	179	-80	-25	4.50	3	15-HETE-d8
31	15-HEPE	317	219	-80	-25	4.33	3	15-HETE-d8
32	18-HEPE	317	215	-80	-25	4.13	3	15-HETE-d8
33	8;9-EpETE	317	69	-80	-25	5.11	3	15-HETE-d8
34	11;12-EpETE	317	167	-80	-15	5.01	3	15-HETE-d8
35	14;15-EpETE	317	207	-80	-15	4.94	3	15-HETE-d8
36	17;18-EpETE	317	215	-80	-15	4.72	3	15-HETE-d8
37	DHA	327	283	-80	-15	7.21	4	DHA-d5
38	4-HDoHE	343	101	-80	-25	5.71	4	15-HETE-d8
39	7-HDoHE	343	141	-80	-25	5.32	4	15-HETE-d8
40	8-HDoHE	343	109	-80	-25	5.34	4	15-HETE-d8
41	10-HDoHE	343	153	-80	-25	5.11	4	15-HETE-d8
42	11-HDoHE	343	149	-80	-25	5.17	4	15-HETE-d8
43	13-HDoHE	343	193	-80	-15	4.99	4	15-HETE-d8
44	14-HDoHE	343	205	-80	-15	5.04	4	15-HETE-d8
45	16-HDoHE	343	233	-80	-25	4.87	4	15-HETE-d8
46	17-HDoHE	343	201	-80	-25	4.90	4	15-HETE-d8
47	20-HDoHE	343	285	-80	-15	4.73	4	15-HETE-d8
48	16;17-EpDPE	343	193	-80	-15	5.57	4	15-HETE-d8
49	19;20-EpDPE	343	241	-80	-15	5.31	4	15-HETE-d8
50	17R-RvD1	375	141	-80	-25	2.09	4	14;15-DHET-d11
51	PD1	359	153	-80	-25	3.00	4	14;15-DHET-d11
52	7S-MaR	359	113	-80	-25	2.91	4	14;15-DHET-d11
53	9-HODE	295	171	-100	-25	4.87	5	9-HODE-d4
54	13-HODE	295	195	-100	-25	4.75	5	9-HODE-d4
55	9;10-EpOME	295	171	-100	-25	5.56	5	9-HODE-d4
56	12;13-EpOME	295	195	-100	-25	5.44	5	9-HODE-d4
57	9-HOTrE	293	171	-100	-25	4.16	5	9-HODE-d4
58	13-HOTrE	293	195	-100	-25	4.16	5	9-HODE-d4
59	13-HOTrEr	293	193	-80	-25	4.29	5	9-HODE-d4

60	5-HETrE	321	115	-100	-25	6.34	5	15-HETE-d8
61	8-HETrE	321	157	-100	-25	5.52	5	15-HETE-d8
62	15-HETrE	321	221	-80	-25	5.27	5	15-HETE-d8
63	AA-d8	311	267	-120	-25	7.40	-	-
64	15-HETE-d8	327	226	-80	-15	4.86	-	-
65	5;6-EET-d11	331	202	-100	-15	5.95	-	-
66	LXA_4-d5	356	115	-80	-25	2.19	-	-
67	PGD_2-d4	355	193	-80	-25	3.18	-	-
68	PGE_2-d4	355	193	-80	-25	3.08	-	-
69	TXB_2-d4	373	173	-80	-35	2.86	-	-
70	15-deoxy-PGJ_2-d4	319	275	-120	-25	5.85	-	-
71	DHA-d5	332	288	-80	-15	7.19	-	-
72	9-HODE-d4	299	172	-100	-25	4.84	-	-
73	14;15-DHET-d11	348	207	-80	-25	3.76	-	-
74	LTC_4-d5	630	272	-80	-35	4.69	-	-
75	LTD_4-d5	500	177	-120	-25	4.86	-	-

HETE: Hydroxyeicosatetraenoic Acid; EET: Epoxyeicosatrienoic Acid; LT: Leukotriene; LXA4: Lipoxin A4; PG: Prostaglandin; TX: Thromboxane; dhk-PG: 13;14-dihydro-15-keto Prostaglandin; HEPE: Hydroxyeicosapentaenoic Acid; EpETE: Epoxyeicosatetraenoic Acid; HDoHE: Hydroxydocosahexaenoic Acid; EpDPE: Epoxydocosapentaenoic Acid; RvD1: Resolven D1; PD1: Protectin D1; MaR: Maresin; HODE: Hydroxyoctadecadienoic Acid; EpOME: EpoxyOctadecenoic Acid; HOTrE: HydroxyOctadecatrienoic Acid; HETrE: Hydroxyeicosatrienoic Acid; DHET: Dihydroxyeicosatrienoic Acid

Table 1: Optimized selected reaction monitoring pairs and parameters of the oxidized fatty acids.

for 10 min before being centrifuged at 9,000 × g for 5 min at 4°C. The upper layer was transferred to a silicon-coated tube, where it was dried under a gentle stream of nitrogen at 40°C to give a residue, which was reconstituted in 100 µL of MeOH. This MeOH solution was then mixed with 900 µL of aqueous HCl (pH 3.5) and placed directly onto the conditioned column. The column was centrifuged at 9,000 × g for 1 min at 4°C and then washed with 300 µL of H_2O and 300 µL of hexane by centrifugation. Finally, the column was placed into a new silicon-coated tube, and the analytes adsorbed onto the column were eluted twice with 300 µL of methyl formate. The eluent was then dried under a gentle stream of nitrogen at 40°C to give a residue, which was reconstituted in 100 µL of MeOH. A 5 µL sample of the reconstituted solution was then introduced into the LC-MS/MS system for analysis.

For the Sep-Pak tC18 cartridge, the cartridge was equilibrated with 12 mL of MeOH and 12 mL of H_2O prior to the extraction process. A 10 µL sample of the IS working solution was then added to 50 µL of the lung homogenate together with 450 µL of MeOH. After vortex mixing, 500 µL of the supernatant was collected and diluted with 4.5 mL of aqueous hydrochloric acid (pH 3.5). This acidified solution was then rapidly loaded onto the conditioned cartridge, which was washed with 12 mL of H_2O. A 6 mL portion of hexane was then added and the analytes were eluted with 9 mL of methyl formate. The resulting eluent was dried under a gentle stream of nitrogen at 40°C to give a residue, which was reconstituted in 100 µL of MeOH. A 5 µL sample of the reconstituted solution was then introduced to the LC-MS/MS system for analysis.

Comparison of mixed-mode SPE with RP SPE using mouse lung homogenate samples

The ratio of the process efficiency between the mixed-mode SPE and RP SPE systems was calculated using mouse lung homogenate samples. Mouse lung homogenate was extracted in 50 µL aliquots according to the Type 3 protocol (Matrix effect and recovery from lung homogenates) or "Lipid extraction using RP SPE" protocol. The extracted samples were analyzed by LC-MS/MS and the ratio of the process efficiency between the mixed-mode SPE and RP SPE systems was calculated using the following equation, with the peak areas of the IS in the mixed-mode SPE and RP SPE systems denoted as F and G, respectively:

$$\text{The ratio of process efficiency (\%)} = \frac{G}{F} \times 100$$

Furthermore, the correlation coefficient of the measured results for the endogenous oxidized fatty acids between the mixed-mode SPE and RP SPE systems was evaluated using 10 and 50 µL aliquots of the mouse lung homogenate.

Lipid analysis by LC-MS/MS

The compounds were separated on an Acquity UPLC system (Waters Corp.). The autosampler and column were maintained at 4 and 60°C, respectively. Separation was achieved with an Acquity UPLC BEH C18 column (100 × 2.1 mm i.d., 1.7 µm; Waters Corp.). Mobile phase A consisted of water/1 mol/L ammonium acetate/5 mmol/L phosphoric acid/FA (990/10/1/1, v/v/v/v), and mobile phase B consisted of acetonitrile/IPA/1 mol/L ammonium acetate/FA (495/495/10/1, v/v/v/v). The flow rate for the separations was set at 0.4 mL/min. The gradient program for methods 1, 3, 4 and 5 was as follows: 0.00-8.00 min (increased from 35 to 80% B), 8.01-11.00 min (increased from 80 to 100% B) and 11.01-13.00 min (decreased from 100 to 35% B). The gradient program for method 2 was as follows: 0-10 min (increased from 25 to 80% B), 10.01-13.00 min (increased from 80 to 100% B) and 13.01-15.00 min (decreased from 100 to 25% B). A FCV-20AH2 system (Shimadzu, Kyoto, Japan) was used as a valve switch to allow for the introduction of the sample to the separation column (between 1-10 min for method 2, and 1-8 min for methods 1, 3, 4, and 5).

Mass spectrometry analyses of oxidized fatty acids were carried out on an API5000 Mass Spectrometer (AB SCIEX, Foster City, CA, USA) equipped with an ESI source. Nitrogen was used as the collision gas for the analysis of all of the metabolites. Oxidized fatty acids were detected in the negative ESI mode with the following source parameters: curtain gas, 15 psi; ion source gas 1, 50 psi; ion source gas 2, 60 psi; ion spray voltage, -4500 V; collision gas, 8 psi; temperature, 500°C; interface heater, on; entrance potential, -10 V; collision cell exit potential, -10 V; resolution Q1, unit; and resolution Q3, unit. The dwell time was set to 25 min for all of the molecules to obtain more than 10 data points per peak. The acquisition and processing of data were carried out with Analyst v1.4.2 (AB SCIEX). Selected reaction monitoring conditions for each molecule are summarized in Table 1.

Results and Discussion

LC-MS/MS has been used previously to allow for the analysis of fatty acids [10,14-17], and the methods described in these studies have been improved in our laboratory. However, despite our best efforts to improve these methods, there are still some issues in need of resolution. With this in mind, the principle aim of the current study was to further optimize the HPLC conditions and extraction method used for the analysis of fatty acids in biological samples.

Optimization of HPLC conditions

A number of issues can arise during the analysis of eicosanoids, including carryover and peak tailing with Cys-LTs and thromboxane B_2 (TXB_2) [18]. These problems can be caused by the adsorption of these molecules onto the metallic surfaces of the analytical system. Although phosphoric acid is generally unsuitable for LC-MS/MS analysis, it has been reported that the inclusion of a small amount of phosphoric acid (e.g., 5 µmol/L) can lead to significant improvements in the peak shapes of lipid mediators such as lysophosphatidic acid and lysophosphatidylserine by preventing the adsorption of these materials onto the metallic surfaces of the analytical system [30]. In this study, a small amount of phosphoric acid was included in the analysis of the oxidized fatty acids, and the subsequent impact on the analysis of Cys-LTs was evaluated in detail.

The extracted ion chromatograms of the Cys-LTs with and without phosphoric acid are shown in Figure 1. In the absence of phosphoric acid, the peak corresponding to leukotriene C_4 (LTC_4) was found to be very broad and the signal-to-noise ratio (S/N) was very low (Figure 1A). In the presence of phosphoric acid, there was a significant improvement in the USP tailing factor of LTC_4 from 12.5 to 1.25, and the S/N also increased from 4.4 to 1484.1 (Figure 1D). The S/N of leukotriene E_4 (LTE_4) also increased (374.2 to 878.6) following the addition of phosphoric acid (Figures 1C and 1F). Although the S/N of leukotriene D_4 (LTD_4) did not change following the addition of phosphoric acid, the peak intensity increased ($6.7e^4$ to $2.6e^5$) (Figures 1B and 1E). Similarly, the peak shape of TXB_2 improved following the addition of phosphoric acid (data not shown). Notably, the addition of phosphoric acid did not appear to have an adverse impact on the other molecules in the system. To the best of our knowledge, this work represents the first reported example of improvements in the USP tailing factors and S/N of Cys-LTs following the addition of phosphoric acid. With more than 10,000 measurements repeated under the same conditions to date, we have observed no problems with the sensitivity or the peak shapes of the oxidized fatty acids analyzed in this way.

Construction of mixed-mode SPE

RP conditions are generally used for lipid extraction and HPLC separation. However, in terms of analysis, it is generally accepted that the combination of different separation modes such as two-dimensional HPLC is more effective for the purification of biological samples than the combination of the same separation modes. Furthermore, the use of the same separation modes of lipid extraction and HPLC can lead to ion suppression effects resulting from the presence of specific types of lipid. Although anion-exchange SPE has been used in terms of the characteristic features of oxidized fatty acids, it is not suitable for the

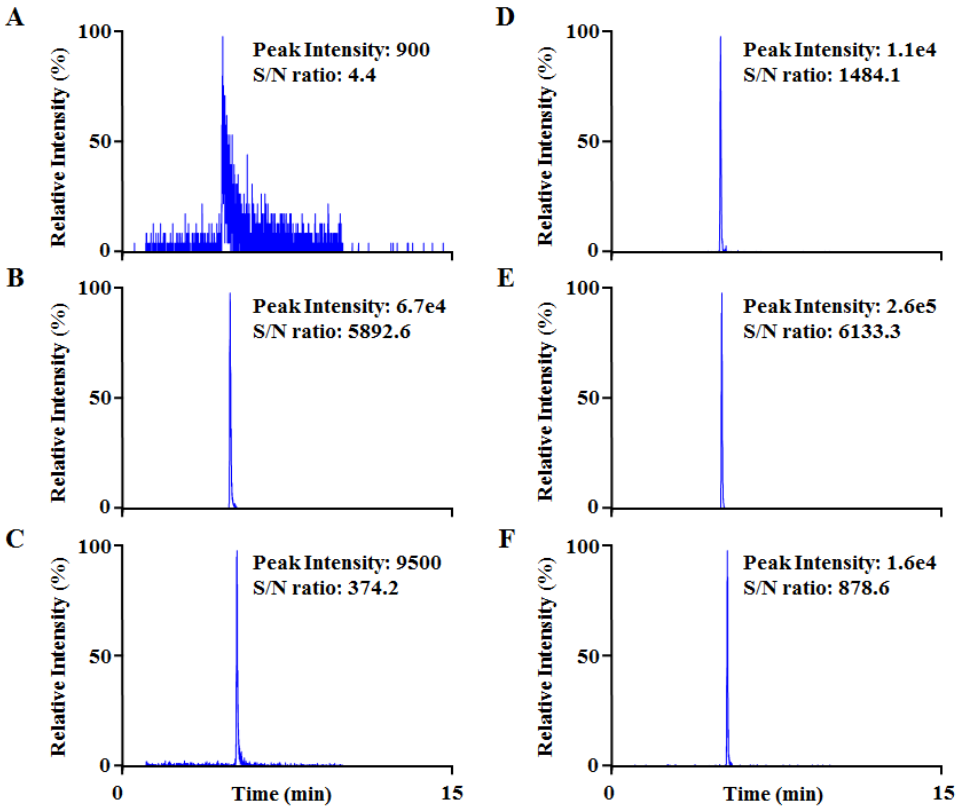

Figure 1: Extracted ion chromatograms of the leukotriene's (10 pg/µL). Leukotriene's were analyzed using measurement method 2 and the selected reaction monitoring transitions for leukotriene C_4, leukotriene D_4 and leukotriene E_4 were 625→272, 495→177 and 438→333, respectively. Extracted ion chromatograms of Leukotriene C_4, leukotriene D_4 and leukotriene E_4 without phosphoric acid (A, B and C, respectively) and with phosphoric acid (D, E and F, respectively) are shown.

extraction of polar lipids such as prostaglandins and thromboxanes [26]. Indeed, the results of our preliminary experiments using an anion-exchange SPE process revealed that the extraction efficiencies of prostaglandins and thromboxanes were <40 % (data not shown). To overcome these issues, we examined the lipid extraction conditions using mixed-mode SPE, which has been used in lipid biology to analyze fatty acid metabolites [27,28]. Although this approach is very attractive, its application has been limited because it can only be applied to a small number of lipids. Furthermore, the details of this method have never been fully clarified. Hence, the versatility of this approach is poor.

The method developed process used in the current study is shown in Figure 2. Using this method under neutral conditions, the dissociated oxidized fatty acids were adsorbed on to the column, which contained octadecyl and trimethylaminopropyl groups that were chemically bonded to monolithic silica. Furthermore, because a spin column was used in the current study, all of the handling procedures (e.g., sample loading, as well as the washing and elution of the oxidized fatty acids) were conducted by centrifugation. Using the spin column, the extraction time was 10 min per sample and the organic solvent requirements were <1 mL per sample.

To verify the utility of this method, the extraction efficiencies of oxidized fatty acids were tested using 62 lipids. The extraction efficiency of each lipid was evaluated in triplicate by LC-MS/MS analysis at concentrations of 3, 10 and 30 pg/μL (containing AA, EPA and DHA all at 30, 100 and 300 pg/μL, respectively) (Table 2). The results revealed that extraction efficiencies of ≥70 % were obtained for 61 of the lipids and Relative Standard Deviations (RSDs) of <15% were obtained for 60 lipids at the three different concentrations tested in the current study. Notably, the extraction efficiencies of prostaglandins and thromboxanes, which were insufficiently extracted by anion-exchange SPE, increased from <40% to >87% compared with anion-exchange SPE (No. 17-24, Table 2). These results therefore demonstrate that the combination of the RP and anion-exchange functions led to a significant improvement in the extraction efficiencies of polar lipids. Only the extraction efficiency of 5,6-epoxyeicosatrienoic acid (5,6-EET) was relatively low (<65%) (No. 11, Table 2). A detailed investigation of the extraction protocol resulted in an increased extraction efficiency for 5,6-EET (≥85 %) following a change in the eluent from 5% NaCl/MeOH (1/9, v/v) to 2% formic acid in MeOH or 5% NaCl/MeOH/isopropanol (2/9/9, v/v/v) (data not shown). This result suggests that the low recovery of 5,6-EET was caused by the adsorption of this material onto the column, and that the extraction efficiencies for molecules with an otherwise poor recovery could be improved by changing the elution conditions. This newly developed extraction method therefore showed good extraction efficiencies for a range of different compounds.

Matrix effect and recovery from lung homogenates

The ME and RE values of this newly developed method were examined using mouse lung homogenates. The ME values for some of the lipids were found to be dependent on the weight of the lung homogenate in the sample. For deuterated LTC_4 (LTC_4-d5), an enhancement of >150% was observed in the ME value when the mass of lung homogenate was increased from 12.5 and 50 mg (No. 12, Table 3). Similar increases in the ME value of >150% were also observed for deuterated 15-hydroxyeicosatetraenoic acid (No. 2, Table 3) and deuterated prostaglandin E_2 (No. 6, Table 3) at 50 mg compared with 12.5 mg. Although enhancements of >130% were observed in the ME values of deuterated AA (AA-d8) (No. 1, Table 3) and deuterated DHA (DHA-d5) (No. 9, Table 3), these increases were found to be independent of the lung homogenate weight. Given that the IS peaks were not observed in samples without IS (data not shown), it was concluded that these results were not being influenced by the interfering peaks derived from the samples. RSD values of <15% were obtained for all lipids at each weight.

For the RE values, a weight-dependent reduction was observed for some of the lipids. However, the RE values were >70% for almost all of the lipids at each weight (Table 4). With the exception of AA-d8 (No. 1, Table 4) and DHA-d5 (No. 9, Table 4) at 50 mg, RSD values of <15% were obtained for all lipids at each weight. Thus, adequate RSD values for the ME and RE values were obtained for each weight using our newly developed method. Consideration of the ME and RE values for the lipids, as well as the concentration of oxidized fatty acids in lung tissue, meant that the weight of lung homogenate used in this study was set to ≤12.5 mg.

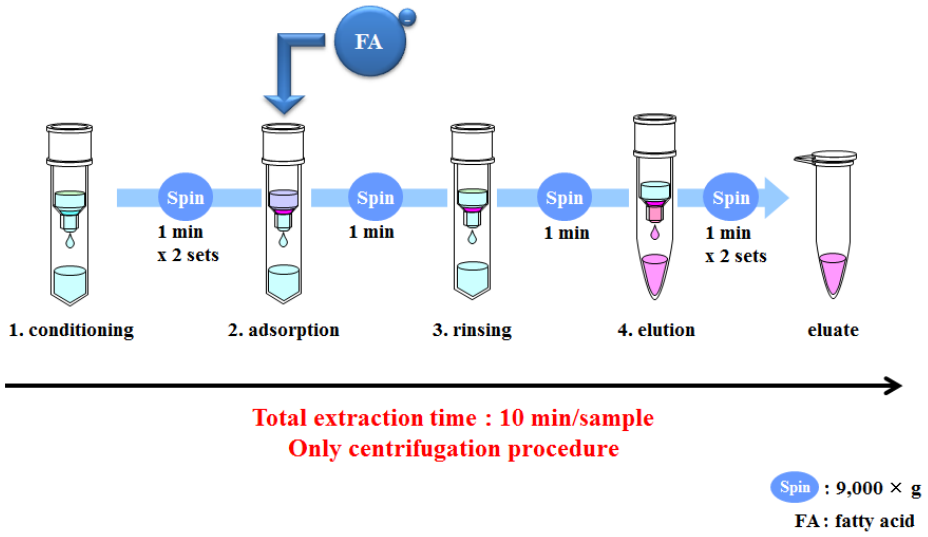

Figure 2: Mixed-mode spin-column extraction.

No.	Lipid	Calibrated range (pg/μL)	r^{2a}	Extraction efficiency[b] (%)			RSD (%)		
				3 pg/μL	10 pg/μL	30 pg/μL	3 pg/μL	10 pg/μL	30 pg/μL
01	AA[c]	10-3000	0.99	74.99 ± 4.59	79.09 ± 5.26	101.28 ± 7.95	6.12	6.65	7.85
02	5-HETE	0.3-100	0.99	99.79 ± 4.37	84.99 ± 2.29	88.95 ± 0.37	4.37	2.69	0.41
03	8-HETE	0.3-100	0.99	97.68 ± 5.73	90.98 ± 2.14	87.76 ± 1.02	5.87	2.35	1.16
04	9-HETE	0.3-100	0.99	97.62 ± 1.20	89.21 ± 0.71	92.20 ± 1.49	1.23	0.80	1.61
05	11-HETE	0.3-100	0.99	93.89 ± 4.26	87.18 ± 2.57	89.61 ± 1.02	4.53	2.95	1.14
06	12-HETE	0.3-100	0.99	93.04 ± 5.76	91.42 ± 2.63	91.31 ± 1.85	6.19	2.88	2.02
07	15-HETE	0.3-100	0.99	96.14 ± 5.60	89.10 ± 2.25	94.49 ± 5.82	5.82	2.53	2.33
08	16-HETE	1-100	0.99	101.72 ± 10.66	95.80 ± 5.53	97.06 ± 2.65	10.48	5.77	2.73
09	17-HETE	0.3-100	0.99	92.70 ± 9.57	91.12 ± 0.89	93.30 ± 2.01	10.32	0.97	2.14
10	18-HETE	0.3-100	0.99	99.94 ± 11.37	88.27 ± 2.18	92.97 ± 0.80	11.38	2.47	0.86
11	5;6-EET	0.3-100	0.99	58.70 ± 6.20	61.05 ± 4.37	62.93 ± 1.82	10.55	7.16	2.89
12	8;9-EET	1-100	0.99	94.86 ± 12.89	78.48 ± 2.60	86.70 ± 0.44	13.59	3.32	0.50
13	11;12-EET	0.3-100	0.99	82.46 ± 4.01	82.70 ± 2.61	85.77 ± 4.35	4.87	3.16	5.07
14	14;15-EET	0.3-100	0.99	94.66 ± 7.23	79.29 ± 1.10	77.35 ± 2.50	7.65	1.39	3.23
15	LTB_4	0.3-100	0.99	95.68 ± 2.81	100.21 ± 3.18	92.38 ± 1.05	2.94	3.18	1.14
16	LXA_4	0.3-100	0.99	106.56 ± 13.70	108.96 ± 0.86	84.62 ± 3.78	12.86	0.78	4.46
17	PGD_2	0.3-100	0.99	95.84 ± 6.71	97.34 ± 1.39	96.25 ± 4.82	7.00	1.43	5.01
18	PGE_2	0.3-100	0.99	96.19 ± 1.36	96.08 ± 6.23	93.36 ± 4.45	1.41	6.49	4.77
19	$PGF_{2\alpha}$	0.3-100	0.99	97.92 ± 6.70	98.55 ± 3.83	94.91 ± 5.43	6.85	3.88	5.72
20	6-keto-$PGF_{1\alpha}$	0.1-100	0.99	96.19 ± 4.51	95.66 ± 2.65	93.88 ± 1.11	4.69	2.77	1.18
21	TXB_2	0.3-100	0.99	100.78 ± 3.06	99.53 ± 3.39	95.91 ± 3.47	3.04	3.40	3.61
22	dhk-PGD_2	0.1-100	0.99	101.95 ± 3.73	101.31 ± 3.66	101.56 ± 0.66	3.66	3.61	0.65
23	dhk-PGE_2	1-100	0.99	105.41 ± 3.44	105.05 ± 1.88	99.81 ± 2.04	3.26	1.79	2.04
24	15-deoxy-PGJ_2	0.3-100	0.99	89.09 ± 3.23	99.15 ± 0.55	87.57 ± 4.10	3.62	0.56	4.68
25	EPA[c]	10-3000	0.99	80.55 ± 5.08	89.71 ± 10.11	110.52 ± 4.73	6.31	11.27	4.28
26	5-HEPE	1-100	0.99	99.77 ± 4.30	96.79 ± 6.73	93.57 ± 6.81	4.31	6.96	7.28
27	8-HEPE	1-100	0.99	109.80 ± 8.16	94.39 ± 4.89	93.18 ± 4.69	7.43	5.18	5.03
28	9-HEPE	1-100	0.99	96.57 ± 7.64	90.80 ± 4.38	92.36 ± 5.07	7.91	4.82	5.49
29	11-HEPE	0.3-100	0.99	99.19 ± 2.24	95.90 ± 5.93	94.78 ± 5.47	2.26	6.19	5.77
30	12-HEPE	1-100	0.99	99.93 ± 5.92	101.10 ± 6.47	97.23 ± 6.39	5.92	6.40	6.57
31	15-HEPE	1-100	0.99	100.64 ± 1.42	104.35 ± 13.61	97.55 ± 8.24	1.41	13.05	8.45
32	18-HEPE	0.3-100	0.99	100.33 ± 7.60	98.18 ± 3.18	98.17 ± 6.97	7.57	3.24	7.10
33	8;9-EpETE	0.3-100	0.99	90.51 ± 3.27	88.52 ± 3.72	86.10 ± 6.95	3.61	4.20	8.07
34	11;12-EpETE	1-100	0.99	94.64 ± 5.26	89.85 ± 6.32	81.35 ± 3.46	5.56	7.04	4.25
35	14;15-EpETE	1-100	0.99	92.12 ± 3.25	75.80 ± 13.27	79.61 ± 4.90	3.52	17.50	6.16
36	17;18-EpETE	1-100	0.99	96.72 ± 2.51	88.80 ± 6.54	87.59 ± 6.28	2.60	7.37	7.17
37	DHA[c]	10-3000	0.99	73.27 ± 2.85	83.30 ± 2.52	97.87 ± 4.64	3.89	3.02	4.74
38	4-HDoHE	0.3-100	0.99	96.00 ± 7.38	106.04 ± 10.56	106.68 ± 9.02	7.68	9.96	8.53
39	7-HDoHE	0.3-100	0.99	96.99 ± 6.10	87.40 ± 5.89	90.07 ± 7.30	6.29	6.74	8.11
40	8-HDoHE	0.3-100	0.99	85.98 ± 4.61	80.24 ± 6.31	85.22 ± 5.93	5.36	7.86	6.96
41	10-HDoHE	0.3-100	0.99	94.78 ± 1.96	92.81 ± 10.99	93.70 ± 6.01	2.07	11.84	6.41
42	11-HDoHE	0.3-100	0.99	98.59 ± 2.39	94.42 ± 7.52	95.94 ± 7.36	2.42	7.97	7.67
43	13-HDoHE	1-100	0.99	105.97 ± 2.32	96.76 ± 9.98	98.40 ± 6.76	2.19	10.32	6.87
44	14-HDoHE	1-100	0.99	97.99 ± 1.00	97.90 ± 10.22	100.60 ± 8.64	1.02	10.44	8.58
45	16-HDoHE	0.3-100	0.99	99.74 ± 2.30	100.80 ± 9.51	101.12 ± 8.18	2.30	9.43	8.09
46	17-HDoHE	1-100	0.99	99.43 ± 9.24	98.27 ± 7.84	101.61 ± 7.63	9.30	7.98	7.51
47	20-HDoHE	0.3-100	0.99	93.27 ± 0.87	99.47 ± 9.45	95.47 ± 7.70	0.93	9.50	8.07
48	16;17-EpDPE	3-100	0.99	97.13 ± 7.26	72.99 ± 6.32	95.56 ± 6.32	7.18	9.95	6.61
49	19;20-EpDPE	1-100	0.99	91.30 ± 8.08	76.37 ± 10.89	87.17 ± 5.90	8.85	14.26	6.77
50	17R-RvD1	0.3-100	0.99	108.10 ± 11.19	93.99 ± 7.27	86.98 ± 12.42	10.35	7.73	14.28
51	PD1	0.3-100	0.99	101.06 ± 2.87	106.55 ± 7.83	106.91 ± 9.91	2.84	7.35	9.27
52	7S-MaR	1-100	0.99	98.00 ± 4.95	101.33 ± 7.19	97.33 ± 10.27	5.05	7.10	10.55
53	9-HODE	0.3-100	0.99	91.84 ± 10.72	93.05 ± 3.12	86.57 ± 5.72	11.67	3.35	6.61
54	13-HODE	0.3-100	0.99	91.96 ± 3.95	93.10 ± 6.12	87.70 ± 7.67	4.30	6.57	8.74
55	9;10-EpOME	0.3-100	0.99	95.63 ± 5.30	93.33 ± 8.68	90.31 ± 7.18	5.54	9.30	7.96
56	12;13-EpOME	0.3-100	0.99	96.08 ± 4.32	94.62 ± 6.00	89.94 ± 5.59	4.49	6.35	6.21
57	9-HOTrE	0.3-100	0.99	87.16 ± 10.54	90.92 ± 7.14	83.39 ± 6.82	12.09	7.86	8.18

58	13-HOTrE	1-100	0.99	83.75 ± 8.43	91.81 ± 8.49	87.59 ± 7.12	10.06	9.25	8.13
59	13-HOTrEr	0.3-100	0.99	88.87 ± 5.59	96.36 ± 9.75	85.78 ± 5.63	6.29	10.12	6.56
60	5-HETrE	0.3-100	0.99	84.24 ± 5.49	90.80 ± 9.07	83.95 ± 4.70	6.52	9.99	5.60
61	8-HETrE	0.3-100	0.99	89.06 ± 7.19	95.02 ± 7.43	86.08 ± 7.43	8.07	8.77	8.64
62	15-HETrE	0.3-100	0.99	83.62 ± 6.36	96.04 ± 14.52	86.37 ± 6.08	7.61	15.12	7.04

[a]Linearity
[b]Extraction efficiency is the average ± SD (*n*=3)
[c]Extraction efficiency is evaluated at 30; 100 and 300 pg/μL.

Table 2: Calibration curve data and extraction efficiencies of oxidized fatty acids .

No.	Lipid	Matrix Effect (%)				RSD (%)			
		6.25 mg	12.5 mg	25 mg	50 mg	6.25 mg	12.5 mg	25 mg	
01	AA-d8	139.55 ± 5.28	136.21 ± 12.79	140.95 ± 4.66	135.96 ± 3.35	3.79	9.39	3.31	2.47
02	15-HETE-d8	106.94 ± 3.19	116.70 ± 6.95	148.11 ± 3.33	171.48 ± 6.34	2.99	5.95	2.25	3.70
03	5;6-EET-d11	99.71 ± 0.95	102.09 ± 3.16	112.58 ± 4.03	104.30 ± 3.57	0.95	3.10	3.58	3.43
04	LXA_4-d5	94.62 ± 2.43	97.62 ± 2.78	101.51 ± 2.65	102.29 ± 0.98	2.57	2.85	2.61	0.95
05	PGD_2-d4	98.26 ± 1.49	105.23 ± 2.03	114.35 ± 1.14	133.47 ± 7.72	1.52	1.93	1.00	5.78
06	PGE_2-d4	100.52 ± 1.51	111.17 ± 1.95	122.88 ± 2.20	153.30 ± 7.95	1.50	1.76	1.79	5.18
07	TXB_2-d4	98.34 ± 1.04	99.96 ± 1.08	103.64 ± 1.28	111.64 ± 1.74	1.06	1.08	1.24	1.56
08	15-deoxy-PGJ_2-d4	96.76 ± 0.96	100.25 ± 1.29	103.80 ± 2.13	112.41 ± 0.29	0.99	1.29	2.05	0.26
09	DHA-d5	147.15 ± 1.98	145.54 ± 8.99	151.32 ± 4.33	152.55 ± 3.23	1.35	6.18	2.86	2.12
10	9-HODE-d4	103.91 ± 2.04	106.60 ± 3.35	116.88 ± 2.16	122.97 ± 3.74	1.97	3.15	1.85	3.04
11	14;15-DHET-d11	95.77 ± 0.29	100.89 ± 3.50	110.57 ± 2.00	129.04 ± 5.13	0.31	3.47	1.81	3.98
12	LTC_4-d5	100.33 ± 10.67	159.32 ± 19.74	179.57 ± 11.56	182.37 ± 22.49	10.63	12.39	6.43	12.33
13	LTD_4-d5	88.28 ± 4.12	94.93 ± 3.91	95.76 ± 3.30	84.90 ± 6.18	4.67	4.12	3.44	7.28

Table 3: Matrix effects for internal standards in mouse lung samples.

No.	Lipid	Recovery (%)				RSD (%)			
		6.25 mg	12.5 mg	25 mg	50 mg	6.25 mg	12.5 mg	25 mg	50 mg
01	AA-d8	106.47 ± 7.44	99.69 ± 9.21	83.03 ± 7.12	104.12 ± 22.09	6.99	9.23	8.58	21.2
02	15-HETE-d8	91.64 ± 2.08	89.00 ± 0.91	90.27 ± 7.12	102.92 ± 12.65	2.27	1.02	7.89	12.29
03	5;6-EET-d11	86.82 ± 3.95	72.07 ± 1.16	62.59 ± 1.80	56.29 ± 4.79	4.55	1.61	2.87	8.50
04	LXA_4-d5	92.68 ± 8.08	87.31 ± 1.27	90.48 ± 5.41	94.15 ± 3.44	8.72	1.46	5.98	3.66
05	PGD_2-d4	89.89 ± 2.60	78.36 ± 3.16	78.04 ± 5.15	67.87 ± 0.66	2.89	4.03	6.60	0.98
06	PGE_2-d4	94.91 ± 6.28	90.30 ± 1.35	90.53 ± 8.96	85.21 ± 3.36	6.61	1.49	9.89	3.95
07	TXB_2-d4	99.39 ± 2.45	92.85 ± 1.76	88.36 ± 8.03	82.84 ± 2.42	2.47	1.90	9.09	2.93
08	15-deoxy-PGJ_2-d4	63.74 ± 3.84	62.14 ± 0.50	69.41 ± 2.83	83.95 ± 4.56	6.03	0.81	4.08	5.43
09	DHA-d5	105.02 ± 11.45	101.57 ± 8.29	84.87 ± 7.65	102.92 ± 17.07	10.90	8.17	9.01	16.67
10	9-HODE-d4	95.48 ± 9.07	89.61 ± 3.06	88.92 ± 5.02	99.04 ± 7.19	9.50	3.42	5.64	7.26
11	14;15-DHET-d11	92.35 ± 3.73	73.72 ± 2.30	83.92 ± 4.49	93.42 ± 5.11	4.04	3.12	5.35	5.47
12	LTC_4-d5	100.74 ± 4.99	87.81 ± 6.59	93.22 ± 3.92	90.86 ± 2.36	4.96	7.50	4.21	2.60
13	LTD_4-d5	83.86 ± 4.20	72.80 ± 4.58	80.32 ± 0.72	83.32 ± 6.18	5.01	6.28	0.90	7.42

Table 4: Recoveries of internal standards in mouse lung samples.

Comparison of mixed-mode SPE with RP SPE using mouse lung homogenate samples

To evaluate our extraction method, the mixed-mode SPE system was compared with RP SPE system using mouse lung homogenate samples. In this study, MonoSpin™ C18 and Sep-Pak tC18 were used as RP SPE devices. Given that the MonoSpin™ C18 column consists of octadecyl groups that are chemically bound to a monolithic silica spin column, it is possible to compare the mixed-mode SPE spin column with the MonoSpin™ C18 column based entirely on the differences in their functional groups. In contrast, Sep-Pak tC18 cartridges are widely used in the field of lipid biology, where they have been reported to exhibit good performance characteristics for the analysis of a broad range of oxylipins in plasma [26]. With this in mind, we evaluated the possibility of using a mixed-mode SPE system by comparison with a Sep-Pak tC18 cartridge. The ratio of the process efficiency values between the mixed-mode SPE and RP SPE systems were calculated for 12 of the ISs using mouse lung homogenate samples. With the exception of 5,6-EET-d11, the process efficiency ratio between the mixed-mode SPE and MonoSpin™ C18 systems was <100% in all cases (Figure 3). Notably, the process efficiency ratio was <60% for five of the ISs. These results therefore suggest that the mixed-mode SPE system was more efficient for the extraction of the lipids from the lung homogenate than the MonoSpin™ C18 spin column device. The mixed-mode SPE system was then compared with Sep-Pak tC18 cartridge. Interestingly, the process efficiency ratio between the mixed-mode SPE and the Sep-Pak tC18 was almost 100% for nearly all of the ISs (Figure 3), which suggested that the mixed-mode SPE system had the same degree of potential as the Sep-Pak tC18 system. In contrast, the process efficiency ratio of LTC_4-d5 was <5% for the MonoSpin™ C18 system and <25% for the Sep-Pak tC18 system. Since the process efficiency ratio of LTC_4-d5 was found to be <5% using the Oasis® HLB cartridge (60 mg/3 cc; Waters Corp.) (data not shown), our results suggested that the extraction efficiency of LTC_4 using the RP SPE system was generally

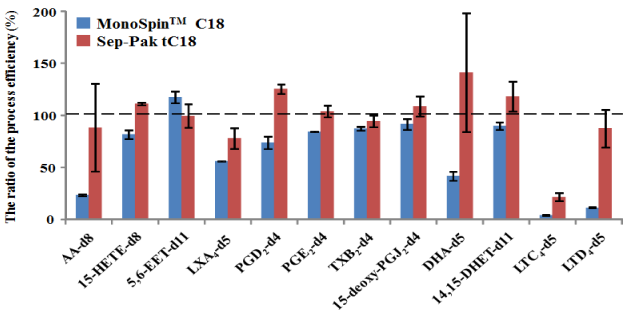

Figure 3: The ratio of process efficiency between mixed-mode SPE and RP SPE. Lipid extractions using the mixed-mode SPE, MonoSpin™ C18 and Sep-Pak tC18 systems were carried out in triplicate, and 12 ISs were analyzed by LC-MS/MS. The peak areas of the 12 ISs were calculated for each method and the ratio of the process efficiencies between the mixed-mode SPE and RP SPE system were calculated according to the "Comparison of mixed-mode SPE with RP SPE using mouse lung homogenate samples" described in the Materials and Methods section. The results for the MonoSpin™ C18 and Sep-Pak tC18 systems are indicated by blue and red bars, respectively.
HETE: Hydroxyeicosatetraenoic Acid; LXA_4: Lipoxin A_4; PG: Prostaglandin; DHET: Dihydroxyeicosatrienoic Acid

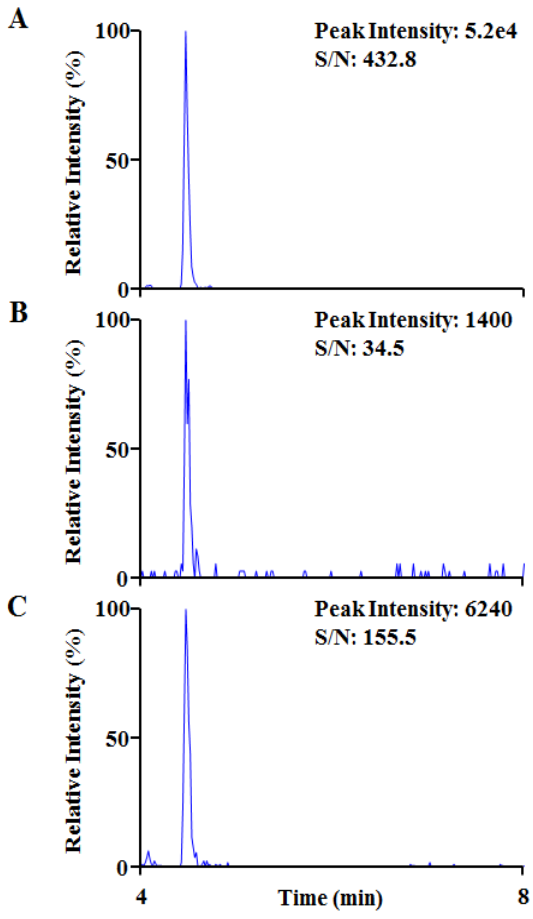

Figure 4: Comparison of leukotriene C_4 in mixed-mode SPE and RP SPE. Leukotriene C_4 in mice lung tissue samples was extracted using mixed-mode SPE, MonoSpin™ C18 and Sep-Pak tC18, and analyzed by LC-MS/MS. Extracted ion chromatograms of leukotriene C_4 in mixed-mode SPE (A), MonoSpin™ C18 (B) and Sep-Pak tC18 (C) are shown.

low. The extracted ion chromatograms of the endogenous LTC_4 extracted from the mouse lung homogenate samples using the different extraction methods are shown in Figure 4. Although the LTC_4 peak could be detected in full using the mixed-mode SPE and the S/N was 432.8, this peak was not sufficiently detected using the MonoSpin™ C18 system and the S/N was about 12-fold lower than the value achieved using the mixed-mode SPE system (432.8 to 34.5). Although there was a 5-fold increase in the S/N using the Sep-Pak tC18 system (34.5 to 155.5) compared with the MonoSpin™ C18 system, the S/N achieved using the Sep-Pak tC18 system was still three times lower than that of the mixed-mode SPE system (155.5 to 432.8). These results suggest that LTC_4 was not being sufficiently purified by the RP SPE system compared with mixed-mode SPE system. The poor performance of this system could be attributed in part to ion suppression from contaminants in the sample matrix. For conventional methods, simply eliminating contaminants that show similar retention behaviors to the compound of interest does not always allow for the resolution of this problem because the lipid extraction and HPLC separation processes are invariably carried out under the same RP conditions. Compared with conventional methods, potential contaminants are removed more effectively using our newly developed method, which involves the combination of mixed-mode and RP separation conditions for the lipid extraction and HPLC separation stages, respectively. Taken together, these results demonstrate that mixed-mode SPE is more effective for the lipid extraction of LTC_4 than RP SPE.

The correlation coefficients for the results measured between the mixed-mode SPE and RP SPE systems using mouse lung homogenate samples are shown in Figures 5 and 6. The proportion of variance was >0.99 for all of the lipids and >0.95 for all of the lipids present at concentrations of <500 pg/mg of tissue. For lipids present at concentrations <100 pg/mg of tissue, the proportion of variance was >0.86 (data not shown). These results therefore show that mixed-mode SPE performed much more effectively than RP SPE. Furthermore, more improvements were achieved for this method compared with the Sep-Pak tC18 system. Using a spin column, the extraction time was dramatically reduced from 1 h per 6 samples to 10 min per 6 samples, and the use of organic solvents was greatly reduced from >20 mL per sample to <1 mL per sample. In addition, the number of samples that could be simultaneously extracted was increased from 10 to 48 samples by changing the manifold to a centrifugal system. By eliminating time-consuming tasks such as those associated with the control of the manifold, the efficiency of the entire operation was markedly improved and the extraction time for 48 samples was less than 1 h. Therefore, this method is simpler, more readily available and more user-friendly than the conventional methods currently available for the analysis of lipids in biological samples.

Conclusions

A new approach has been developed for the analysis of oxidized fatty acids using LC–MS/MS combined with mixed-mode extraction with a spin column. The addition of a small amount of phosphoric acid to the mobile phase suppressed the adsorption of the Cys-LTs onto the metallic surfaces of the analytical system, which led to a dramatic improvement in the USP tailing factor and S/N of the Cys-LTs. This modified extraction method is suitable for various oxidized fatty acids including prostaglandins and thromboxanes. In addition, this method can be adapted to molecules that show poor recovery by changing the elution conditions. This new method was also compared with a conventional method using mouse lung homogenate samples, and the results showed that molecules exhibiting a poor recovery using conventional methods, such as LTC_4, were detected mush more

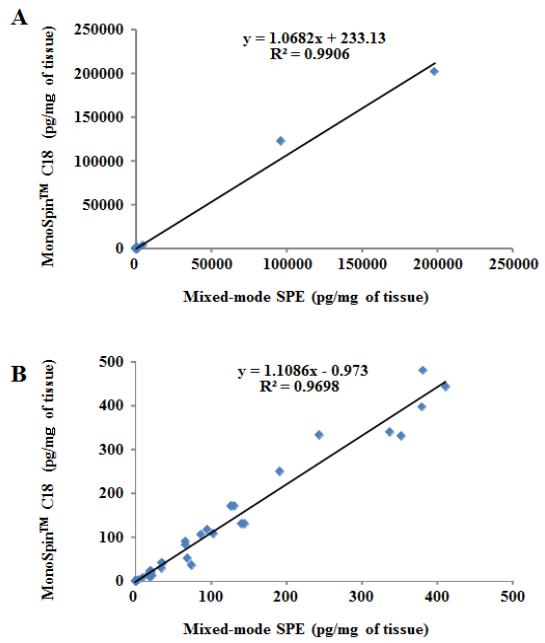

Figure 5: Scatter plot of the quantitative values of the mixed-mode SPE and MonoSpin™ C18 systems. Forty-five different metabolites derived from DHA and AA in mice lung tissue samples were analyzed using mixed-mode SPE and MonoSpin™ C18. The average values of the measurements (*n*=3) for each method were calculated for all lipids. All lipids (A) and lipid present at <500 pg/mg of tissue (B) are shown. R^2: proportion of variance.

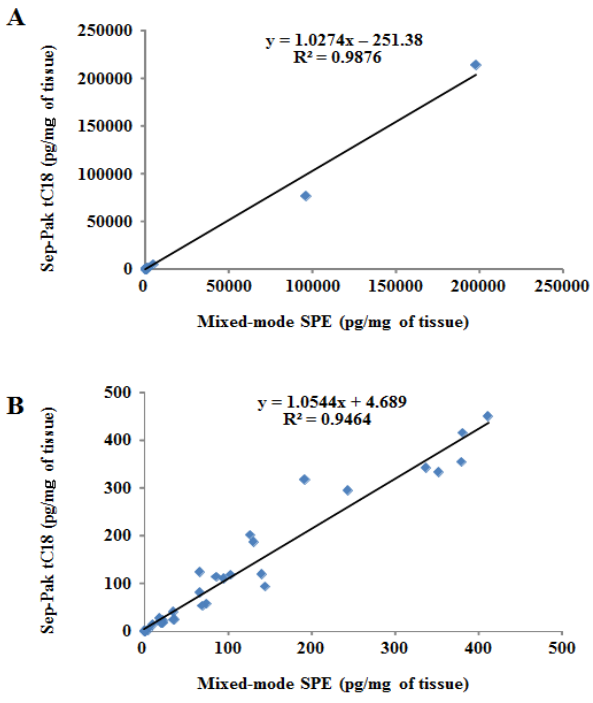

Figure 6: Scatter plot of the quantitative values of the mixed-mode SPE and Sep-Pak tC18 systems. Forty-five different metabolites derived from DHA and AA in mice lung tissue samples were analyzed using mixed-mode SPE and Sep-Pak tC18. The average values of the measurements (*n*=3) for each method were calculated for all lipids. All lipids (A) and lipid present at <500 pg/mg of tissue (B) are shown. R^2: proportion of variance.

effectively using our newly developed method because of its improved sample purification characteristics. We have already used this system in this context for lung, brain, kidney, plasma, and urine samples. Further work is needed to expand on the target lipid molecules, validate the analytical method and investigate the application of this method to a wide variety of tissue samples. We believe that this newly developed protocol is a useful tool for lipid biology and drug discovery research.

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