Quantitative determination of esterified eicosanoids and related oxygenated metabolites after base hydrolysis[1]

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Abstract **Eicosanoids and related metabolites (oxylipins) possess potent signaling properties, elicit numerous important physiologic responses, and serve as biomarkers of disease. In addition to their presence in free form, a considerable portion of these bioactive lipids is esterified to complex lipids in cell membranes and plasma lipoproteins. We developed a rapid and sensitive method for the analysis of esterified oxylipins using alkaline hydrolysis to release them followed by ultra-performance LC coupled with mass spectrometric analysis. Detailed evaluation of the data revealed that several oxylipins are susceptible to alkaline-induced degradation. Nevertheless, of the 136 metabolites we examined, 56 were reproducibly recovered after alkaline hydrolysis. We classified those metabolites that were resistant to alkaline-induced degradation and applied this methodology to quantify metabolite levels in a macrophage cell model and in plasma of healthy subjects. After alkaline hydrolysis of lipids, 34 metabolites could be detected and quantified in resting and activated macrophages, and 38 metabolites were recovered from human plasma at levels that were substantially greater than in free form. By carefully selecting internal standards and taking the observed experimental limitations into account, we established a robust method that can be reliably employed for the measurement of esterified oxylipins in biological samples.**—Quehenberger, O., S. Dahlberg-Wright, J. Jiang, A. M. Armando, and E. A. Dennis. **Quantitative determination of esterified eicosanoids and related oxygenated metabolites after base hydrolysis.** ***J. Lipid Res.*** **2018.** 59: **2436–2445.**

Supplementary key words lipidomics • mass spectrometry • oxidized lipids • oxylipins

Eicosanoids and related metabolites, sometimes referred to as oxylipins, are a group of structurally diverse metabolites that derive from the oxidation of PUFAs, including arachidonic acid, linoleic acid, α and γ linolenic acid, dihomo γ linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid. They are locally acting bioactive signaling lipids that regulate a diverse set of homeostatic and inflammatory processes (1, 2). Given the important regulatory functions in numerous physiological and pathophysiological states, the accurate measurement of eicosanoids and other oxylipins is of great clinical interest and lipidomics is now widely used to screen effectively for potential disease biomarkers (3).

The biosynthesis of eicosanoids and oxylipins involves the action of multiple enzymes organized into a complex and intertwined lipid-anabolic network (4). Generally, the enzymatic formation of eicosanoids requires free fatty acids as substrates; thus, the pathway is initiated by the hydrolysis of phospholipids (PLs) by phospholipase A_2 upon physiological stimuli (5). The hydrolyzed PUFAs are then processed by three enzyme systems: cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 enzymes. Each of these enzyme systems produces unique collections of oxygenated metabolites that function either as end-products or as intermediates for a cascade of downstream enzymes. The resulting eicosanoids exhibit diverse biological activities, half-lives, and utilities in regulating many

This work was supported by National Institute of General Medical Sciences Grant R01 GM20501 and LIPID MAPS "Glue Grant" U54 GM069338 and National Institute of Diabetes and Digestive and Kidney Diseases Grants R01 DK105961 and P30 DK063491. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. E.A.D. is a co-founder, director, and consultant to LipoNexus, Inc., which has licensed intellectual property from the University of California at San Diego.

Manuscript received 23 August 2018 and in revised form 4 October 2018.

Published, JLR Papers in Press, October 15, 2018
DOI https://doi.org/10.1194/jlr.D089516

Abbreviations: COX, cyclooxygenase; CV, coefficient of variance; EET, epoxyeicosatrienoic acid; ISTD, internal standard; LOX, lipoxygenase; LT, leukotriene; MRM, multiple reaction monitoring; PG, prostaglandin, PL, phospholipid; PSTD, primary standard; SPE, solid phase extraction; TX, thromboxane; UPLC, ultra-performance LC.

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[S] The online version of this article (available at http://www.jlr.org) contains a supplement.

physiologic processes in health and disease, including the immune response, inflammation, and homeostasis (6–10). Additionally, nonenzymatic processes can produce oxidized PUFA metabolites via free radical reactions giving rise to isoprostanes and other oxidized fatty acids (11).

Eicosanoids are either secreted and signal through G protein-coupled receptors in an autocrine or paracrine fashion or act intracellularly via various peroxisome proliferator-activating receptors (4, 12, 13). For optimal biological activity, these mediators need to be present in their free nonesterified form. However, a number of studies reported that a portion of eicosanoids are naturally esterified and can also be contained in cell membrane lipids, including PLs, in the form of esters (14–16). The role of esterified eicosanoids is not clear but they may be signaling molecules in their own right or serve as a cellular reservoir for rapid release upon cell stimulation (17, 18).

Two potential mechanisms for the formation of eicosanoid-containing PLs have been proposed: *i*) direct oxidation of PUFAs on the intact PLs; and *ii*) reacylation of preformed free oxylipins into lysoPLs. COXs require free fatty acid as substrate and show little activity toward PUFAs in intact PLs (19). A number of subsequent studies support the concept that prostaglandins (PGs) are first formed enzymatically and then incorporated into PLs by the sequential actions of long-chain acyl-CoA synthases and lysoPL acyltransferases (20, 21). Additionally, preformed fatty acid epoxides, including the regioisomers of epoxyeicosatrienoic acid (EET), are effectively incorporated primarily into the PL fraction of cellular lipids, presumably via CoA-dependent mechanisms (22).

In contrast, mammalian 12/15 LOX can act directly on PLs to generate esterified HETE isomers, including esterified 12-HETE and 15-HETE (23, 24). Similarly, the endocannabinoid 2-arachidonylglycerol is a substrate for COX-2 and is metabolized to PGH_2 glycerol ester as effectively as free arachidonic acid (25). The final products derived from this direct PL oxygenation pathway include esterified PGs as well as 11-HETE and 15-HETE. PUFAs contained in PLs can also be oxidized by nonenzymatic reactions. Free radical peroxidation reactions observed under conditions of oxidative stress can freely proceed on intact PLs resulting in the formation of isoprostanes (26).

Previously, we and others applied LC-MS/MS protocols to test whether plasma levels of oxylipins can be used as biomarkers to differentiate the progressive form of nonalcoholic fatty liver disease, termed nonalcoholic steatohepatitis, from the milder form termed nonalcoholic fatty liver. In that study, we identified a panel of nonesterified oxylipins that when used together is able to discriminate nonalcoholic steatohepatitis from nonalcoholic fatty liver with a high degree of certainty (27). Another study used an approach that included an alkaline hydrolysis step with the aim of measuring the sum total of free and esterified oxylipins (28). Of the markers monitored, products derived from free radical-mediated oxidation of linoleic acid were reported to be significantly elevated in nonalcoholic steatohepatitis. These results differed significantly from our findings, but can in part be explained by the difference in the experimental approach, as we measured the free oxylipins present in plasma, not those appearing after alkaline hydrolysis (27). In order to quantitatively capture the sum total of esterified and free oxylipins, all plasma samples need to be hydrolyzed, which requires strong alkaline conditions to quantitatively release the oxidized PUFAs before analysis. However, neither any specific experimental conditions nor systematic testing of the effect of strong bases on eicosanoid stability were reported in the later study (28). In contrast, in the present study, we have specifically determined the stability of the oxylipins under the hydrolysis conditions employed, and compiled a list of metabolites that can be reproducibly measured in biological samples.

From previous studies in our and other laboratories, we know that eicosanoids and specifically PGs are sensitive to alkaline-induced degradation. The objective of the current study was to develop precise conditions to minimize degradation of lipid metabolites during alkaline treatment and to identify specific eicosanoids and related oxidized PUFAs that are released intact from esterified lipids and which can be quantitatively included in searches for potential biomarkers.

MATERIALS AND METHODS

Reagents

All solvents were ultra-performance LC (UPLC) grade and were purchased from Fisher Scientific (Waltham, MA). All primary standards (PSTDs) for standard curves and deuterated internal standards (ISTDs) were purchased from Cayman Chemicals (Ann Arbor, MI) or Enzo Life Sciences (Farmingdale, NY). Strata-X polymeric reversed phase columns were purchased from Phenomenex (Torrance, CA). Human plasma was purchased from Gemini Bio Products (West Sacramento, CA).

Cell culture

RAW 264.7 cells (ATCC) were used in all cell experiments. Briefly, 4x1E5 cells were plated into each well of a 6-well plate and cultured overnight in 2 ml of DMEM containing 10% FBS (Atlanta Biologicals, Flowery Branch, GA) and were grown overnight at 37°C and 5% CO_2. For the experiment, the culture medium was exchanged with 2 ml of DMEM without FBS, and the cells were primed with Kdo2-lipid A (Avanti Polar Lipids, Alabaster, AL) at 100 ng/ml for 4 h, then stimulated with ATP (5 mM) for an additional 20 h. At the end of the incubation period, DMEM was removed and cells were harvested into 1 ml of PBS, counted, homogenized, and both cell homogenate and medium fractions were frozen. For analysis, we used 500 μl of the cell homogenates.

Lipid extraction

Free eicosanoids. For the extraction of free eicosanoids, 50 μl of plasma, 500 μl of cell homogenates, or the PSTD collection consisting of 136 individual standards were spiked with 100 μl of the ISTD mix (1 ng of each of 26 deuterated standards in ethanol) and diluted with Dulbecco's PBS to give a 10% total ethanol concentration (29). Eicosanoids were then isolated by solid phase extraction (SPE) using Strata-X polymeric reversed phase columns. The columns were activated with consecutive washes of 3 ml of 100% methanol and 3 ml of water. The samples were

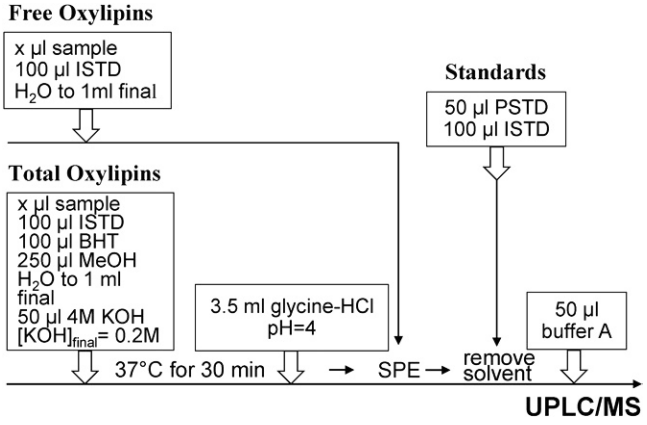

Fig. 1. Experimental flow chart for the analysis of free and esterified eicosanoids and other oxylipins.

loaded and washed with 3 ml of 10% methanol. Eicosanoids were then eluted with 1 ml of 100% methanol, dried under vacuum, and dissolved in 50 µl of buffer A consisting of water/acetonitrile/acetic acid (60/40/0.02, v/v/v). Samples were immediately analyzed using UPLC-MS/MS. A complete list of all PSTDs used for standard curves and deuterated standards and their assignments for normalization is provided in supplemental Table S1.

Total eicosanoids. To extract total eicosanoids, 50 µl of plasma, 500 µl of cell homogenates, or 50 µl of the PSTDs mix were spiked with ISTDs (in 100 µl of ethanol) and added to a mixture consisting of 100 µg of butylated hydroxytoluene (in 100 µl ethanol), 250 µl methanol, 50 µl KOH (4 M), and water to a final volume of 1 ml (**Fig. 1**). The mixture was kept for 30 min at 37°C to hydrolyze the esterified eicosanoids. Following hydrolysis, 3.5 ml of glycine-HCl buffer (0.1 mM, pH 4) were added. The free eicosanoids were then isolated by SPE and analyzed according to the protocol for the free eicosanoids, as described above. An unadulterated mix of pure PSTDs and ISTDs that was not subjected to hydrolysis conditions or SPE served as a control to estimate recoveries.

Separation and quantification of eicosanoids

Chromatographic separation. Separation was performed on an Acquity UPLC system (Waters, Milford, MA), equipped with a RP C18 BEH shield column (2.1 × 100 nm; 1.7 µm; Waters). For the separation of eicosanoids, a binary buffer system was used consisting of buffer A (described above) and buffer B composed of acetonitrile/2-propanol (50/50, v/v). At a flow rate of 0.5 ml/min, buffer A was held at 100% for 1 min followed by a gradient over 3 min to 55% buffer B, then further increased over 1.5 min to 100% buffer B and kept at this level for 0.5 min. The starting conditions were reconstituted in 1 min. The column was kept at 40°C and the sample manager at 4°C. The samples (10 µl) were injected via partial loop injection using needle overfill mode. To minimize carryover, needle washes were carried out between samples.

MS data acquisition. Data were collected on an AB/Sciex 6500 QTRAP hybrid triple quadrupole mass spectrometer (Sciex, Framingham, MA) using negative electrospray and scheduled multiple reaction monitoring (MRM) mode. The source settings were as follows: curtain gas (CUR = 20 psi), nebulizer gas (GS1 = 30 psi), turbo heater gas (GS2 = 20 psi), electrospray voltage (TEM = −4,500 V), source temperature (500°C), and collision gas (CAD = medium).

Quantitation. Eicosanoids were quantified by the stable isotope dilution method. Briefly, identical amounts of ISTDs were added to each sample and to all the PSTDs. Nine point standard curves were generated for each of the 136 PSTDs, ranging from 0.03 ng to 10 ng. To calculate the amount of eicosanoids in a sample, ratios of peak areas between endogenous eicosanoids and matching deuterated internal eicosanoids were calculated. Ratios were converted to absolute amounts by linear regression analysis of standard curves. Currently, we quantify most eicosanoids at low femtomole levels.

To determine recovery values of the PSTDs under alkaline hydrolysis conditions, MS peak areas were compared before and after the addition of base. All measurements were performed in triplicate or five replicate measurements and the data are reported as averaged values. The coefficient of variance (CV) determines the precision of this quantitation method.

PL measurements

The PL measurements were carried out by LC-MS using a multiplex approach as previously described (30). Briefly, 50 µl of human plasma were hydrolyzed as described above and extracted according to Bligh and Dyer (31). As a control, nonhydrolyzed plasma was included. The organic solvent was removed and the lipids were reconstituted in buffer A (isopropanol/hexane/water = 59/40/1, v/v/v, with 10 mM ammonium acetate) and analyzed on a Waters Acquity UPLC-Sciex 6500 QTrap mass spectrometer system. The lipids were separated on a silica column (2.1 × 150 mm; 3 µm; Phenomenex) using a binary solvent gradient from 100% A to 100% B (isopropanol/hexane/water = 50/40/10,

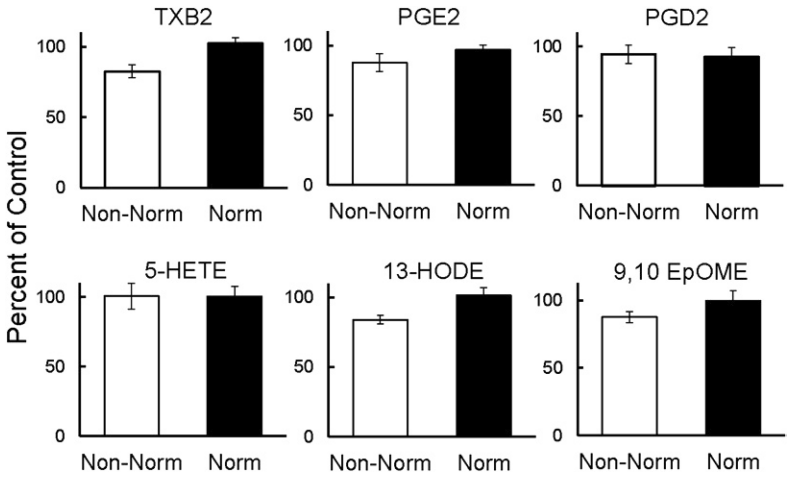

Fig. 2. Recoveries of oxylipin standards in their free form after SPE. A collection of primary oxylipin standards was analyzed with and without SPE purification. Recoveries were determined by comparing MS intensities obtained with standards after undergoing SPE purification with MS intensities of standards without SPE purification that were analyzed in parallel and served as controls. The open bars show non-normalized recoveries (Non-Norm), which were calculated from the raw MS peak areas without normalization to ISTDs; the closed bars show the same data set but normalized to ISTD (Norm). All data are expressed as percent of untreated controls. The mean and SD of triplicate measurements are displayed. Shown is a representative subset of standards (see supplemental Table S1 for the complete data set).

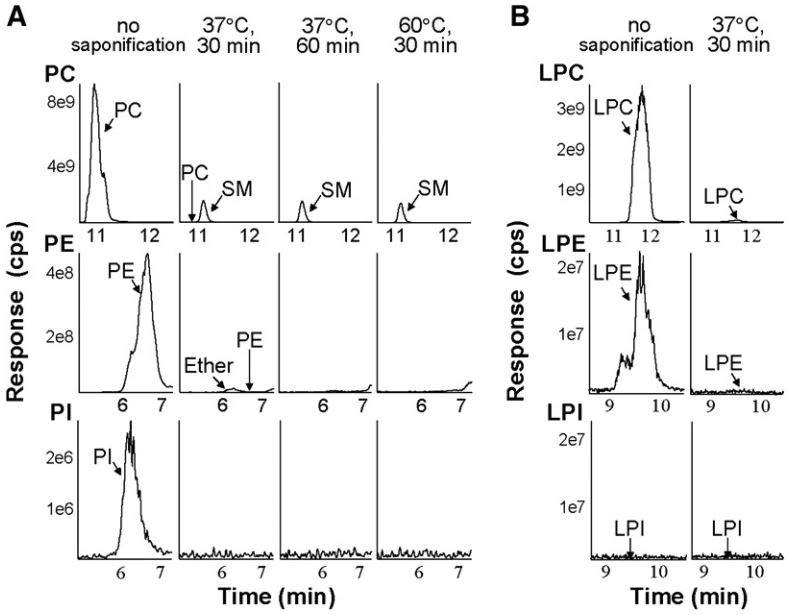

Fig. 3. Alkaline hydrolysis efficiency. To determine the hydrolysis efficiency, lipids in human plasma were hydrolyzed with 0.2 N potassium hydroxide for the indicated times and temperatures. Remaining intact PLs (A) and lysoPLs (B) were then measured by LC-MS. Shown are the chromatograms for the PL classes, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI), as well as the respective lyso-lipids. The mildest condition tested (37°C, 30 min) was sufficient to effectively hydrolyze all PLs and lysoPLs. Ether, ether-linked PE.

v/v/v, with 10 mM ammonium acetate) over 16 min. PL molecular species (432 isobaric species in total) were identified by MS using precursor ion and neutral loss scans in positive and negative modes. For the PL class analysis, the sum total of individual molecular species within each class was used.

RESULTS AND DISCUSSION

Quantitation of eicosanoid-related oxylipins in their free nonesterified form

Eicosanoids are important lipid metabolites that are involved in a number of physiological processes at the cellular level. As with fatty acids, they can exist either in the free form or esterified to complex lipids, such as PLs. The objective of the current study was to develop precise conditions that allow the quantitative measurement of both free and esterified oxylipins. To achieve this, we first examined the recovery of free eicosanoids during the prepurification step prior to LC-MS analysis using a defined set of quantitation standards consisting of 161 authentic metabolites that were mixed at precisely measured concentrations. Included in the standard cocktail were also 26 deuterated analogs that can be used to offset any potential losses during workup. The standard mix was then divided into two aliquots and analyzed by LC-MS with or without SPE prepurification. **Figure 2** shows the recovery of a subset of eicosanoids that were selected based on their metabolic pathway. In order to assess the degree of any potential losses during sample preparation and SPE purification, we plotted the raw MS data after SPE without normalization as percent recovery compared with the raw MS data obtained with standards set that did not undergo SPE purification. As can be seen, the recovery of these lipid metabolites in their free nonesterified form and undergoing our standard purification procedure was largely quantitative, ranging between 90% and 100%, even without normalization to ISTDs (Fig. 2). Any potential losses were minimal and could easily be offset with the application of our routine normalization procedure using deuterated eicosanoid analogs as ISTDs. A complete list of the recoveries for all metabolites as well as ISTD assignments, pertinent technical information, and instrument settings are provided in the supplemental Table S1.

Optimization of hydrolysis conditions to preserve oxylipin structure

A number of studies reported that a portion of eicosanoids are naturally esterified and can be contained in

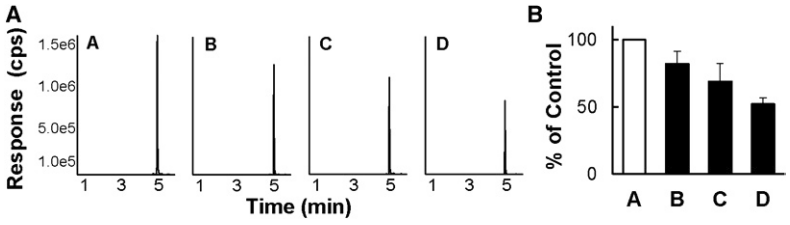

Fig. 4. Recovery of 7-hydroxy-docosahexaenoic acid (7-HDoHE) subjected to alkaline conditions. The 7-HDoHE is shown as a representative example to test the stability of related hydroxylated PUFAs. Three conditions were tested: A, no saponification; B, 37°C for 30 min; C, 37°C for 60 min; and D, 60°C for 30 min. The mass spectral response for 7-HDoHE is shown in panel A. The average relative intensity of triplicate measurements is shown in panel B. The untreated control was set at 100%. As can be seen, there was a time- and temperature-dependent decline in the recovery of 7-HDoHE.

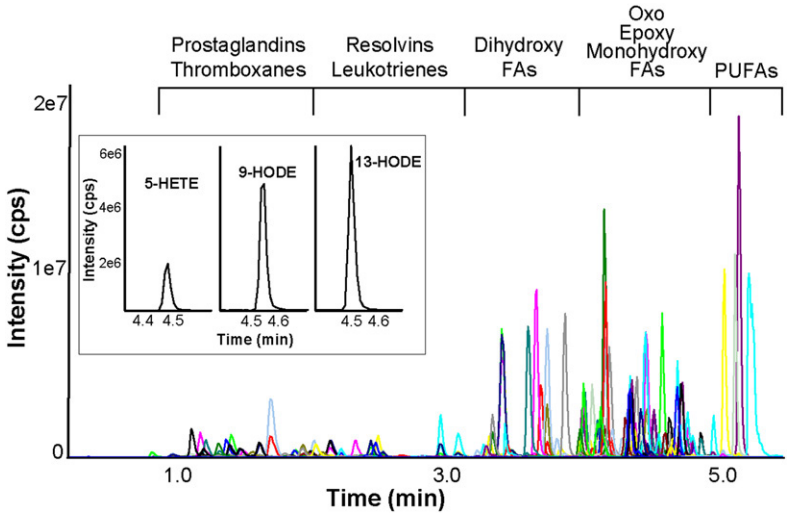

Fig. 5. MRM chromatogram of oxylipin standards subjected to alkaline hydrolysis conditions. A set of pure oxylipin standards (136 metabolites) was subjected to alkaline hydrolysis conditions, purified by SPE, and analyzed by LC-MS. Shown is the complete MRM chromatogram of all 136 metabolites. Inset: As an example and to demonstrate peak quality, the MRMs for 5-HETE, 9-HODE, and 13-HODE were extracted and shown individually.

membrane lipids in the form of esters. To profile quantitatively all eicosanoids incorporated into the various lipid fractions using an approach that preserves the intact molecule represents an enormous technical challenge. An alternative approach is to release the eicosanoids first by hydrolysis and then measure the metabolites in their free form. Several laboratories have applied alkaline hydrolysis for this purpose; however, the hydrolysis conditions varied considerably as they were often optimized for the analysis of certain subclasses of eicosanoids, including isoprostanes, fatty acid alcohols, ketones, and epoxides (26, 32–35). Considering that many oxylipins are unstable under extreme alkaline or acidic conditions (36–38), it is important to balance hydrolysis efficiency and structural preservation of the analytes. To achieve this, we explored mild alkaline conditions for their efficacies to hydrolyze oxylipins esterified to complex lipids, including PLs. We established the optimal base concentration at 0.2 M KOH and tested the hydrolysis efficiency at this concentration on human plasma at various temperatures and incubation times. The majority of base-stable metabolites that are generated by enzymes, including the fatty acid epoxides, are contained in PLs (22). Thus, we focused on the PL fraction to measure hydrolysis efficiency. The mass chromatograms for several PL classes taken before and after base hydrolysis indicated that the mildest condition, 0.2 N KOH at 37°C for 30 min, was sufficient to hydrolyze >95% of the plasma PLs (**Fig. 3**). There was some remaining sphingomyelin, which contains N-linked fatty acids that are more resistant to hydrolysis, even at 60°C. No lysoPLs were detectable post hydrolysis, which indicates that the PLs were not converted to the lyso moieties and the hydrolysis step effectively released all sn1 and sn2 fatty acids (Fig. 3B). We also subjected some selected oxylipin standards to the same conditions and observed that at 37°C and 30 min, the base-induced destruction of these metabolites was least, as exemplified by the recovery of intact 7-hydroxydocosahexaenoic acid (**Fig. 4**). Like the nondeuterated metabolites, the degradation of the deuterated ISTDs increased similarly with increasing temperature and time. As a result, the sensitivity and precision of the analysis decreases proportionally. Our data show that the hydrolysis condition of 0.2 N KOH at 37°C for 30 min provides the optimal balance between hydrolysis efficiency and structural preservation of the analytes. Deviating from these conditions augments metabolite degradation and low abundance metabolites may fall below the lower limit of detection. Furthermore, reproducibility decreases with increasing degradation and, consequently, the precision of the analysis deteriorates.

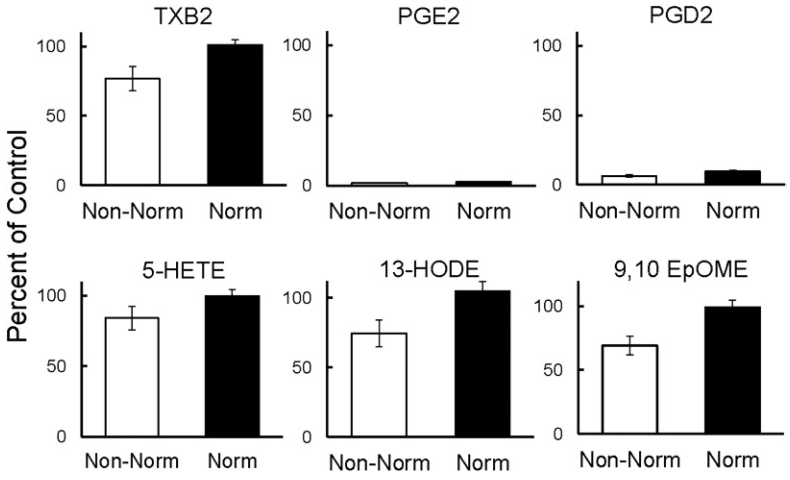

Fig. 6. Recoveries of oxylipins under alkaline hydrolysis conditions. A set of pure oxylipin standards (136 metabolites) was subjected to alkaline hydrolysis conditions, purified by SPE, and analyzed by LC-MS. Recoveries were determined by comparing MS intensities of the standards after alkaline treatment with MS intensities of the untreated standards that were analyzed in parallel and served as controls. The open bars show non-normalized recoveries (Non-Norm), which were calculated from the raw MS peak areas without normalization to ISTDs; the closed bars show the same data set but normalized to ISTD (Norm). All data are expressed as percent of untreated controls. The mean and SD of five replicate measurements are displayed. Shown is a representative subset of standards (see supplemental Table S2 for the complete data set).

TABLE 1. List of eicosanoids and related oxylipins that can be reliably measured after alkaline hydrolysis

Pathway	Analyte	Day 1 Recovery (%)	Day 2 Recovery (%)	Day 3 Recovery (%)	Average Recovery (%)	CV (%)
COX	11 HEPE	94	82	81	85	10
	11 HETE	96	98	102	98	8
	12 HHTRE	86	91	102	93	11
	PGF_{1a}	110	94	104	102	12
	d17 6k PGF_{1a}	117	92	117	108	14
	6k PGF_{1a}	105	87	99	97	10
	PGF_{2a}	102	92	105	100	11
	2,3 dinor 11b PGF_{2a}	104	103	94	101	9
	dh PGF_{2a}	109	92	89	96	13
	20oh PGF_{2a}	110	107	90	103	11
	PGF_{3a}	97	91	92	93	6
	TXB_1	100	105	101	102	6
	TXB_2	101	110	95	103	7
	TXB_3	98	117	94	103	11
LOX	5 HETE	100	93	89	94	8
	8 HETE	99	101	106	102	7
	12 HETE	100	105	98	100	7
	Tetranor 12-HETE	114	99	118	113	11
	15 HETE	102	106	95	100	9
	5 HETrE	116	105	97	106	10
	8 HETrE	105	104	97	101	7
	15 HETrE	105	110	98	105	9
	9 HODE	92	104	101	99	8
	13 HODE	118	116	103	111	11
	9 HOTrE	98	109	109	106	9
	13 HOTrE (y)	113	116	105	110	12
	LTB_4	91	100	93	95	7
	14 HDoHE	97	93	95	95	7
	17 HDoHE	107	101	91	99	10
	7(R) Maresin-1	101	80	86	88	12
CYP	9,10 EpOME	99	105	107	104	7
	12,13 EpOME	98	100	98	99	7
	18 HEPE	90	80	83	84	10
	5,15 diHETE	108	83	108	99	15
	19,20 diHDPA	111	85	93	97	15
	8,9 diHETrE	100	88	84	90	12
	11,12 diHETrE	113	94	119	110	15
	14,15 diHETrE	100	99	85	95	12
	9,10 diHOME	107	97	103	103	6
	12,13 diHOME	107	103	101	103	5
	8,9 EET	97	93	94	96	15
	11,12 EET	106	98	97	100	5
	14,15 EET	104	94	102	101	8
	14,15 EpETE	87	85	93	88	9
Non-enzyme	4 HDoHE	106	86	91	94	12
	7 HDoHE	87	81	80	82	9
	8 HDoHE	97	100	90	95	10
	10 HDoHE	99	107	98	101	7
	11 HDoHE	101	96	91	95	12
	13 HDoHE	120	110	93	107	13
	16 HDoHE	100	99	93	97	6
	20 HDoHE	100	102	94	99	7
	9 HETE	104	106	101	102	9
	8 iso PGF2a III	103	91	103	99	9
	2,3 dinor 8-iso PGF2a	99	98	91	96	10
	8-iso PGF3a	96	105	96	98	12

The set of PSTDs was supplemented with ISTDs, subjected to alkaline hydrolysis conditions, and analyzed by LC-MS. An identical set of untreated standards was analyzed in parallel and served as control. The analyses were performed in five replicates on each of three consecutive days. Recoveries after alkaline treatment were calculated by comparing the mass spectral intensities with those of untreated standards and after normalization to ISTDs. Shown is the list of metabolites that were recovered at 80–120% and with a CV of ⩽15%. A list of all abbreviations is provided on the website (http://www.lipidmaps.org/data/standards/standards.php?lipidclass=LMFA).

Analysis of eicosanoids after alkaline treatment

Next, we expanded our stability tests and examined our entire library of PSTDs for their resistance to base-induced degradation (**Fig. 5**). As shown in **Fig. 6** and supplemental Table S2, many of the eicosanoids are susceptible to base-induced degradation, as indicated by the changes in their mass spectral intensities. The data shown represent the average of five replicate measurements performed on a single day. In particular, PGs and leukotrienes (LTs) were virtually undetectable. The exceptions were PGF_{2a} and LTB_4, which were resistant to degradation (supplemental Table S2). Additionally, fatty acids containing hydroxy,

TABLE 2. Total eicosanoids and other oxylipins in stimulated RAW macrophages that satisfy our selection criteria for accurate measurement

		Total Oxylipins				Free Oxylipins				Esterified	
		Stimulated		Control		Stimulated		Control			
Pathway	Analyte	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Stimulated	Control
		pmol/1E6 cells				*pmol/1E6 cells*				%	%
COX	11 HEPE	ND	ND	0.18	0.02	ND	ND	ND	ND	ND	100
	11 HETE	2.48	0.43	0.33	0.03	1.84	0.27	0.35	0.06	26	0
	PGF_{2a}	1.36	0.15	0.29	0.02	1.09	0.11	0.29	0.01	19	0
LOX	5 HETE	1.85*	0.48	1.25	0.05	0.16*	0.04	0.21	0.05	92	83
	8 HETE	0.23	0.03	0.42	0.07	0.03*	0.01	0.02	0.00	89	94
	12 HETE	0.33	0.03	0.18	0.01	ND	ND	ND	ND	100	100
	15 HETE	1.07	0.16	0.28	0.03	0.59	0.13	0.21	0.06	45	25
	5 HETrE	0.33	0.02	0.19	0.03	0.07*	0.01	0.07	0.01	80	62
	8 HETrE	0.14*	0.05	0.11	0.02	ND	ND	0.02	0.01	100	83
	15 HETrE	0.13	0.03	0.03	0.00	0.08	0.01	0.02	0.01	37	8
	9 HODE	0.66	0.14	0.23	0.04	0.71	0.29	0.19	0.03	0	16
	13 HODE	0.23	0.01	0.11	0.03	0.18	0.04	0.05	0.01	19	51
	13 HOTrE (y)	0.16	0.04	0.03	0.00	ND	ND	ND	ND	100	100
	14 HDoHE	0.40	0.04	0.15	0.02	0.14	0.01	0.02	0.01	66	86
CYP	9,10 EpOME	0.78	0.15	0.94	0.17	0.13	0.02	0.05	0.01	83	95
	12,13 EpOME	0.68	0.13	0.79	0.12	0.15	0.03	0.05	0.01	77	94
	18 HEPE	ND	ND	0.15	0.02	ND	ND	ND	ND	ND	100
	11,12 diHETrE	0.07	0.03	0.11	0.02	ND	ND	ND	ND	100	100
	19,20 diHDPA	0.12	0.05	0.16	0.02	ND	ND	0.02	0.00	100	89
	9,10 diHOME	0.08	0.01	0.03	0.02	0.05	0.03	0.01	0.00	46	72
	12,13 diHOME	0.07	0.01	0.04	0.01	0.05	0.02	0.01	0.00	34	69
	8,9 EET	0.69*	0.30	0.87	0.14	ND	ND	0.03	0.02	100	97
	11,12 EET	1.56	0.32	2.52	0.28	ND	ND	ND	ND	100	100
	14,15 EET	2.03	0.61	3.66	0.74	ND	ND	0.04	0.01	100	99
	14,15 EpETE	ND	ND	0.32	0.16	ND	ND	ND	ND	ND	100
Non-enzyme	4 HDoHE	2.01	0.15	2.53	0.26	0.16	0.00	0.08	0.01	92	97
	7 HDoHE	0.43*	0.05	0.41	0.06	ND	ND	ND	ND	100	100
	8 HDoHE	0.93	0.31	1.43	0.36	ND	ND	0.1	0.00	100	93
	10 HDoHE	0.20	0.04	0.10	0.02	ND	ND	0.03	0.01	100	75
	11 HDoHE	0.23*	0.04	0.22	0.03	0.06*	0.03	0.04	0.02	76	83
	13 HDoHE	0.63	0.17	0.09	0.02	0.24	0.08	0.06	0.01	62	36
	16 HDoHE	0.25	0.01	0.10	0.02	0.03*	0.01	0.02	0.01	86	82
	20 HDoHE	0.67	0.12	0.39	0.06	ND	ND	0.07	0.03	100	81
	9 HETE	0.29	0.05	0.55	0.11	ND	ND	ND	ND	100	100

RAW macrophages were stimulated with KdO2-lipid A and ATP and the total eicosanoids were measured in lipid extracts after saponification (Stimulated). As a control, unstimulated cells were processed identically (Control). For comparison, the levels of cell-associated free metabolites were measured in parallel. The mean and SD of triplicate measurements are displayed. The esterified fraction is expressed as percent of the total (Esterified). ND, not detected. All metabolites changed significantly ($P < 0.05$) upon stimulation, except the ones marked with an asterisk (*). A list of all abbreviations is provided on the website (http://www.lipidmaps.org/data/standards/standards.php?lipidclass=LMFA).

di-hydroxy, or epoxy groups showed resistance to degradation to various degrees. Even though some degradation may have occurred during the saponification step, when we normalized the spectral data of the nondeuterated eicosanoids to their deuterated analogs, we were able to neutralize the loss for many of the eicosanoids. For example, the nonnormalized "raw" recovery of thromboxane (TX) B_2 was about 78%. However, when we normalized the value to its deuterated analog measured under identical conditions, the recovery was calculated to be 100%. Similarly, the normalized recoveries were quantitative for 5-HETE, 13-HODE, and 9,10-EpOME, compared with the nonnormalized recoveries of about 70–80% based on raw mass spectral peak areas (Fig. 6). As a class, the epoxides and diols are quite stable under basic conditions (33), and many of them were recovered quantitatively from the standard mix under alkaline hydrolysis conditions (**Table 1**). For some eicosanoids, normalization to ISTDs did not improve the overall recovery. This applies specifically to the ones with more complex structural elements, including PGs and LTs as well as ketones (supplemental Table S2).

Most naturally occurring PGs have a considerable potential for hydrolysis, dehydration, or isomerization, depending on their immediate environment (39). PGs contain multiple hydroxyl groups and keto groups and a rigid five-member prostane ring. The resulting β-hydroxy ketone system is unstable and readily undergoes dehydration under acidic or basic conditions to A- or B-type PGs (36, 40). Alternatively, PGD and PGE can oxidize to the 9,11-diketones, PGK_1, and PGK_2, which were generated and increased about 3-fold during alkaline hydrolysis. Furthermore, bicyclo PGE_2, a base-catalyzed breakdown product of PGE_2 and 13,14-dihydro-15-keto PGE_2 (dhk PGE_2) was found to be substantially increased after alkaline hydrolysis (supplemental Table S2). These breakdown or conversion products cannot be reliably measured after alkaline hydrolysis and should not be included in quantitative analyses of esterified eicosanoids.

Precision of the method

For the method to be applicable to biological samples, it has to be accurate and reliable. For this purpose, we

TABLE 3. Total eicosanoids and other oxylipins in human plasma that satisfy our selection criteria for accurate measurement

		Total Oxylipins		Free Oxylipins		
Pathway	Analyte	Mean (pmol/ml)	SD (pmol/ml)	Mean (pmol/ml)	SD (pmol/ml)	Esterified (%)
COX	11 HEPE	335.03	40.59	104.94	17.67	69
	11 HETE	4,692.85	1,011.69	2,400.56	1,112.21	49
	PGF_{2a}	20.94	2.11	12.49	2.38	40
LOX	5 HETE	8,447.45	579.75	4,493.63	547.71	47
	8 HETE	9,495.39	1,504.95	1,750.21	207.60	82
	Tetranor 12-HETE	42.49	4.08	6.97	1.46	84
	12 HETE	10,988.43	1,631.18	1,865.15	179.14	83
	15 HETE	7,386.67	744.52	1,883.51	44.28	75
	5 HETrE	143.86	27.52	103.80	7.52	28
	8 HETrE	7,057.36	1,139.55	1,317.45	130.96	81
	15 HETrE	1,076.25	171.56	280.08	7.38	74
	9 HODE	17,145.92	3,441.52	6,628.18	721.65	61
	13 HODE	14,788.06	564.63	6,065.74	1,056.66	59
	9 HOTrE	565.75	155.94	114.86	3.47	80
	13 HOTrE (y)	244.55	17.07	86.16	7.68	65
	14 HDoHE	1,459.56	206.03	467.66	207.09	68
	17 HDoHE	1,888.64	297.49	359.21	54.17	81
CYP	9,10 EpOME	19,274.99	857.23	6,024.43	1,114.28	69
	12,13 EpOME	23,762.05	1,515.63	8,998.90	1,662.62	62
	18 HEPE	300.29	39.73	103.15	9.80	66
	5,15 diHETE	57.97	7.73	66.89	32.40	0
	8,9 diHETrE	14.37	3.10	4.21	0.61	71
	11,12 diHETrE	4.89	1.34	1.35	0.42	72
	14,15 diHETrE	11.91	0.40	3.78	0.67	68
	9,10 diHOME	27.07	1.45	7.22	1.03	73
	12,13 diHOME	10.27	1.17	6.41	1.03	38
	8,9 EET	8,719.22	1,600.98	1,308.17	179.90	85
	14,15 EET	90.28	9.69	26.41	2.82	71
	14,15 EpETE	6,405.69	469.56	1,871.33	20.30	71
Non-enzyme	4 HDoHE	827.04	142.28	262.82	47.55	68
	7 HDoHE	314.59	44.92	76.16	15.12	76
	8 HDoHE	11,437.32	1,610.95	1,187.05	110.70	90
	10 HDoHE	64.55	10.68	23.63	4.41	63
	11 HDoHE	1,700.85	311.31	337.79	23.51	80
	13 HDoHE	670.10	108.67	176.54	60.99	74
	16 HDoHE	1,046.17	144.11	257.29	9.26	75
	20 HDoHE	1,392.57	83.96	168.07	0.57	88
	9 HETE	44,523.25	1,009.61	5,479.52	355.19	88

For comparison, the levels of the free metabolites isolated without saponification were measured in parallel. The mean and SD of triplicate measurements are displayed. The esterified fraction is expressed as percent of the total (esterified). A list of all abbreviations is provided on the website (http://www.lipidmaps.org/data/standards/standards.php?lipidclass=LMFA).

compiled a panel of eicosanoids that were either resistant to base-induced degradation or suffered only minor destruction and were reproducibly recovered. Each metabolite in our library of standards was measured in five replicate measurements on three consecutive days with and without saponification, normalized to ISTDs, and averaged. The recovery after alkaline hydrolysis was calculated and the precision was expressed as the coefficient of variation.

For practical purposes, we used a cut-off point of 80–120% normalized recovery and a precision (CV) of 15% or less to assemble a list of eicosanoids that can be reproducibly measured (Table 1). Of the 136 eicosanoids in the standard mix that we used for this purpose (supplemental Table S2), 56 metabolites satisfied these criteria and were included in the list. The 3 day average was close to 100% recovery for most of these metabolites. These data indicated that the assay is reproducible and, with careful selection of ISTDs for normalization and neutralization of potential losses, the method is useful for the analysis of selected esterified eicosanoids.

Application of the method to measure esterified eicosanoids in biological samples

To establish usefulness, we applied this protocol to the analysis of esterified eicosanoids in biological samples, including RAW cells, a cell model of mouse macrophages (41, 42), and human plasma (43). For the cell model, mouse RAW macrophages were activated with the Toll-like receptor agonist Kdo2-lipid A, and ATP and eicosanoids were analyzed in their free and esterified form in both stimulated and unstimulated control cells. Considering the limitations outlined in Table 1 and using the algorithm for the identification of stable metabolites, we identified and quantified a number of eicosanoids that were present in their free and esterified form (**Table 2**). In total, we detected 34 metabolites that met the criteria for inclusion. In the unstimulated control cells, the COX-derived metabolites, 11-HETE and PGF_{2a}, were present only in their free form. In contrast, metabolites formed by cytochrome P450 and nonenzymatic pathways were mainly found in their esterified form. As can be seen, most eicosanoids increased

during stimulation. The eicosanoid ratio (esterified vs. free) in the stimulated cells is a reflection of de novo synthesis, hydrolysis by phospholipase A_2, and release into the extracellular medium. Of course it is possible that some eicosanoids are tightly bound to receptors or carrier proteins even during SPE purification, but are released upon base hydrolysis and would therefore be included in the "esterified" measure. Additionally, it is well-established that PGs, including PGF_{2a}, are formed and rapidly secreted during stimulation with Toll-like receptor agonists and ATP (41). Consequently, there may be a relative increase in the esterified portion due to secretion of metabolites in their free form. Of note, the data contain the cell-associated free eicosanoids and do not include the secreted fraction.

We next examined esterified eicosanoids in human plasma. In all, we recovered 38 eicosanoids after base hydrolysis (**Table 3**). For comparison, we also measured these metabolites in their free form as previously reported (3, 43). The dynamic range for the total eicosanoids spans several orders of magnitude from about 5 to 44,500 pmol/ml and, on average, about 70% of all eicosanoids were esterified. A potential matrix effect adds to the complexity of the measurement. The matrix effect was determined previously by spiking the ISTD mixture into a plasma sample (29). The results showed that the recovery of ISTDs that are resistant to alkaline-induced degradation is greater than 80%, indicating that matrix effects and ion suppression are minimal. The plasma measurements were carried out on 50 μl of plasma of which the equivalent of 10 μl was injected into the UPLC/MS for an analysis. Thus, the lower limits of quantification for eicosanoid measurements in biological material are in the low femtomole range.

CONCLUSION

In this study, we assessed the suitability of base hydrolysis to quantify esterified eicosanoids in biological material. We showed that about 75% of all cell-associated eicosanoids were esterified. Globally this percentage remained largely unchanged between control and Kdo2-lipid A/ATP-stimulated cells, even though some selected metabolites increased either in their free or esterified form. In a previous report, we demonstrated the presence of free eicosanoids in human plasma (43) and found that the levels are correlated with various inflammatory and metabolic diseases, including nonalcoholic fatty liver disease (27). In the current study, we expanded our method to include esterified eicosanoids in the analysis. Overall, we fine-tuned the alkaline hydrolysis conditions to minimize metabolite degradation, adjusted the prepurification steps to enhance metabolite recovery, optimized the assignment of ISTDs to compensate for potential losses, and established an algorithm based on recovery and reproducibility to compile a table of metabolites that can be accurately measured. When taking these limitations into consideration, this method can now be successfully applied to accurately measure the sum total, i.e., free and esterified oxylipins, in human plasma and a variety of biological samples.

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