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RESEARCH PAPER

Reversed phase UHPLC/ESI-MS determination of oxylipins in human plasma: a case study of female breast cancer

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Abstract

The ultrahigh-performance liquid chromatography-mass spectrometry (UHPLC/MS) method was optimized and validated for the determination of oxylipins in human plasma using the targeted approach with selected reaction monitoring (SRM) in the negative-ion electrospray ionization (ESI) mode. Reversed phase UHPLC separation on an octadecylsilica column enabled the analysis of 63 oxylipins including numerous isomeric species within 12-min run time. The method was validated (calibration curve, linearity, limit of detection, limit of quantification, carry-over, precision, accuracy, recovery rate, and matrix effect) and applied to 40 human female plasma samples from breast cancer patients and age-matched healthy volunteers (control). Thirty-six oxylipins were detected in human plasma with concentrations above the limit of detection, and 21 of them were quantified with concentrations above the limit of quantitation. The concentrations determined in healthy controls are in a good agreement with previously reported data on human plasma. Quantitative data were statistically evaluated by multivariate data analysis (MDA) methods including principal component analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA). S-plot and box plots showed that 13-HODE, 9-HODE, 13-HOTrE, 9-HOTrE, and 12-HHTrE were the most upregulated oxylipin species in plasma of breast cancer patients.

Keywords Oxylipins · Eicosanoids · UHPLC/MS · Breast cancer · Statistical analysis · Human plasma

Introduction

Breast cancer, a malignant tumor resulting from an uncontrolled growth of cells of the mammary gland, represents the most common tumor in women [1] with minor occurrence in men as well. Cancer is caused by mutations or other abnormalities in genes responsible for regulating the cellular growth and proliferation. Breast cancer is initiated by somatic (85–90%) or inherited germ-line (5–10%) mutations. Cells of the ducts draining milk from lobules to nipple or in cells of the lobules (milk-producing glands) usually are the origin of breast cancer. Histologically, epithelial mammary tumors can be distinguished into non-invasive and invasive. Histological subtypes of invasive breast cancer include tubular, medullary, mucinous, papillary, and cribriform variants [2]. Rather than a single disease entity, breast cancer represents a spectrum of malignant tumors affecting one organ [3]. Based on the expression of hormone receptors and human epidermal growth factor receptor (HER)-2, tumors with different biology and clinical presentation that require different therapeutic strategies may be defined [4]. Currently, the diagnosis of breast cancer is usually only based on imaging techniques (usually mammography, with biopsy being mandated in case of suspected malignancy). Biomarkers play an increasingly important role in the management of cancer patients [3]. Lipids are an essential integral part of cell membranes and lipid composition can change during an uncontrolled growth of tumor cells, as reported for breast tumor cell lines [5], breast [5, 6], and kidney tumor tissues [7, 8]. Lipids may represent potential drugs as well as targets for novel therapies [9, 10].

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Oxylipins represent a class of bioactive lipids formed from polyunsaturated fatty acids (PUFA), such as arachidonic acid (AA), linoleic acid (LA), α-linolenic acid (αLnA), eicosapentaenoic acid (EPA), dihomo-γ-linolenic acid (DGLnA), adrenic acid (AdA), and docosahexaenoic acid (DHA). Oxylipins are associated with a number of physiological functions, including inflammation, apoptosis, cell proliferation, blood clotting, regulation of blood pressure, reproduction, diuresis, and modulation of the immune and nervous systems. Changes in the oxylipin metabolism have been linked with a range of pathological conditions, including ischemic heart diseases, cancer, obesity, diabetes, or arthritis [11–15]. Eicosanoids, octadecanoids, and docosanoids are major groups of oxylipins. Eicosanoids are the most well-known oxylipins, being formed from 20-carbon PUFA (AA, DGLnA, and EPA), octadecanoids from 18-carbon PUFA (LA and αLnA), and docosanoids from 22-carbon PUFA (DHA and AdA). PUFA are formed by the elongation and desaturation of αLnA and LA (essential fatty acids) into longer-chain PUFA or from the diet [15]. Oxylipins are also known as short half-life substances acting locally and being generated in situ when needed and not being stored. However, not all oxylipins have a short half-life, as evidenced by the constant concentrations of both free and esterified oxylipins in kidney, ileum, adipose, and liver tissues [16, 17].

Oxylipins are generated by either enzymatic pathway using cyclooxygenase (COX), lipoxygenase (LOX), cytochrome P450 (CYP450) enzymes, or non-enzymatic pathway. First, PUFA are released from glycerophosholipids by the action of phospholipase A_2 (PLA_2) enzyme (Fig. 1). The location of polyunsaturated acyls is mostly in *sn-2* position of the glycerol backbone of glycerophospholipids [11–13, 18–20]. At least 15 groups of PLA_2 enzyme with different biological roles have been reported so far, which can be sorted in five categories including secreted PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), calcium-independent PLA_2, platelet-activating factor acetyl hydrolase, and lysosomal PLA_2. $cPLA_2$ enzyme with the supplementary role of $sPLA_2$ is considered as the principal enzyme for PUFA release from glycerophospholipids and subsequent eicosanoid biosynthesis [21, 22].

COX enzyme produces prostanoids (prostaglandins and thromboxanes) with one or more double bonds and 5-carbon ring at 8- to 12-carbon. COX enzyme also produces selected hydroxy-FA, such as 11-hydroxy-eicosatetraenoic acid (11-HETE) from AA or 13-hydroxy-docosahexaenoic acid (13-HDoHE) from DHA. LOX enzyme catalyzes the formation of hydroxy-FA and the metabolites including leukotrienes, lipoxins, resolvins, protectins, and hepoxilins. Oxylipins with epoxygenase or ω-hydroxylase activity are formed by CYP450 enzyme; for example, EPA can be converted into epoxy-eicosatetraenoic acid (EpETE) by this enzyme [15]. Isoprostanes are an example of non-enzymatic pathway, and they are generated from arachidonic acid [21].

These enzymatic and non-enzymatic pathways produce hundreds of oxylipin isomers with similar structures, but possible different physico-chemical properties. This fact makes the analysis of oxylipins very difficult and challenging. Other major issue is the concentration in plasma or serum, which is the lowest among all endogenous lipid metabolites (pmol/mL) [11].

The most frequently used technique for the analysis of oxylipins is the coupling of liquid chromatography and mass spectrometry (LC/MS), especially targeted tandem mass spectrometry using SRM with the triple quadrupole (QqQ) mass analyzer being the best choice for this type of scan events. As oxylipins possess a carboxylic group, negative-ion ESI is the most sensitive for their analysis. The separation of oxylipins is typically performed on an octadecylsilica (C18) column, which provides the highest selectivity for the separation of isobaric oxylipins. For example, Dennis et al. [13] developed an UHPLC/MS method for the analysis of 184 eicosanoids and 26 deuterated internal standards in 5 min, whereby Kortz et al. [14] determined 94 oxidized metabolites and 7 PUFAs using LC/MS. Komaba et al. [23] developed an on-line two-dimensional reversed phase LC/MS-MS method for the simultaneous determination of prostaglandins E2, F2α, and 13, 14-dihydro-15-keto prostaglandin F2α. In the past, oxylipins have been analyzed by immunoassay techniques, such as radioimmunoassay (RIA) and enzyme immunoassays (EIA), or gas chromatography-mass spectrometry (GC/MS), but these methods have serious limitations in the identification and quantitation of multiple oxylipins or involve a time-consuming derivatization step of labile oxylipins for GC/MS [11–13]. For example, Shono et al. [24] analyzed PGE2 by a heterologous enzyme immunoassay using a stable enzyme-labeled hapten mimic or Reinke [25] monitored thromboxane in body fluids, where oxylipin 11-dehydrothromboxane B2

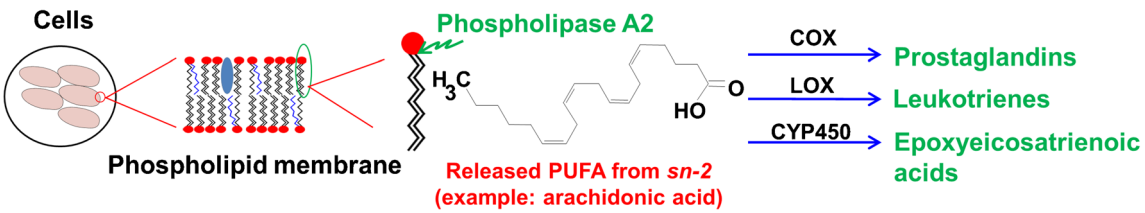

Fig. 1 Scheme of biosynthesis of eicosanoids derived from arachidonic acid (AA) via cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450) pathways

were analyzed by a specific enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody. GC/MS and GC/MS/MS [26] were applied to assess in vivo synthesis of prostaglandins, leukotrienes, thromboxane, and isoprostanes in humans.

The aim of this paper was the development of a sensitive targeted method using UHPLC/MS with SRM transitions for the quantitative analysis of a large number of oxylipins, including the full validation. This method was applied to real plasma samples of breast cancer patients and healthy volunteers to elucidate possible differentiation of both groups by MDA.

Materials and methods

Chemicals and standards

Acetonitrile, 2-propanol, ethanol (all HPLC/MS grade), and acetic acid (≥ 99.99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was prepared with a Milli-Q Reference Water Purification System (Molsheim, France). Sixty-three oxylipin standards and 14 deuterated internal standards were purchased from Cayman Chemical (Ann Arbor, MI, USA). The full list of purchased oxylipin standards is shown in Table S1 in the Electronic Supplementary Material (ESM). Plasma of female breast cancer patients (20 samples) and healthy volunteers (20 samples) were obtained from the Department of Surgery, Atlas Hospital Zlín in the Czech Republic, based on the institutional ethical agreement and signed information consent (ESM Table S2).

Sample preparation

For the optimization of SRM method, the stock solution of oxylipin standards at the concentration 5 ng/μL was prepared in ethanol and stored at − 80 °C until use. Samples of human plasma were prepared by solid phase extraction (SPE) using Strata-X polymeric reversed-phase cartridges (8B-S100-FBJ-T, Phenomenex, Aschaffenburg, Germany). Plasma (250 μL) was deproteinized with 1 mL of acetonitrile, vortexed and centrifuged at 6000 rpm for 10 min at ambient temperature. The supernatant was diluted 10 times with water for next steps of SPE. Before SPE, cartridges were washed with 3 mL of methanol and then equilibrated with 3 mL of water. Then, the sample was loaded, with a subsequent wash with 3 mL of 10% aqueous methanol. Oxylipins were eluted with 3 mL of methanol, and then dried under nitrogen at ambient temperature and redissolved in 50 μL of mobile phase A (acetonitrile–water–acetic acid (45/55/0.02, *v*/*v*/*v*) mixture). For the quantitation of oxylipins, 10 μL of a mixture of internal standards was added to human plasma before the extraction to prepare finale concentrations of all standards 10 pg/μL except for d_4-LTB4 and d_4-9,10-DiHOME (5 pg/μL).

RP-UHPLC/ESI-MS conditions

UHPLC/MS analyses were performed on a liquid chromatograph Agilent 1290 Infinity series (Agilent Technologies, Waldbronn, Germany) coupled with AB Sciex 6500 QTRAP—hybrid quadrupole-linear ion trap mass spectrometer (Sciex, Framingham, MA, USA). The following conditions were used for the separation of oxylipins: Acquity UPLC BEH C18 VanGuard Precolumn (5 × 2.1 mm × 1.7 μm), Acquity UPLC BEH C18 column (150 × 2.1 mm × 1.7 μm), flow rate 0.4 mL/min (during analysis 0–12 min) and 0.5 mL/min (wash step 12.5–14.5 min), injection volume 5 μl, column temperature 40 °C, and mobile phase gradient: 0 min—2% B, 12 min—62% B, 12.5–14.5 min—99% B, and 15–20 min—2% B, where A was a mixture of acetonitrile–water–acetic acid (45/55/0.02, *v*/*v*/*v*), and B was a mixture of acetonitrile–2-propanol (50/50, *v*/*v*).

Mass spectrometer was equipped with a Turbo V ion source. Oxylipins were measured in negative-ion ESI mode with the following conditions: capillary voltage − 4 kV, temperature 525 °C, curtain gas (CUR) 20 psi, nebulizer gas (GS1) 50 psi, turbo gas (GS2) 50 psi, and declustering potencial (DP) − 80 V. Oxylipins were analyzed using SRM and optimized collision energy for each oxylipin (see Table 1 for details).

Method validation

The stock solution of 14 internal (deuterated) oxylipin standards at the concentration of 0.1 mg/mL was prepared in ethanol. Calibration standard samples were prepared by the dilution of the IS stock solution (1:1) with dilution factors of 10, 20, 50, 100, 500, 1000, 2500, 5000, and 10,000, and every calibration sample level contained the same volume of pooled plasma. The limit of detection (LOD) and the limit of quantification (LOQ) were determined based on signal to noise ratio (S/N) as $S/N = 3$ and $S/N = 10$, respectively. Ten-times diluted and 500-times diluted calibration standard samples were injected 6 times to evaluate the repeatability (relative standard deviation, RSD, %) of peak areas. The carry-over for all IS was also measured after the calibration standard sample at high concentration level.

Pooled plasma was spiked with 5 endogenous oxylipin standards (8,15-DiHETE, 11-HEPE, LTB4, 7-HDoHE, and PGF2α) and LPE (14:0) standard to prepare the final concentration of 12 pg/μL used as the quality control (QC) sample during the validation and the quantitative study of plasma

Table 1 SRM conditions with PUFA precursors and metabolic pathways for 63 oxylipin standards and 14 deuterated oxylipin internal standards

Lipid class	Oxylipin species	PUFA precursor	Metabolic pathway	Precursor ion $[M-H]^-$	Main product ion	t_R [min]	CE [V]	IS
FA and conjugates	tetranor-12-HETE	AA	CYP450	265	109	5.29	− 16	D_4-9,10-DiHOME
Octadecanoids	9-HOTrE	αLnA	LOX	293	171	6.12	− 20	D_6-20-HETE
	13-HOTrE	αLnA	LOX	293	195	6.36	− 21	D_6-20-HETE
	13-OxoODE	LA	LOX	293	113	7.94	− 26	D_8-15-HETE
	9-OxoODE	LA	LOX	293	185	8.34	− 27	D_8-12-HETE
	13-HODE	LA	LOX	295	195	7.65	− 25	D_4-13-HODE
	9-HODE	LA	LOX	295	171	7.82	− 23	D_4-9-HODE
	12(13)-EpOME	LA	CYP450	295	195	9.26	− 18	D_{11}-14,15-EET
	9(10)-EpOME	LA	CYP450	295	171	9.48	− 19	D_{11}-8,9-EET
	12,13-DiHOME	LA	CYP450	313	183	4.67	− 30	D_4-12,13-DiHOME
	9,10-DiHOME	LA	CYP450	313	201	4.99	− 30	D_4-9,10-DiHOME
Eicosanoids	12-HHTrE	AA	COX	279	179	5.47	− − 17	D_4-9,10-DiHOME
	15-HEPE	EPA	LOX	317	219	6.74	−17	D_6-20-HETE
	11-HEPE	EPA	LOX	317	167	6.78	− 22	D_6-20-HETE
	5-HEPE	EPA	LOX	317	255	7.61	− 17	D_4-13-HODE
	14(15)-EpETE	EPA	CYP450	317	207	8.17	− 18	D_8-12-HETE
	12-OxoETE	AA	LOX	317	273	8.61	− 19	D_8-12-HETE
	15-OxoETE	AA	LOX	317	273	8.70	− 21	D_8-15-HETE
	5-OxoETE	AA	LOX	317	203	9.75	− 22	D_7-5-OxoETE
	15-HETE	AA	LOX	319	219	7.95	− 17	D_8-15-HETE
	11-HETE	AA	LOX	319	167	8.38	− 17	D_8-12-HETE
	12-HETE	AA	LOX	319	179	8.62	− 20	D_8-12-HETE
	8-HETE	AA	LOX	319	155	8.64	− 21	D_8-12-HETE
	9-HETE	AA	LOX	319	167	8.83	− 22	D_8-12-HETE
	5-HETE	AA	LOX	319	257	9.28	− 18	D_8-5-HETE
	11,12-EET	AA	CYP450	319	167	9.90	− 17	D_{11}-11,12-EET
	5,6-EET	AA	CYP450	319	191	10.37	− 17	D_{11}-11,12-EET
	15-HETrE	DGLnA	LOX	321	221	8.84	− 22	D_8-5-HETE
	5-HETrE	DGLnA	LOX	321	259	10.97	− 19	D_{11}-11,12-EET
	tetranor-PGDM	AA	COX	327	247	0.88	− 22	D_4-PGF2α
	PGJ2	AA	COX	333	189	3.21	− 20	D_4-PGF2α
	PGB2	AA	COX	333	235	3.21	− 25	D_4-PGF2α
	PGA2	AA	COX	333	271	3.60	− 20	D_4-PGF2α
	15-deoxy-12,14 PGD2	AA	COX	333	271	4.44	− 20	D_4-PGF2α
	8,15 DiHETE	AA	LOX	335	235	3.76	− 21	D_4-LTB4
	6-trans LTB4	AA	LOX	335	195	4.13	− 22	D_4-LTB5
	LTB4	AA	LOX	335	195	4.39	− 20	D_4-LTB6
	5,15-DiHETE	AA	LOX	335	173	4.50	− 18	D_4-LTB7
	5,6-DiHETE	AA	LOX	335	145	5.47	− 26	D_4-9,10-DiHOME
	14,15-DiHETrE	AA	CYP450	337	207	5.35	− 22	D_4-9,10-DiHOME
	5,6-DiHETrE	AA	CYP450	337	145	7.19	− 23	D_4-13-HODE
	PGH2	AA	COX	351	271	1.77	− 20	D_4-PGF2α
	PGE2	AA	COX	351	271	1.77	− 23	D_4-PGF2α
	11β-PGE2	AA	COX	351	271	1.87	− 19	D_4-PGF2α
	15-keto-PGF2	AA	COX	351	219	1.92	− 23	D_4-PGF2α
	PGD2	AA	COX	351	271	1.93	− 23	D_4-PGF2α
	13,14-dh-15-k-PGE2	AA	COX	351	315	2.41	− 21	D_4-PGF2α
	8-iso-PGF2	AA	COX	353	193	1.42	− 28	D_4-PGF2α

Table 1 (continued)

Lipid class	Oxylipin species	PUFA precursor	Metabolic pathway	Precursor ion $[M-H]^-$	Main product ion	t_R [min]	CE [V]	IS
	(+/−) 5-iPF2α-VI	AA	non-enzymatic	353	115	1.58	− 24	D_4-PGF2α
	PGF2α	AA	COX	353	309	1.62	− 30	D_4-PGF2α
	13,14-dh-15-k-PGF2	AA	COX	353	291	2.35	− 33	D_4-PGF2α
	6-keto-PGF1	AA	COX	369	245	1.13	− 30	D_4-PGF2α
	TXB2	AA	COX	369	195	1.44	− 21	D_4-PGF2α
Docosanoids	20-HDoHE	DHA	CYP450	343	281	7.37	− 20	D_4-13-HODE
	17-HDoHE	DHA	LOX	343	281	7.91	− 21	D_8-15-HETE
	10-HDoHE	DHA	LOX	343	153	8.29	− 21	D_8-12-HETE
	14-HDoHE	DHA	LOX	343	205	8.30	− 20	D_8-12-HETE
	11-HDoHE	DHA	LOX	343	149	8.47	− 18	D_8-12-HETE
	7-HDoHE	DHA	LOX	343	281	8.67	− 18	D_8-5-HETE
	8-HDoHE	DHA	LOX	343	189	8.79	− 18	D_8-5-HETE
	4-HDoHE	DHA	LOX	343	281	9.51	− 15	D_{11}-14,15-EET
	19,20-DiHDPE	DHA	CYP450	361	273	5.29	− 23	D_4-9,10-DiHOME
	Resolvin D1	DHA	LOX	375	141	2.27	− 21	D_4-PGF2α
Deuterated IS	D_4-13-HODE			299	198	7.65	− 25	
	D_4-9-HODE			299	172	7.82	− 23	
	D_4-9,10-DiHOME			317	203	4.40	− 30	
	D_4-12,13-DiHOME			317	185	4.67	− 30	
	D_7-5-oxoETE			323	209	9.50	− 22	
	D_6-20-HETE			325	281	6.46	− 20	
	D_8-15-HETE			327	226	7.95	− 17	
	D_8-12-HETE			327	184	8.62	− 20	
	D_8-5-HETE			327	265	9.28	− 18	
	D_{11}-14,15-EET			330	175	8.90	− 17	
	D_{11}-8,9-EET			330	155	9.65	− 17	
	D_{11}-11,12-EET			330	167	9.90	− 17	
	D_4-LTB_4			339	197	4.39	− 20	
	D_4-PGF2α			357	313	1.55	− 30	

FA fatty acid, *PUFA* polyunsaturated fatty acid, *AA* arachidonic acid, *αLnA* α-linolenic acid, *LA* linoleic acid, *EPA* eicosapentaenoic acid, *DGLnA* dihomo-γ-linolenic acid, *DHA* docosahexaenoic acid, *COX* cyclooxygenase, *LOX* lipoxygenase, *CYP450* cytochrome P450, and *IS* internal standard used for the quantitation

samples. The extraction efficiency was investigated using extracts of pooled sample spiked with IS before the extraction and extracts of pooled sample spiked after the extraction at two concentration levels (high and low level) [27, 28]. The high level (HL) was prepared by adding 30 μL of mixture of IS (final concentration 30 pg/μL except for d_4-LTB4 and d_4–9,10-DiHOME, where concentrations were 15 pg/μL) and the low level (LL) 5 μL of mixture of IS (final concentration 5 pg/μL except d_4-LTB4 and d_4–9,10-DiHOME, where concentrations were 2.5 pg/μL) to the pooled sample (see the "Sample preparation" section). The matrix effect was examined by comparing peak areas of solution of pure IS and extracts of 6 individual plasma samples spiked with IS after the extraction. The precision was calculated from pooled plasma samples spiked before the extraction at low and high concentration levels. The within-run precision (accuracy) was measured using 3 pooled plasma samples for low and high concentration levels in a single run. The between-run precision (accuracy) was determined on three days among three different runs. The precision was expressed by RSD (%). The accuracy was studied using pooled plasma samples spiked after the extraction (high and low level), where concentrations were compared with the nominal values obtained from the calibration curves and expressed as percentage. The stability was investigated using three individual plasma samples spiked by IS before the extraction. These samples were left in the autosampler and reinjected after 4, 8, and 24 h. The stability was calculated by the comparison of peak areas at 0 h vs. 4, 8, and 24 h in the autosampler, and results were expressed by percentage.

Data analysis

The Analyst 1.6.2 software allowed the determination of peak areas of investigated oxylipins. SIMCA software version 13.0 (Umetrics AB, Umeå, Sweden) was used for the statistical data analysis. Particular box plots were constructed in R free software environment [29] using readxl and ggplot2 packages.

Results and discussion

Optimization and validation of RP-UHPLC/ESI-MS method

The optimization of UHPLC/MS method has started from conditions described in prior study [11] with minor improvements. The full optimization of column type, mobile phases with additives, gradient, and flow rate for the qualitative analysis of 46 oxylipins has been reported previously [11], which is now extended to a wider range of oxylipin species (i.e., 9-HOTrE, 9(10)-EpOME, 12(13)-EpOME, 9,10-DiHOME, 11-HEPE, 14(15)-EpETE, 15-OxoETE, 12-OxoETE, 5-OxoETE, 8-HETE, 9-HETE, 5-HETrE, 17-HDoHE, 10-HDoHE, 11-HDoHE, 7-HDoHE, 8-HDoHE, 20-HDoHE, 11β-PGE2, and (±)-5-iPF2α-VI). The whole list of oxylipin standards used during the method development is shown in Table 1. Figure 2 shows chromatograms of the separation of 63 oxylipin standards (Fig. 2a) and 14 deuterated oxylipin internal standards (Fig. 2b). Other modifications are the use of a VanGuard Pre-column to increase the column life-time and an improved sample preparation protocol, which significantly reduced contamination of the mass spectrometer during the analysis of biological samples, because our previously developed method suffered from serious problems with the contamination of mass spectrometer during the analysis of larger sample sets, which is corrected by presented improvements of sample preparation presented in this work.

SPE technique is the best choice for the extraction of oxylipins from biological samples, such as human plasma, due to their low concentrations. SPE has a better ability to eliminate the contamination than liquid/liquid extraction (LLE) techniques, which improves the sensitivity in biological samples [13]. The polymeric Strata-X reversed-phase column has been used for SPE of oxylipins [11] with minor modifications. Two hundred fifty microliters of human plasma has been used for the isolation of oxylipins. Acetonitrile was applied for the denaturation due to the high efficiency in protein precipitation. The order of effectiveness of deproteinization for most common organic solvents was published as follows: acetonitrile>acetone>ethanol>methanol [30].

The most common approach for the quantitative analysis of oxylipins in biological samples is targeted UHPLC/MS with SRM. All oxylipins have been measured in the negative-ion mode, and each oxylipin standard has been optimized for SRM, including the selection of SRM pairs for each oxylipin (m/z values for precursor and product ions, respectively) and collision energies. MS/MS spectra of all oxylipins have been measured at various collision energies (from − 35 to − 15 V) to select the most sensitive SRM transition. Figure S1 (see ESM) shows MS/MS spectra of all 63 oxylipin standards at optimized collision energies (see Table 1), and Fig. S2 (see ESM) shows MS/MS spectra of 12-HETE at various collision energies to illustrate the optimization of collision energy. As the final SRM transition for the targeted method, the SRM transition with the highest sensitivity is used. Neutral losses of H_2O ($\Delta m/z = 18$—dehydration) and CO_2 ($\Delta m/z = 44$—decarboxylation) were observed for all oxylipin MS/MS spectra. The loss of water is common for all compounds containing oxygen; therefore, SRM transition is not very specific and should be avoided, if possible. For most oxylipins, the most abundant fragmentation pathway is related to the hydroxyl group, respectively, to the carbonyl group. Optimized SRM parameters for all standards are listed in Table 1.

The UHPLC/MS method was validated for 14 deuterated internal oxylipin standards in line with the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) recommendations [27, 28] and included the determination of extraction recovery, matrix effect, linearity of calibration curve, carry-over, stability, within-run, and between-run precision (accuracy). The validation was performed with pooled sample prepared as a mixture of human plasma from healthy volunteers and breast cancer patients (ESM Tables S3 and S4). The calibration curve was prepared for all 14 deuterated IS with a minimum of 6 concentration levels. The coefficient of determination (R^2) of calibration curves ranges from 0.9991 to 0.9996 for all IS, which confirms a good linearity of this method. The repeatability values expressed as RSD are from 0.46 to 2% (10× diluted calibration sample) and from 0.35 to 5.96% (500× diluted calibration sample), which illustrates very good repeatability. The blank sample was measured after the high concentration level of the calibration curve (10× diluted calibration sample) in order to determine the carry-over, which is less than 0.5% for all IS. The extraction recovery for all IS ranges from 71.2 to 102.8% for the low concentration level and from 84.3 to 98.3% for the high concentration level. Within-run and between-run precision (for LL and HL) is expressed by RSD and values for these parameters are within 15% for all 14 internal standards; only 20-HETE shows worse precision for LL. Accuracy values are also within ± 15% except for PGF2α and 20-HETE at the LL with accuracy values ± 20%. These results confirm the applicability for the determination of oxylipins in human plasma samples. Values of matrix effect are in the range from 107 to 162%, reflecting the ion enhancement. The ion enhancement/

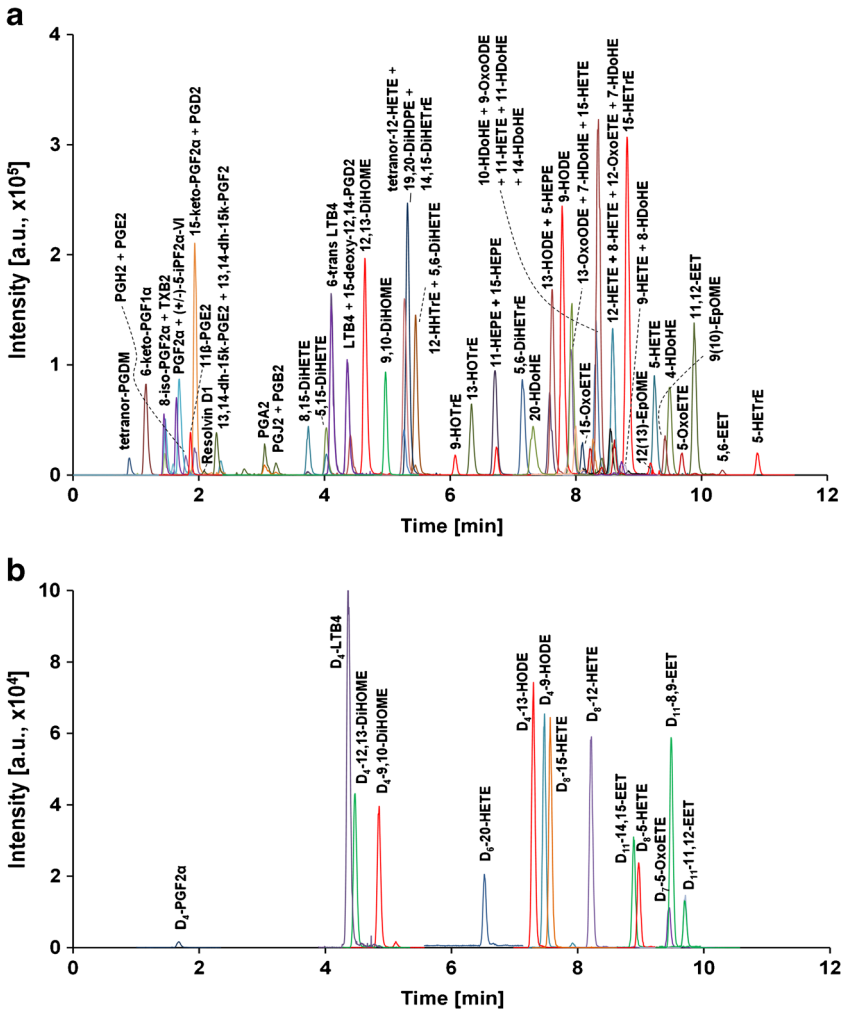

Fig. 2 Extracted ion chromatograms in RP-UHPLC/ESI-MS analysis of **a** mixture of 63 oxylipin standards and **b** mixture of 14 deuterated oxylipin internal standards using negative-ion mode and SRM scans. Other details in the "Materials and methods" section

suppression effects for certain retention windows are compensated by the use of 14 deuterated IS added to individual samples.

On other hand, small ion suppression is observed for PGF2α (67.9%). The stability of standards was between 95.5 and 112% after 4 h, between 94.5 and 113% after 8 h,

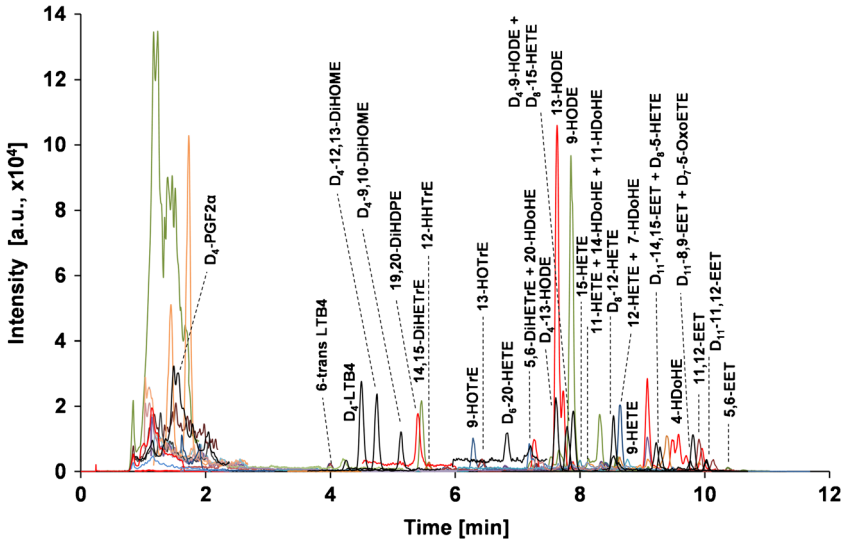

Fig. 3 Extracted ion chromatograms in RP-UHPLC/ESI-MS analysis of human plasma sample. Other details in the "Materials and methods" section

Table 2 Comparison of concentrations of oxylipins in human plasma samples determined in the present study and reported in the literature (pmol/mL)

Sample type	Human female plasma [14]	Human plasma [16]	Human male plasma [19]	Human plasma [32]	Human plasma [33]	Human serum [34]	Human male serum [35]	Human male serum [36]	Human plasma [37]	Human plasma [38]	Human plasma [39]	Human female plasma [40]	Average values from literature [14, 16, 19, 32–39]	Human female plasma[a]
Number of subjects	22	10	6	10	100	26	20	10	70	10	100	40		20
9-HOTrE	–	0.41 ± 0.07	–	0.35 ± 0.47	0.50 ± 0.05	–	0.44 ± 0.07	0.38 ± 0.10	1.19 ± 0.91	–	–	NQ-0.72	0.56	4.00 ± 2.12
13-HOTrE	–	0.33 ± 0.05	–	–	0.49 ± 0.02	–	0.44 ± 0.07	0.38 ± 0.07	1.11 ± 0.74	–	–	NQ-1.54	0.55	2.04 ± 1.11
13-HODE	7.43		–	13.27 ± 12.07	10.60 ± 1.28	20.03 ± 6.01	8.30 ± 0.82	6.80 ± 1.10	58.20 ± 28.00	10.00 ± 1.30	–	1.71–22.84	16.83	16.50 ± 5.97
9-HODE	7.22	8.50 ± 1.34	–	7.03 ± 6.14	6.80 ± 1.67	22.80 ± 6.05	6.00 ± 0.72	5.10 ± 0.88	11.00 ± 6.10	9.40 ± 1.80	–	2.25–17.84	9.32	18.13 ± 7.16
12-HHTrE	0.94	–	–	1.06 ± 0.78	2.03 ± 0.28	–	–	–	–	0.30 ± 0.14	–	–	1.09	0.51 ± 0.60
15-HETE	1.85	1.09 ± 0.18	4.08 ± 1.11	1.32 ± 0.35	0.80 ± 0.02	–	1.30 ± 0.19	1.40 ± 0.29	2.04 ± 1.20	0.91 ± 0.22	2.40 ± 0.64	NQ-1.92	1.72	0.93 ± 0.50
11-HETE	0.72	0.79 ± 0.14	0.99 ± 0.33	–	0.73 ± 0.06	–	0.69 ± 0.13	0.73 ± 0.21	0.40 ± 0.36	0.95 ± 0.19	–	NQ-0.84	0.75	0.88 ± 0.45
12-HETE	–	0.72 ± 0.13	9.75 ± 7.82	5.47 ± 4.04	4.22 ± 0.29	28.19 ± 7.09	22.10 ± 5.20	19.80 ± 7.00	3.95 ± 3.30	1.00 ± 0.20	6.80 ± 1.50	NQ-1.95	10.20	13.96 ± 13.47
9-HETE	–	0.27 ± 0.05	1.03 ± 0.38	–	1.70 ± 0.08	–	0.18 ± 0.01	0.20 ± 0.01	0.17 ± 0.16	0.78 ± 0.30	–	NQ-0.57	0.62	1.18 ± 0.36
11,12-EET	–	0.24 ± 0.04	0.51 ± 0.70	–	–	–	1.30 ± 0.17	0.18 ± 0.05	1.02 ± 1.40	–	–	–	0.65	3.47 ± 2.84
5,6-EET	–	–	–	–	0.11 ± 0.02	–	–	–	–	–	–	–	0.11	1.35 ± 0.82
6-trans LTB4	–	–	–	–	0.11 ± 0.03	–	–	–	< 0.10	–	–	–	0.11	0.26 ± 0.08
LTB4	–	–	< 0.03	–	0.04 ± 0.01	–	–	–	< 0.10	–	–	–	0.04	0.24 ± 0.20
14,15-DiHETrE	1.46	0.81 ± 0.12	4.31 ± 1.78	0.95 ± 0.31	1.45 ± 0.07	–	0.69 ± 0.04	0.67 ± 0.03	0.60 ± 0.18	0.54 ± 0.05	–	–	1.28	3.17 ± 1.35
5,6 DiHETrE	0.99	–	1.15 ± 0.67	0.33 ± 0.09	0.61 ± 0.06	–	–	–	0.19 ± 0.09	0.19 ± 0.03	–	–	0.58	0.52 ± 0.28
20-HDoHE	–	–	–	–	0.23 ± 0.02	–	–	–	–	1.00 ± 0.48	–	–	0.62	1.28 ± 0.65
14-HDoHE	–	–	–	2.81 ± 2.21	1.64 ± 0.13	–	–	–	–	0.57 ± 0.13	–	NQ-1.92	1.67	2.81 ± 3.53
11-HDoHE	–	–	–	–	0.18 ± 0.01	–	–	–	–	0.57 ± 0.22	–	–	0.38	0.48 ± 0.47
7-HDoHE	–	–	–	–	0.131 ± 0.02	–	–	–	–	0.40 ± 0.18	–	–	0.27	9.03 ± 10.82
4-HDoHE	–	–	–	–	5.30 ± 1.31	–	–	–	–	0.56 ± 0.18	–	NQ-0.33	2.93	3.34 ± 2.03
19,20-DiHDPE	–	2.58 ± 0.38	–	1.71 ± 0.52	1.23 ± 0.39	–	2.10 ± 0.15	2.10 ± 0.20	0.81 ± 0.42	1.20 ± 0.14	–	–	1.68	1.79 ± 1.03

[a] Oxylipin concentrations in healthy human female plasma samples measured in the present study with the standard deviations for the whole data set

and between 92.3 and 116.7% after 24 h in the autosampler. Results from validation are summarized in Tables S3 and S4 (see ESM). The monitoring of QC peak areas during the method validation confirms the signal stability (ESM Fig. S3).

Determination of oxylipins in human plasma samples

The quantitation of oxylipins is a difficult task due to the trace concentrations. The selection of appropriate isotopically labeled IS is a crucial point in the analysis. Before the detection and quantitation, the separation of oxylipins is essential due to the large number of isomeric oxylipins, e.g., differing only in the position of double bond or hydroxyl group, which is the reason why we cannot use the same concept for the quantitation as for phospholipids and sphingolipids, where one class of lipids elutes in one peak and just one internal standard is used for each class of lipids [31]. In the case of oxylipins, deuterated IS are necessary, because they are chemically and structurally related to oxylipins analogs, but they have different precursor/product pair for SRM scans, and they are not naturally occurring in plasma. The serious issue is an acquirement of deuterated IS, because they are not commercially available for all oxylipins, and they are expensive. For the quantitation of 63 oxylipins, we choose 14 deuterated IS covering the whole retention window of our method. The typical chromatogram with reconstructed ion current for individual oxylipins in real human plasma sample is shown in Fig. 3 with 21 quantified oxylipins and 14 deuterated IS. Table S5 (see ESM) provides the full data with concentrations of individual quantified oxylipins in all studied samples, which have been used for the statistical evaluation.

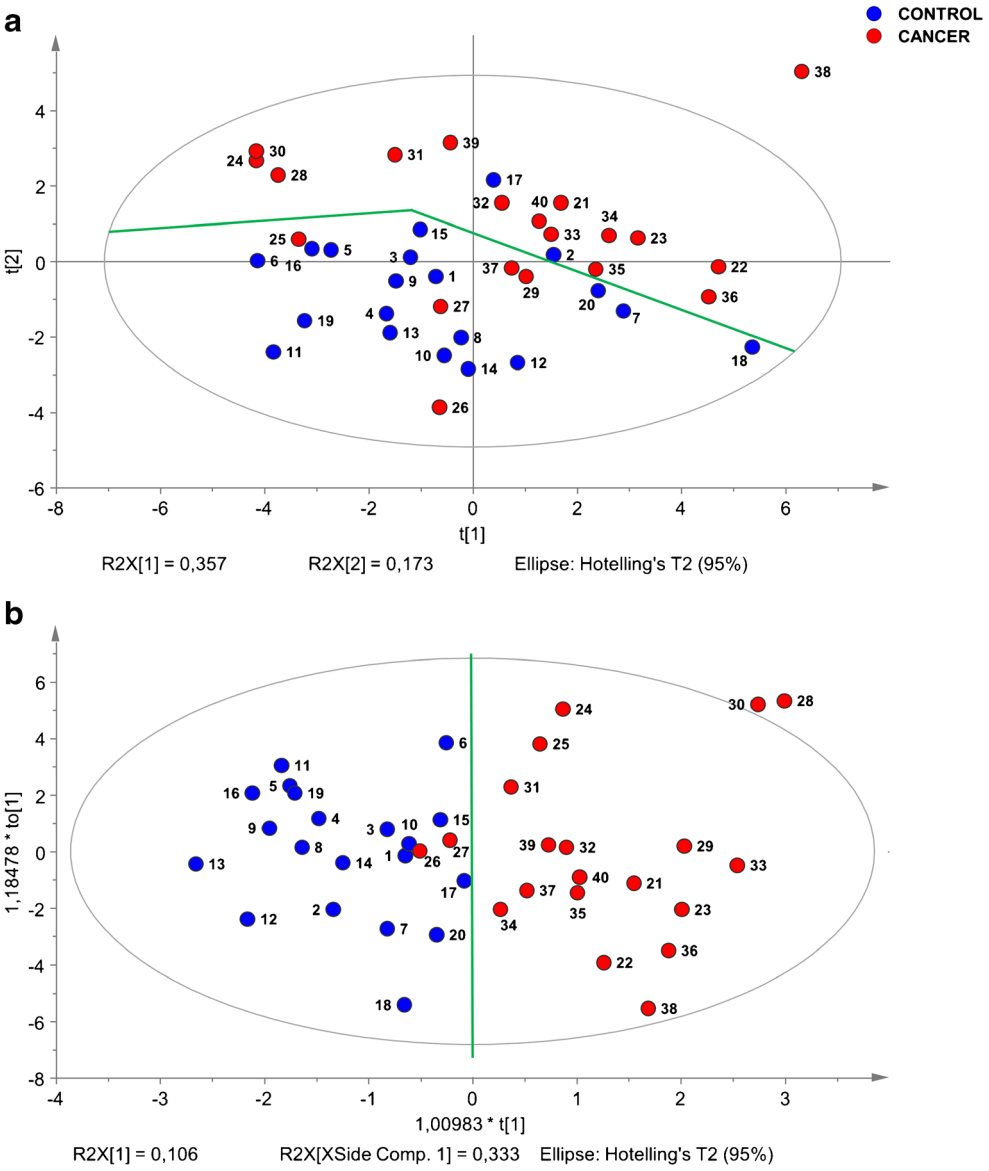

Fig. 4 Multivariate data analysis models for 20 breast cancer patients (red dots) and 20 healthy controls (blue dots) with labeled numbers of individual subjects according to ESM Table S2: (a) unsupervised PCA and (b) supervised OPLS-DA

Oxylipins PGF2α, 9-HODE, 13-HODE, 9,10-DiHOME, 12,13-DiHOME, 5-OxoETE, 20-HETE, 5-HETE, 15-HETE, 12-HETE, 8,9-EET, 11,12-EET, 14,15-EET, and LTB4 have own deuterated analogs for the quantitation. Other oxylipins have to be quantified using the nearest deuterated IS (see Table 1 for details); for example, we used D_4-13-HODE IS for the quantitation not only for 13-HODE, but also for 5,6-DiHETrE, 20-HDoHE, and 5-HEPE. In this work, we quantified oxylipins in 40 samples of human plasma from healthy volunteers and breast cancer patient. The breast cancer affects mostly women, so we measured human plasma samples only from women (ESM Table S2). We also carefully selected samples according to the age of women. Thus, the mean of age of breast cancer patients was 55.5 (interval from 47 to 66 years) and for healthy volunteers 56.5 (interval from 46 to 66 years).

The mixture of deuterated IS was added to each sample at known concentration before the extraction, and the amount of oxylipins in human plasma was obtained by comparing peak areas of analyte and deuterated IS, and concentrations of oxylipins are expressed in absolute values (pmol/mL). The full list of quantified oxylipins is shown in Table S6 (see ESM) with the mean concentrations in human female plasma of breast cancer patients and healthy volunteers together with statistical parameters (fold change, *p* values, *T* values, and VIP (variable importance in projection) values), where *p* values are calculated using *T* test and fold change as a ratio of mean concentrations of oxylipin species in breast cancer human plasma to healthy controls. We are able to quantify 21 oxylipins in human plasma including 6-trans LTB4, LTB4, 19,20-DiHDPE, 14,15-DiHETrE, 12-HHTrE, 13-HOTrE, 9-HOTrE, 5,6-DiHETrE, 20-HDoHE, 14-HDoHE, 11-HDoHE, 7-HDoHE, 4-HDoHE, 13-HODE, 9-HODE, 15-HETE, 12-HETE, 11-HETE, 9-HETE, 11,12-EET, and 5,6-EET. The concentration of most oxylipins in breast cancer patients' plasma is increased compared to healthy controls, which is in agreement with the previous report [32]. The comparison of determined concentrations of 21 oxylipins for healthy volunteers with those reported in literature is

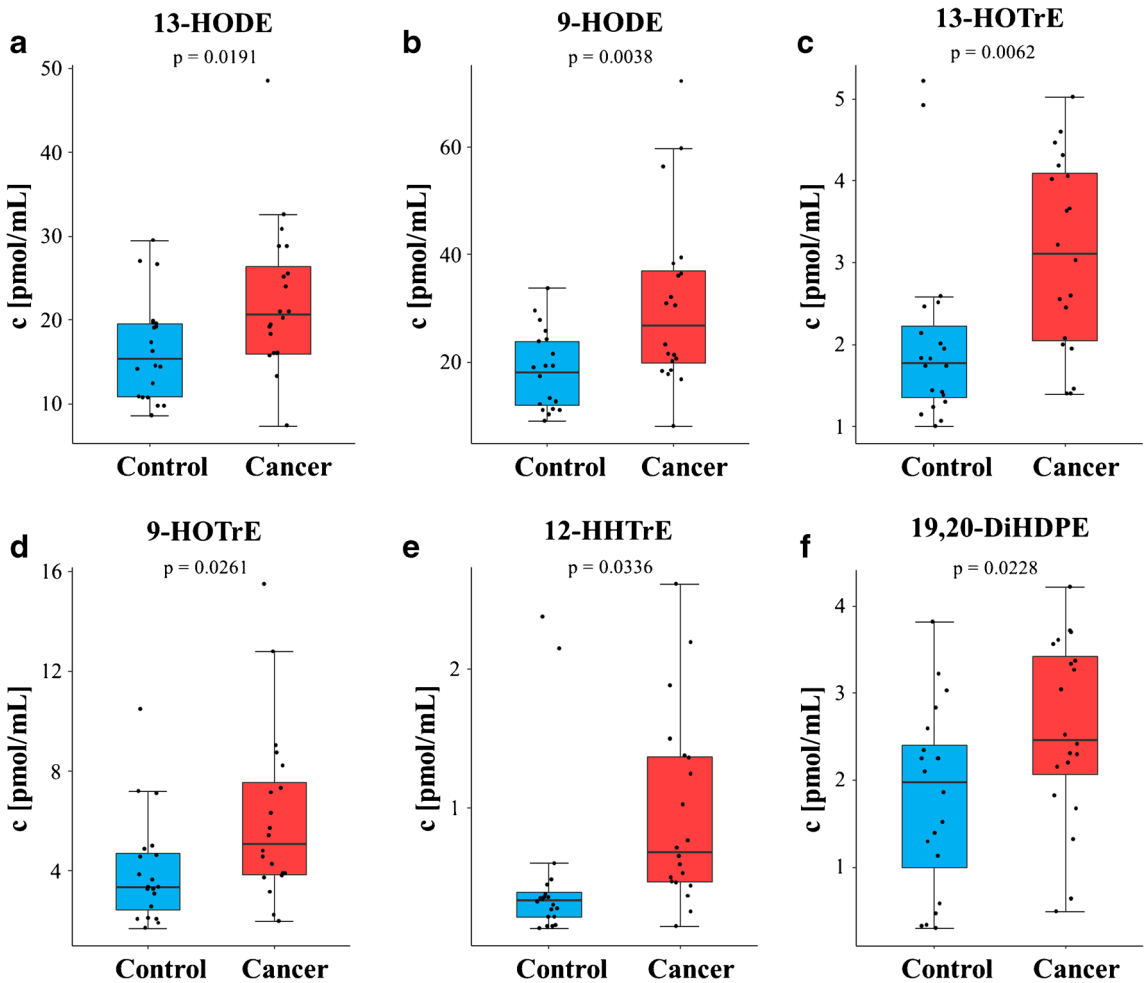

Fig. 5 Box plots of the most upregulated oxylipins in human plasma of breast cancer patients: **a** 13-HODE, **b** 9-HODE, **c** 13-HOTrE, **d** 9-HOTrE, **e** 12-HHTrE, and **f** 19,20-DiHDPE

shown in Table 2, which is an important proof that our determined concentrations in plasma can be correlated with previous works [14, 16, 19, 32–40]. It should not be overlooked that in some cases differences among reported values from some papers are not negligible; therefore, we have calculated mean values from the literature for comparison with our values for healthy volunteers (Table 2). We are also able to detect more oxylipins above the LOD but below the LOQ; therefore, they cannot be quantified: tetranor 12-HETE, 5,6-DiHETE, 15-HEPE, 5-HEPE, 14,15-EpETE, 10-HDoHE, 15-HETrE, 5-OxoETE, 12,13-DiHOME, 9,10-DiHOME, 13-OxoODE, 9-OxoODE, 5-HETE, 9(10)-EpOME, and 12(13)-EpOME.

Multivariate data analysis of oxylipin quantitative data

Lipidomic data were statistically evaluated by MDA methods, such as unsupervised PCA and supervised OPLS-DA. The complete list of determined concentrations of 21 oxylipins for all human subjects is provided in Table S5 (see ESM). The unit variance (UV) scaling and the logarithmic transformation were used before the statistical analysis. Models are described using Q^2 and R^2 parameters [6]. Fractions of the sum of squares all X and Y (just X for PCA) predicted by the model according to the cross validation are described by parameter Q^2 and fractions of the sum of squares of all X and Y (just X for PCA) that the model can explain using the latent variables, are described by R^2. Unsupervised PCA model had 4 components, parameter $R^2(X)$ had value 0.711, Q^2 0.365 (Fig. 4a). OPLS-DA model has 1 + 3 + 0 components, $R^2(X)$ 0.606, $R^2(Y)$ 0.680, and Q^2 0.203. The supervised OPLS-DA model (Fig. 4b) brings the improvement of group separation, where red dots represent cancer samples and blue dots control samples. The receiver operating characteristics (ROC) curve (ESM Fig. S4) for OPLS-DA model provided 90% sensitivity, 100% specificity, and 95% accuracy.

S-plot was created from OPLS-DA model providing the information about most up- or downregulated lipid species in human breast cancer plasma samples (ESM Fig. S5). Upregulated oxylipin species are in the upper right corner and downregulated oxylipin species are in the bottom left corner. Oxylipin species with a low statistically significance are found in the middle part of S-plot. Box plots of the most upregulated lipid species (13-HODE, 9-HODE, 13-HOTrE, 9-HOTrE, 12-HHTrE, and 19,20-DiHDPE) are shown in Fig. 5. p values are lower than 0.05 only for 6 from 21 determined oxylipins, which is in correlation with the fact that Q^2 values are relatively low, and therefore, the robustness of such statistical models may be also lower. Four of 5 most upregulated oxylipins belong to the family of octadecanoids from by lipoxygenase (LOX) pathways from linoleic acid (9-HODE and 13-HODE) and linolenic acid (9-HODE and 13-HODE) [15]. There is a lack of the literature information of biological interpretation of observed changes in cancer.

Conclusions

We developed and validated UHPLC/ESI-MS method for the determination of 63 oxylipins in human plasma, which was applied to a small case study of breast cancer patients. We reported here concentrations for 21 oxylipins determined by our high-throughput quantitative assay in female plasma from 20 breast cancer patients and 20 age-matched healthy controls. The concentration of some oxylipins are upregulated for cancer patients; in some cases, the increase is statistically significant (13-HODE, 9-HODE, 13-HOTrE, 9-HOTrE, and 12-HHTrE) based on p values > 0.05 and fold changes in the range 1.5–2. Unsupervised PCA indicates only minor but visible grouping of cancer and control groups, but supervised OPLS-DA improves the group resolution with rather good ROC characteristics. It is important to point out that this work is just small pilot case study with relatively low number of human subjects, and the potential for early cancer diagnosis and therapy monitoring should be carefully evaluated after larger prospective cohort in the future.

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Compliance with ethical standards

The study was approved by the institutional ethical committee. All patients and healthy volunteers signed informed consent.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Pelengaris S, Khan M. The molecular biology of cancer: a bridge from bench to bedside. 2nd ed. Oxford: Wiley-Blackwell; 2013.
2. Weiss MC. Breast cancer information and support [Internet]. Breast cancer.org. 2018. Available from: https://www.breastcancer.org/. Accessed 26 July 2018.
3. Melichar B. Laboratory medicine and medical oncology: the tale of two Cinderellas. Clin Chem Lab Med. 2013;51:99–112.

4. Melichar B, Hornychova H, Kalabova H, Basova H, Mergancova J, Urminska H, et al. Increased efficacy of a dose-dense regimen of neoadjuvant chemotherapy in breast carcinoma: a retrospective analysis. Med Oncol. 2012;29:2577–85.
5. Cifkova E, Lisa M, Hrstka R, Vrana D, Gatek J, Melichar B, et al. Correlation of lipidomic composition of cell lines and tissues of breast cancer patients using hydrophilic interaction liquid chromatography/electrospray ionization mass spectrometry and multivariate data analysis. Rapid Commun Mass Spectrom. 2017;21:253–63.
6. Cifkova E, Holcapek M, Lisa M, Vrana D, Gatek J, Melichar B. Determination of lipidomic differences between human breast cancer and surrounding normal tissues using HILIC-HPLC/ESI-MS and multivariate data analysis. Anal Bioanal Chem. 2015;407: 991–1002.
7. Jirasko R, Holcapek M, Khalikova M, Vrana D, Student V, Prouzova Z, et al. MALDI orbitrap mass spectrometry profiling of dysregulated sulfoglycosphingolipids in renal cell carcinoma tissues. J Am Soc Mass Spectrom. 2017;28:1562–74.
8. Hajek R, Lisa M, Khalikova M, Jirasko R, Cifkova E, Student V, et al. HILIC/ESI-MS determination of gangliosides and other polar lipid classes in renal cell carcinoma and surrounding normal tissues. Anal Bioanal Chem. 2018;410:6585–94.
9. Melichar B, Konopleva M, Hu W, Melicharova K, Andreeff M, Freedman RS. Growth-inhibitory effect of a novel synthetic triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-oic acid, on ovarian carcinoma cell lines not dependent on peroxisome proliferator-activated receptor-g expression. Gynecol Oncol. 2004;93:149–54.
10. Krop IE, Mayer IA, Ganju V, Dickler M, Johnston S, Morales S, et al. Pictilisib for estrogen receptor-positive, aromatase inhibitor-resistant, advanced or metastatic breast cancer (FERGI): a randomised, double-blind, placebo-controlled trial. Lancet Oncol. 2016;17:811–21.
11. Berkecz R, Lisa M, Holcapek M. Analysis of oxylipins in human plasma: comparison of utrahigh-performance liquid chromatography and ultrahigh-performance supercritical fluid chromatography coupled to mass spectrometry. J Chromatogr A. 2017;1511:107–21.
12. Strassburg K, Huijbrechts AML, Kortekaas KA, Lindeman JH, Pedersen TL, Dane A, et al. Quantitative profiling of oxylipins through comprehensive LC-MS/MS analysis: application in cardiac surgery. Anal Bioanal Chem. 2012;404:1413–26.
13. Wang Y, Armando AM, Quehenberger O, Yan C, Dennis EA. Comprehensive ultra-performance liquid chromatographic separation and mass spectrometric analysis of eicosanoids metabolites in human samples. J Chromatogr A. 2017;1359:60–9.
14. Kortz L, Dorow J, Becker S, Thiery J, Ceglarek U. Fast liquid chromatography-quadrupole linear ion trap-mass spectrometry analysis of polyunsaturated fatty acids and eicosanoids in human plasma. J Chromatogr B. 2013;927:209–13.
15. Gabbs M, Leng S, Devassy JG, Monirujjaman M, Aukema HM. Advances in our understanding of oxylipins derived from dietary PUFAs. Adv Nutr. 2015;6:513–40.
16. Schebb NH, Ostermann AI, Yang J, Hammock BD, Hahn A, Schuchardt JP. Comparison of the effects of long-chain omega-3 fatty acid supplementation on plasma levels of free and esterified oxylipins. Prostaglandins Other Lipid Mediat. 2014;113-115:21–9.
17. Yamada H, Oshiro E, Kikuchi S, Hakozaki M, Takahashi H, Kinura K. Hydroxyeicosapentaenoic acids from the Pacific krill show high ligand activities for PPARs. J Lipid Res. 2014;55:895–904.
18. Massey KA, Nicolaou A. Lipidomics of oxidized polyunsaturated fatty acids. Free Radic Biol Med. 2013;59:45–55.
19. Shinde DD, Kim KB, Oh KS, Abdalla N, Liu KH, Bae SK, et al. LC-MS/MS for the simultaneous analysis of arachidonic acid and 32 related metabolites in human plasma: basal plasma concentrations and aspirin-induced changes of eicosanoids. J Chromatogr B. 2012;911:113–21.
20. Song J, Liu X, Wu J, Meehan MJ, Blevitt JM, Dorrestein PC, et al. A highly efficient, high-throughput lipidomics platform for the quantitative detection of eicosanoids in human whole blood. Anal Biochem. 2013;433:181–8.
21. Buczynski MW, Dumlao DS, Dennis EA. Thematic review series: proteomics. An integrated omics analysis of eicosanoids biology. J Lipid Res. 2009;50:1015–38.
22. Burke JE, Dennis EA. Phospholipase A_2 structure/function, mechanism, and signaling. J Lipid Res. 2009;50(Suppl):S237–42.
23. Komaba JJ, Matsuda D, Shibakawa K, Nakade S, Hashimoto Y, Miyata Y, et al. Development and validation of an online-two-dimensional reversed-phase liquid chromatography-tandem mass spectrometry method for the simultaneous determination of prostaglandin E2, F2α, and 13,14-dihydro-15-keto prostaglandin F2α levels in human plasma. Biomed Chromatogr. 2009;23:315–23.
24. Shono F, Yokota K, Horie K, Yamamoto S, Yamashita K, Watanabe K, et al. A heterologous enzyme immunoassay of prostaglandin E2 using a stable enzyme-labeled hapten mimic. Anal Biochem. 1988;168:284–91.
25. Reinke M. Monitoring thromboxane in body fluids: a specific ELISA for 11-dihydrothromboxane B2 using a monoclonal antibody. Am J Physiol Endocrinol Metab. 1992;262:E658–62.
26. Tsikas D. Application of gas chromatography-mass spectrometry and gas chromatography-tandem mass spectrometry to assess in vivo synthesis of prostaglandins, thromboxane, leukotrienes, isoprostanes and related compounds in humans. J Chromatogr B. 1998;717:201–45.
27. Agency EMA. Committee for Medicinal Products for Human Use (CHMP) [Internet]. European medicines agency. 2011. Available from: https://www.ema.europa.eu/en/committees/committee-medicinal-products-human-use-chm. Accessed 10 July 2018.
28. Food U, Administration D. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research: Rockville. 2001.
29. The R foundation. The R project for statistical computing [Internet]. R-project.org. 2018. Available from: https://www.r-project.org/. Accessed 26 July 2018.
30. Blanchard J. Evaluation of the relative efficacy of various techniques for deproteinizing plasma samples prior to high-performance liquid chromatographic analysis. J Chromatogr B. 1981;226:455–60.
31. Cifkova E, Hajek R, Lisa M, Holcapek M. Hydrophilic interaction liquid chromatography-mass spectrometry of (lyso)phosphatidics, (lyso)phosphatidylserines and other lipid classes. J Chromatogr A. 2016;1439:65–73.
32. Loomba R, Quehenberger O, Armando A, Dennis EA. Polyunsaturated fatty acid metabolites as novel lipidomic biomarkers for noninvasive diagnosis of nonalcoholic steatohepatitis. J Lipid Res. 2015;56:185–92.
33. Quehenberger O, Armando AM, Brown AH, Milne SB, Myers DS, Merrill AH, et al. Lipidomics reveals a remarkable diversity of lipids in human plasma. J Lipid Res. 2010;51:3299–305.
34. Ferreiro-Vera C, Mata-Granados JM, Priego-Capote F, Quesada-Gómez JM, Luque de Castro MD. Automated targeting analysis of eicosanoids inflammation biomarkers in human serum and in the exometabolome of stem cells by SPE-LC-MS/MS. Anal Bioanal Chem. 2011;399:1093–103.
35. Schuchardt JP, Schmidt S, Kressel G, Dong H, Willenberg I, Hammock BD, et al. Comparison of free serum oxylipin concentrations in hyper- vs. normolipidemic men. Prostaglandins Leukot Essent Fatty Acids. 2013;89:19–29.
36. Schuchardt JP, Schmidt S, Kressel G, Dong H, Willenberg I, Hammock BD, et al. Modulation of blood oxylipin levels by long-chain omega-3 fatty acid supplementation in hyper- and normolipidemic men. Prostaglandins Leukot Essent Fatty Acids. 2014;90:27–37.

37. Psychogios N, Hau DD, Peng J, Guo AC, Mandal R, Bouatra S, et al. The human serum metabolome. PLoS One. 2011;6:e16957.
38. Caligiuri SPB, Aukema HM, Ravandi A, Pierce GN. Elevated levels of pro-inflammatory oxylipins in older subjects are normalized by flaxseed consumption. Exp Gerontol. 2014;59:51–7.
39. Bowden JA, et al. Harmonizing lipidomics: NIST interlaboratory comparison exercise for lipidomics using standard reference material 1950 – metabolites in frozen human plasma. J Lipid Res. 2017;58:2275–88.
40. Yuan ZX, Majchrzak-Hong S, Keyes GS, Iadarola MJ, Mannes AJ, Ramsden CE. Lipidomic profiling of targeted oxylipins with ultra-performance liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem. 2018;140:6009–29.