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Targeting esterified oxylipins by LC–MS - Effect of sample preparation on oxylipin pattern

Annika I. Ostermann[1], Elisabeth Koch[1], Katharina M. Rund, Laura Kutzner, Malwina Mainka, Nils Helge Schebb*

Chair of Food Chemistry, Faculty of Mathematics and Natural Sciences, University of Wuppertal, Gaußstraße 20, Wuppertal, Germany

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ABSTRACT

A major part of oxygenated metabolites of polyunsaturated fatty acids – i.e. eicosanoids and other oxylipins – in biological samples is found in the esterified form. Yet, their biological role is only poorly understood. For quantification of esterified oxylipins in biological samples current protocols mostly apply alkaline hydrolysis with or without prior lipid extraction to release oxylipins into their free form which can be subsequently quantified via liquid chromatography-mass spectrometry.

Herein, a detailed protocol for precise and reproducible quantification of esterified oxylipins in plasma is presented comprising i) extraction of lipids and removal of proteins with *iso*-propanol, ii) base hydrolysis with potassium hydroxide to saponify lipids and iii) solid phase extraction of the liberated oxylipins on C8/anion exchange mixed mode material. Unequal extraction of internal standards and lipid classes during lipid extraction before hydrolysis led to distorted concentrations, emphasizing that the choice of solvent used in this step is important to minimize discrimination. Regarding the hydrolysis conditions, at least 30 min incubation at 60 °C is required with 0.1 M KOH in sample. Drying of the SPE cartridges is a critical parameter since autoxidation processes of PUFA, which are present in high concentrations after cleavage, lead to artificial formation of epoxy fatty acids. With the developed protocol, inter-day, intra-day and inter-operator variance was < 21% for most oxylipins including hydroxy-, dihydroxy-, and epoxy-PUFA. The applicability of the developed methodology is demonstrated by investigating the changes in the oxylipin pattern following omega-3 fatty acid feeding to rats.

1. Introduction

Eicosanoids and other oxylipins are oxygenated metabolites of polyunsaturated fatty acids (PUFA) which are formed endogenously in a network of enzymatic and autoxidative processes termed arachidonic acid (ARA) cascade. The biology of unesterified – i.e. free – oxylipins has been investigated in numerous studies over the past decades [1–3]. However, only a small portion of oxylipins in plasma (of humans) is found in the free form while the major part of oxylipins, especially epoxy- and hydroxy-PUFA as well as isoprostanes and –furanes, are bound in lipids [4,5], e.g. phospholipids and triglycerides [5,6], and lipoproteins [3,7,8]. Although physiological effects of esterified oxylipins have been described [9] and some reports exist investigating the profile of free vs. bound oxylipins [4], the pattern of oxylipins in lipoprotein fractions [7,8] or changes in esterified oxylipins during dietary interventions [10,11], their biological role still remains largely unknown [2,6].

Although liquid chromatography-tandem mass spectrometry (LC-

Abbreviations: ACN, acetonitrile; ALA, alpha linolenic acid; ARA, arachidonic acid; BMI, body mass index; BHT, butylated hydroxytoluene; BSA, bovine serum albumine; CE, cholesterol ester; COX, cyclooxygenase; CYP, cytochrome P450; DiHDPE, dihydroxy docosapentaenoic acid; DiHETE, dihydroxy eicosatetraenoic acid; DiHETrE, dihydroxy eicosatrienoic acid; DHA, docosahexaenoic acid; EA, ethylacetate; EpETE, epoxy eicosatetraenoic acid; EpETrE, epoxy eicosatrienoic acid; EPA, eicosapentaenoic acid; EpDPE, epoxy docosapentaenoic acid; FFA, free fatty acid; HDHA, hydroxy docosahexaenoic acid; HEPE, hydroxy eicosapentaenoic acid; HETE, hydroxy eicosatetraenoic acid; HOAc, acetic acid; IS, internal standard; iProp, *iso*-propanol; IsoP, isoprostane; LA, linoleic acid; LLE, liquid-liquid extraction; LOX, lipoxygenase; LT, leukotriene; MTBE, methyl *tert* butyl ether; n3-DPA, n3 docosapentaenoic acid; *n*Hex, *n*-hexane; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, prostaglandin; PL, phospholipid; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; Rv, resolvin; RBC, red blood cells; SPE, solid phase extraction; SPM, specialized pro-resolving lipid mediator; TG, triglyceride; Tx, thromboxane

* Corresponding author at: Chair of Food Chemistry, Faculty of Mathematics and Natural Sciences, University of Wuppertal, Gaußstr. 20, 42119 Wuppertal, Germany.

E-mail address: nils@schebb-web.de (N.H. Schebb).

[1] Authors contributed equally.

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MS/MS) based methods exist to quantify selected lipids with oxylipin moieties [12,13], esterified oxylipins are most commonly quantified indirectly using LC–MS/MS following cleavage from the lipids as a sum of free and esterified oxylipins [4,14–22]. While other approaches have been described, e.g. treatment with phospholipase A2 [23], alkaline hydrolysis is mostly used to cleave the ester bond. However, sample treatment before hydrolysis, hydrolysis conditions and extraction procedures differ hugely [24]. Different procedures are described directly hydrolyzing the sample following addition of organic solvent [4,11,14,16,21,22]. In other protocols liquid-liquid extraction (LLE) is used to extract lipids from plasma before hydrolysis [17,18,20] – or liquid-solid extraction in case of tissue [19] – or proteins are at first precipitated and lipids are subsequently extracted by LLE [15]. In some though not in all methods the extraction solvent or the sample is acidified prior extraction [15,18,19].

Alkaline hydrolysis is conducted using 0.2-0.5 M potassium hydroxide and 0.5–5 M sodium hydroxide or 0.1 M sodium carbonate in the sample [4,11,14–16,19–22] and hydrolysis conditions range from 4 °C [19,20] to 90 °C [15] for 20 to 60 min [4,14–16,18,21] or overnight (18 h) [19,20,22]. Also, two step procedures are described in which free oxylipins are generated by the addition of water following trans-esterification to methyl esters using sodium methoxide [17]. For the extraction of the resulting free oxylipins solid phase extractions [4,14,16,17,21,22] or LLE [15,18,20] are used.

While protocols are widely applied [3,24], only limited information on the impact of the different experimental procedures for the cleavage of lipids on the determined oxylipin concentration is available. Thus, it is impossible to deduce the best procedure for reproducible and precise analysis from the literature. Therefore, we herein describe a sample preparation strategy for the quantification of total oxylipins (i.e. sum of free and esterified) following base hydrolysis and provide detailed data on sample preparation steps, extraction efficiency and variability. The final standard operation procedure (SOP) was applied to investigate the effects of feeding an omega-3 PUFA (n3-PUFA) rich diet on the profile of total oxylipins in rat plasma.

2. Materials and methods

2.1. Materials

Oxylipin standards and deuterated oxylipin internal standards were purchased from Cayman Chemical (local distributor: Biomol, Hamburg, Germany). For the sample preparation 15 different internal standards (IS) covering all relevant oxylipin classes were used (SI Fig. S1). The purity of the standards was checked according to Hartung et al. [25]. LC–MS-grade methanol (MeOH), acetonitrile (ACN), *iso*-propanol (iProp) and acetic acid (HOAc) were purchased from Fisher Scientific (Schwerte, Germany). *n*-Hexane (*n*Hex, HPLC grade) was obtained from Carl Roth (Karlsruhe, Germany) and ethanol (EtOH, absolute, p.a.) from Merck (Darmstadt, Germany). All other chemicals were purchased from Sigma Aldrich (Schnelldorf, Germany) or VWR (Darmstadt, Germany). Nitrogen (5.0) was supplied from a nitrogen tank (Westfalen Gas, Münster Germany) of similar quality generated by a Nitrogen Generator (Fey Druckluft, Laatzen, Germany). Pooled human EDTA-plasma was obtained following centrifugation (15 min, 4 °C, 1200 x *g*) of EDTA-blood and mixing of the plasma supernatants from 4 to 6 healthy male and female volunteers aged between 25–38 years. Plasma was immediately stored at −80 °C until analysis.

2.2. LC–MS analysis of total oxylipins

In the following the final protocol developed in this study is reported. Details of all investigated conditions are reported in the SI.

Internal standards and additives (10 µL 0.2 mg/mL butylated hydroxytoluol (BHT), 100 µM indomethacin and 100 µM *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB) [29] in methanol) were added to 100 µL freshly thawed plasma and samples were vortexed briefly. 400 µL ice cold *iso*-propanol (−30 °C) were added, vortexed and samples were frozen at −80 °C for at least 30 min in a pre-cooled sample rack. Samples were thawed briefly at room temperature, vortexed and centrifuged (4 °C, 10 min, 20 000 x *g*). The supernatant was hydrolyzed at 60 °C for 30 min using 100 µL 0.6 M KOH in MeOH/water (75/25, *v/v*). Following hydrolysis, samples were put on ice and neutralized with ~20 µL 25% aqueous HOAc.

Extraction of oxylipins was conducted as described [28] with modifications. Bond Elut Certify II cartridges (Agilent, Waldbronn, Germany) were prepared with one column volume (~ 3 mL) of each, EA/*n*Hex (75/25, *v/v*) with 1% HOAc, MeOH and 0.1 M Na_2HPO_4 buffer (adjusted to pH 6.0 with HOAc in water/MeOH (95/5, *v/v*)). The neutralized sample was diluted on the cartridge with 2 mL 0.1 M aqueous Na_2HPO_4 buffer (adjusted to pH 6.0 with HOAc). The pH was checked in a representative sample per batch using pH stripes (5.1–7.2 scale, Macherey-Nagel, Düren, Germany) and adjusted to pH 6.0 if necessary with diluted HOAc. In our experience, the same amount of acid can be used for neutralization of similar samples (e.g. plasma from different individuals). Samples were washed with one column volume ultrapure water (18 MΩ) as well as MeOH/water (50/50, *v/v*) and dried with vacuum (-200 mbar, 30 s). Oxylipins were eluted using 2 mL of EA/*n*Hex (75/25, *v/v*) with 1% HOAc in prepared glass tubes containing 6 µL of 30% methanolic glycerol.

Samples were evaporated to dryness using a vacuum concentrator (1 mbar, 30 °C, ~70 min; Christ, Osterode am Harz, Germany) and the residue was reconstituted in 50 µL MeOH containing 40 nM of 1-(1-(ethylsulfonyl)piperidin-4-yl)-3-(4-(trifluoromethoxy)phenyl)urea used as IS2 to calculate recovery rates of IS as a measure for extraction efficiency. Samples were centrifuged sharply (4 °C, 10 min, 20 000 x *g*). 5 µL of the supernatant was analyzed by means of LC–MS/MS (QTRAP, Sciex, Darmstadt, Germany) as described [28,30]. The oxylipins are detected in scheduled selected reaction monitoring mode following fragmentation using commonly applied transitions [31].

2.3. Effect of n3-PUFA feeding on the oxylipin pattern in rats

Male 6-week old Sprague-Dawley rats (n = 6; 200–210 g) obtained from Charles River Laboratories International Inc. (Sulzfeld, Germany) were fed for 23 days with either a standard diet containing sunflower oil (Control) or an n3-PUFA rich diet (EPA + DHA) containing sunflower oil with 1% EPA and 1% DHA ethyl esters. Both diets contained 10% total fat and were described in detail in [32]. Animals had access to food and water *ad libitum*. Rats were sacrificed by cardiac puncture following anesthesia with xylazine/ketamine (66/5 mg/kg BW). Blood was collected and plasma was generated by centrifugation (10 min at 855 x *g* 4 °C). Plasma was directly frozen in liquid nitrogen and stored at −80 °C until analysis. All animal experiments were approved by the animal welfare service of the state of Lower Saxony (Oldenburg, Germany; 33.9-42502-04-13/1134, 28.05.2013).

2.4. Data analysis

Statistical analysis was performed using Graphpad Prism software (version 8.0, San Diego, CA; USA). All other calculations were done in Microsoft Excel (version 2010 and 365, Redmond, WA, USA).

3. Results

LLEs ($CHCl_3$/MeOH, MTBE/MeOH and EA/nHex) with acidified solvents yielded high recovery rates for all oxylipins (> 65%, slightly lower with MTBE/MeOH) and lipid classes (≥85%, except PC C21:0 and PS C16:0 with EA/*n*Hex (58% and 68% respectively)) from BSA (50 mg/ml in phosphate buffered saline, PBS, Fig. 1) while *direct hydrolysis* yielded low recovery rates for different analytes e.g. resolvins, leukotrienes and hydroxy-PUFA (SI Fig. S2).

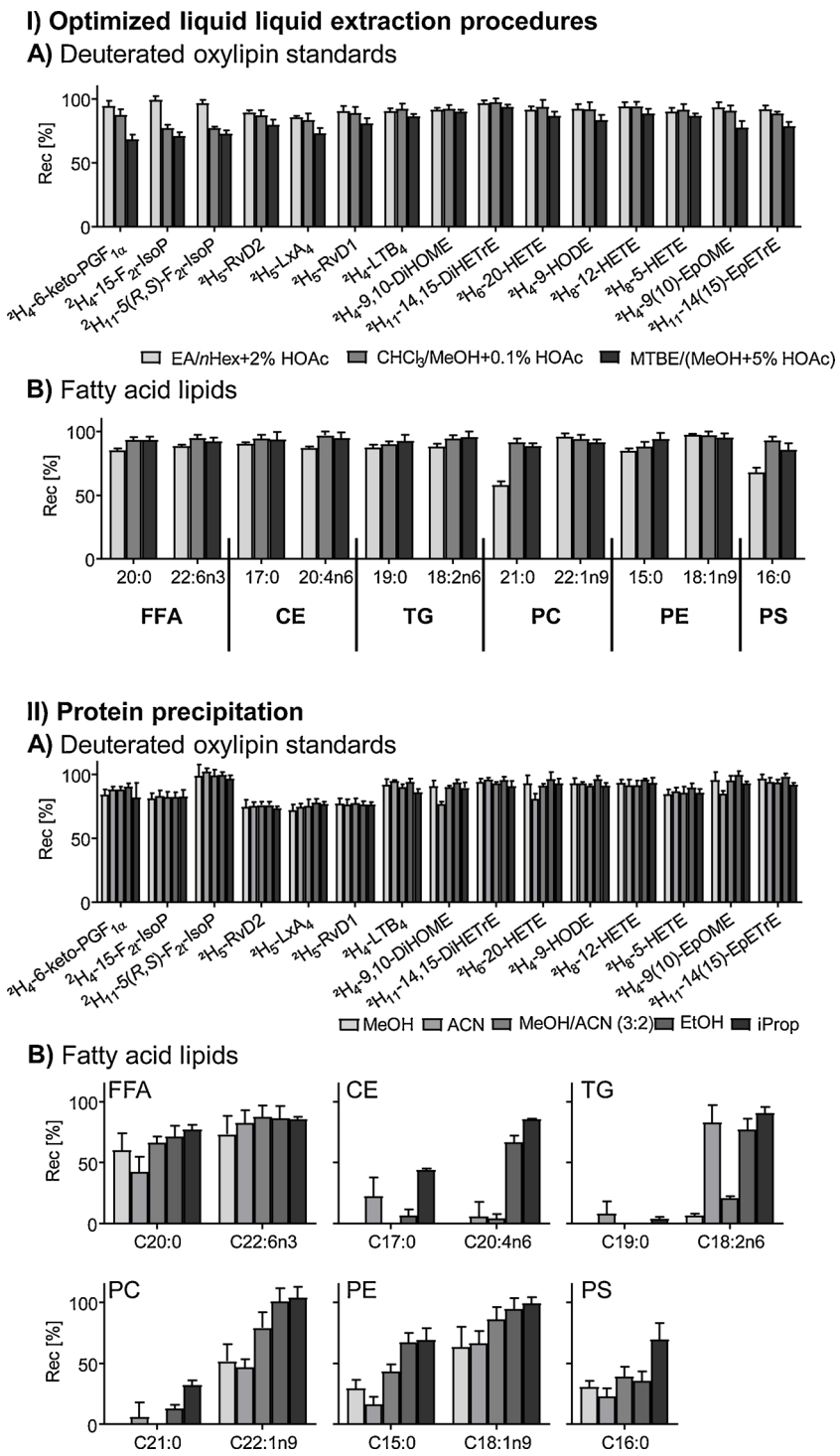

Fig. 1. Extraction efficiency of **(I)** optimized LLE and **(II)** protein precipitation. Shown are recoveries of (**A**) deuterated oxylipin standards (to mimic extraction of free oxylipins) and (**B**) lipids of fatty acids as surrogate for esterified oxylipins (to mimic extraction of lipids) from 100 µL BSA (50 mg/ml in PBS; mean ± SD; n = 4 (LLE) and n = 3 (protein precipitation)). LLEs were performed as described in SI Table S1 with acidified solvents and protein precipitation was performed with 400 µL organic solvent. Oxylipins were directly analyzed with LC-MS following LLE and extracted by SPE (on Bond Elut Certify II material) after protein precipitation.

Decreasing the content of acid in the LLE extraction solvent resulted in insufficient extraction of oxylipins with all extraction protocols and of saturated PC and PS using EA/*n*Hex (SI Fig. S3). Increased recovery of IS with acidified solvents was also observed in plasma (SI Fig. S4).

Using *protein precipitation*, all tested solvents led to acceptable and similar recovery rates of deuterated oxylipin standards (Fig. 1 II A). For all lipid classes (except FFA), a general trend was observable: lipids containing saturated fatty acids were insufficiently extracted while better extraction was achieved for lipids with unsaturated moieties (Fig. 1 II B). The only solvent yielding good recovery rates (i.e. ≥ 85%) for all lipids containing unsaturated fatty acids was *iso*-propanol. Accordingly, calculated plasma concentrations of hydroxy- and epoxy-PUFA were higher using *iso*-propanol (SI Fig. S5).

A *direct comparison of LLE with acidified solvents versus protein precipitation* for extraction of lipids from plasma revealed differences in calculated concentrations of hydroxy-PUFA, with protein precipitation

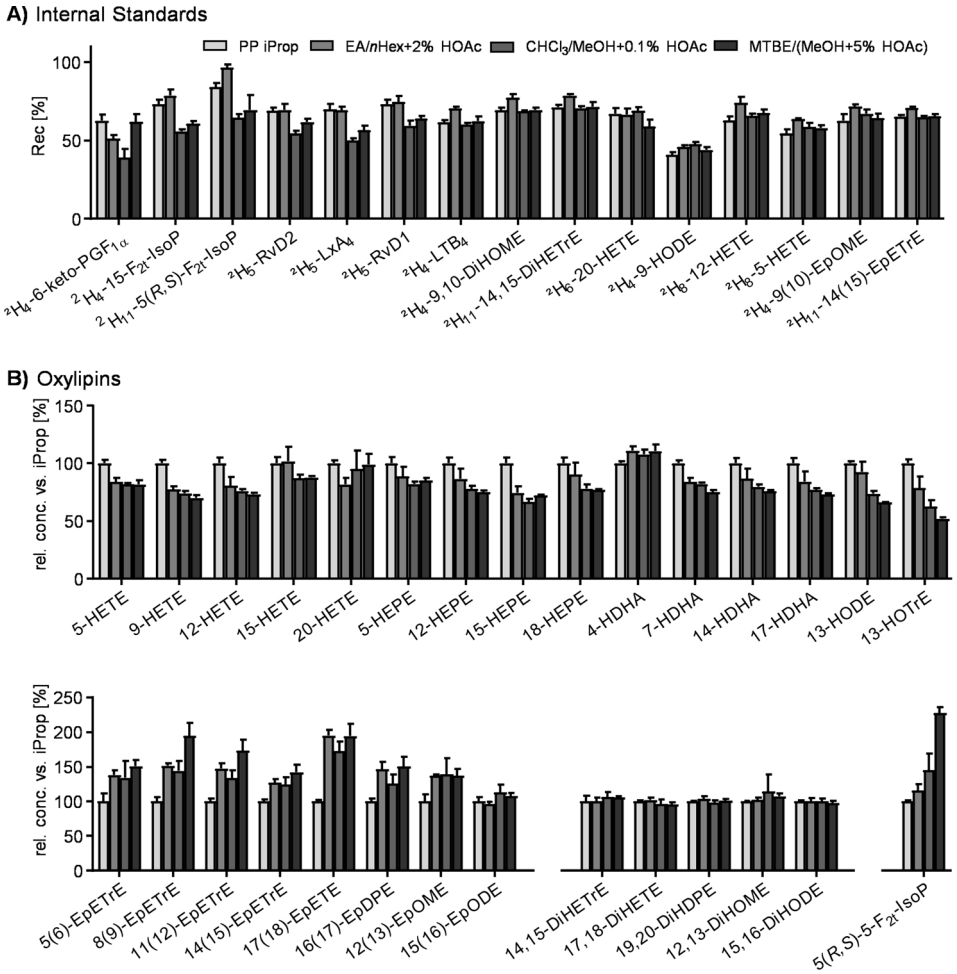

Fig. 2. Extraction of lipids from plasma using optimized LLE procedures with acidified solvents or protein precipitation with *iso*-propanol. Shown are **(A)** recoveries of internal standards as well as **(B)** relative concentrations of representative oxylipins in 100 µL plasma following protein precipitation with *iso*-propanol (PP iProp, 1 + 4) or LLE (mean ± SD; n = 3, SI Table S1).

resulting in higher concentrations compared to LLE. In contrast, epoxy-PUFA and 5(*R,S*)-5-F_{2t}-IsoP were higher with LLE (Fig. 2). Only concentrations of dihydroxy-PUFA were similar with all investigated extraction procedures. It should be noted that inter-day variance (two days) with LLE was higher (up to ~30% for some hydroxy- and epoxy-PUFA) compared to protein precipitation (< 15%).

Lower sample volume or higher base concentration during hydrolysis resulted in higher concentrations of epoxy-PUFA and several hydroxy-PUFA (especially 4-HDHA) (up to ~30%, Fig. 3). *Hydrolysis times* of 30–45 min yielded comparable oxylipin concentrations while hydrolysis for 15 min resulted in lower concentrations of hydroxy-PUFA (Fig. 3). After 30 min of hydrolysis, 70% of PGE_2 was converted to PGB_2, while no residual PGE_2 could be found (SI Fig. S9).

During *SPE* on silica-based cartridge material (Bond Elut Certify II), drying of the stationary phase prior to elution of the sample (either with nitrogen positive pressure or vacuum) led to increased concentrations of epoxy-PUFA (Fig. 4 I, SI Fig. S6) at longer drying times. Use of a neat fatty acid standard (ARA) revealed that specifically *cis*-epoxy-PUFA were formed while only a slight increase of the corresponding *trans*-epoxy-PUFA was observed (Fig. 4 II). Comparing *cis*- and *trans*-epoxy-PUFA plasma concentrations also revealed a higher increase in *cis*-compared to *trans*-epoxy-PUFA (SI Fig. S6). In contrast, oxylipin concentrations were not altered with longer drying time using polymer-based cartridge material (Oasis MAX) (Fig. 4 I). Using controlled drying times, SPE on Oasis MAX (2 min) and Bond Elut Certify II (30 s) led to comparable concentrations for oxylipins (Fig. 4 I and Fig. 5). However, intra- and interday variances – especially for epoxy-PUFA – were higher for Oasis MAX cartridges (Fig. 5 and SI Fig. S7).

Modifications of the procedure affect oxylipin concentration: Increasing the sample volume from 100 µL to 200 µL while maintaining the organic content of the sample on the cartridge ≤25% and a sample volume on column of 3 mL (one column volume) resulted either in losses of hydroxy- or epoxy-PUFA (SI Fig. S8).

Feeding rats for 23 days with a diet containing 1% of each DHA and EPA ethyl esters increased the total plasma concentration of n3-PUFA oxylipins compared to the control group receiving sunflower oil based chow (Table 1). EPA metabolites were increased 38-82-fold (absolute increase from 0.6-3 nM to 35-190 nM), while increase in DHA metabolites was 4.5–7.5-fold (absolute increase from 3-18 nM to 25-110 nM). ARA derived oxylipins (except 20-HETE) were reduced 0.4-0.3-fold (absolute decrease from 65-190 nM to 19-130 nM) compared to the control group.

4. Discussion

In biological samples such as plasma and tissues esterified oxylipins are commonly quantified following base hydrolysis as part of a sum parameter comprising free and esterified oxylipins. Here, we present a detailed sample preparation approach for the quantification of total oxylipins in plasma.

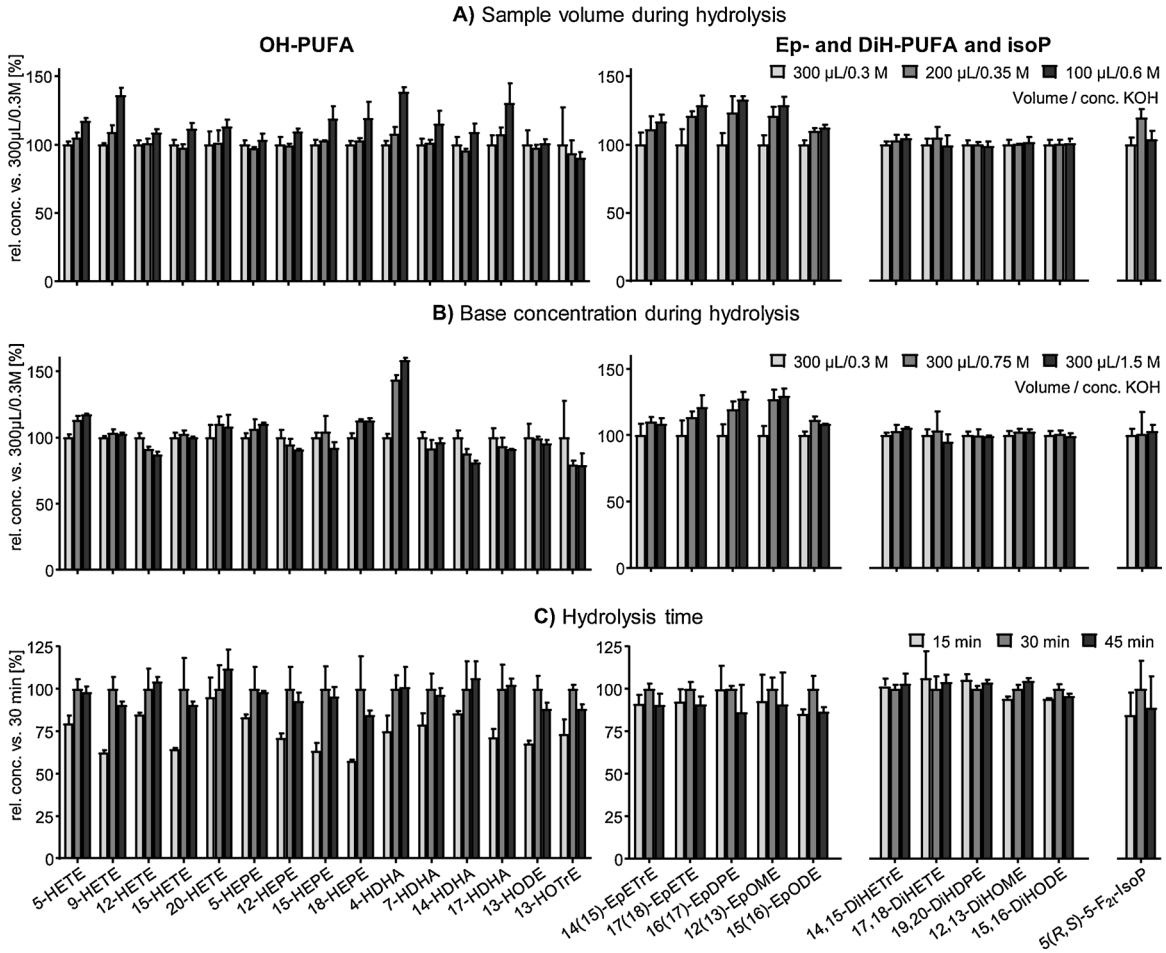

Fig. 3. Optimization of hydrolysis conditions. Shown are relative concentrations of a representative set of analytes in 100 µL plasma following protein precipitation with *iso*-propanol (400 µL, 1 + 4) and hydrolysis (mean ± SD; n = 3). A) Sample volume was varied keeping ~0.1 M KOH during hydrolysis (30 min). B) Base concentration was varied with constant sample volume (800 µL, 30 min) C) Hydrolysis time was varied using 0.1 M KOH in sample.

4.1. Extraction of lipids prior hydrolysis

Lipids are commonly directly hydrolyzed in the biological matrix without prior extraction steps [4,11,14,16,21,22]. To increase solubility of the analytes in the sample, organic solvent – mostly MeOH (25–56% of the sample volume [4,14,16,21]) – is added. In our hands, this approach led to inacceptable recovery rates of internal standards, e.g. < 60% for hydroxy-PUFA (using 75% MeOH during hydrolysis at a concentration of 1.5 M sodium hydroxide; SI Fig. S2).

Three LLE protocols for the extraction of lipids prior to hydrolysis were tested: i) extraction with ethyl acetate and *n*-hexane which is used as elution solvent in the SPE procedure used routinely in the lab for the extraction of free oxylipins (EA/*n*Hex, [28]), ii) extraction according to Folch et al. with chloroform and methanol, a standard protocol for lipid extraction ($CHCl_3$/MeOH, [26]) and iii) methyl *tert*-butyl ether and methanol which is an established alternative lipid extraction procedure allowing to omit the use of halogenated organic solvents (MTBE/MeOH, [27]). It is not surprising that both, $CHCl_3$/MeOH and MTBE/MeOH were well suited for the extraction of lipids and nonpolar oxylipins. Of note, with EA/*n*Hex, extraction of phosphatidylcholine and phosphatidylserine with saturated fatty acid moieties was insufficient. With increasing polarity of the analytes extraction efficiency decreased with all protocols, presumably due to increasing amounts of the analytes being present in plasma in the deprotonated form. Consequently, acidification of the extraction solvent resulted in enhanced extraction power.

Pronounced differences in calculated concentrations of total hydroxy- and epoxy-PUFA as well as IsoP were observed when comparing acidified and non-acidified extraction solvents. This can be explained by the huge differences in recovery rates of lipids (the predominant form of oxylipins in plasma [7]) and the non-esterified oxylipin IS. Accordingly, absolute areas which only reflect the extraction of lipids were more comparable (SI Fig. S4). Since no esterified isotope labeled oxylipins are commercially available, these results emphasize the importance of equal extraction efficiency among all lipid and oxylipin classes to ensure reliable and reproducible quantification of esterified oxylipins. This is not only crucial in the context of IS availability, but also considering differences in distribution of esterified oxylipins among lipid classes in biological samples of different origin (e.g. plasma vs. tissue).

Protein precipitation is a less labor-intensive alternative to remove proteins and extract lipids from biological matrices. Organic solvents have been widely used [33–36] for this purpose. All investigated approaches (*iso*-propanol, acetonitrile, methanol and methanol/acetonitrile (3/2, *v/v*) and ethanol) were well-suited to extract free oxylipin IS from the biological matrix (Fig. 1II A). However, there were pronounced differences for the different lipid classes (Fig. 1II B) with *iso*-propanol yielding overall the best extraction efficiency.

Comparing the most effective protein precipitant – *iso*-propanol – and optimized LLEs with acidified solvents, oxylipin concentrations in plasma were in the same range (Fig. 2). However, in terms of simplicity and sample handling time, protein precipitation is the preferred method.

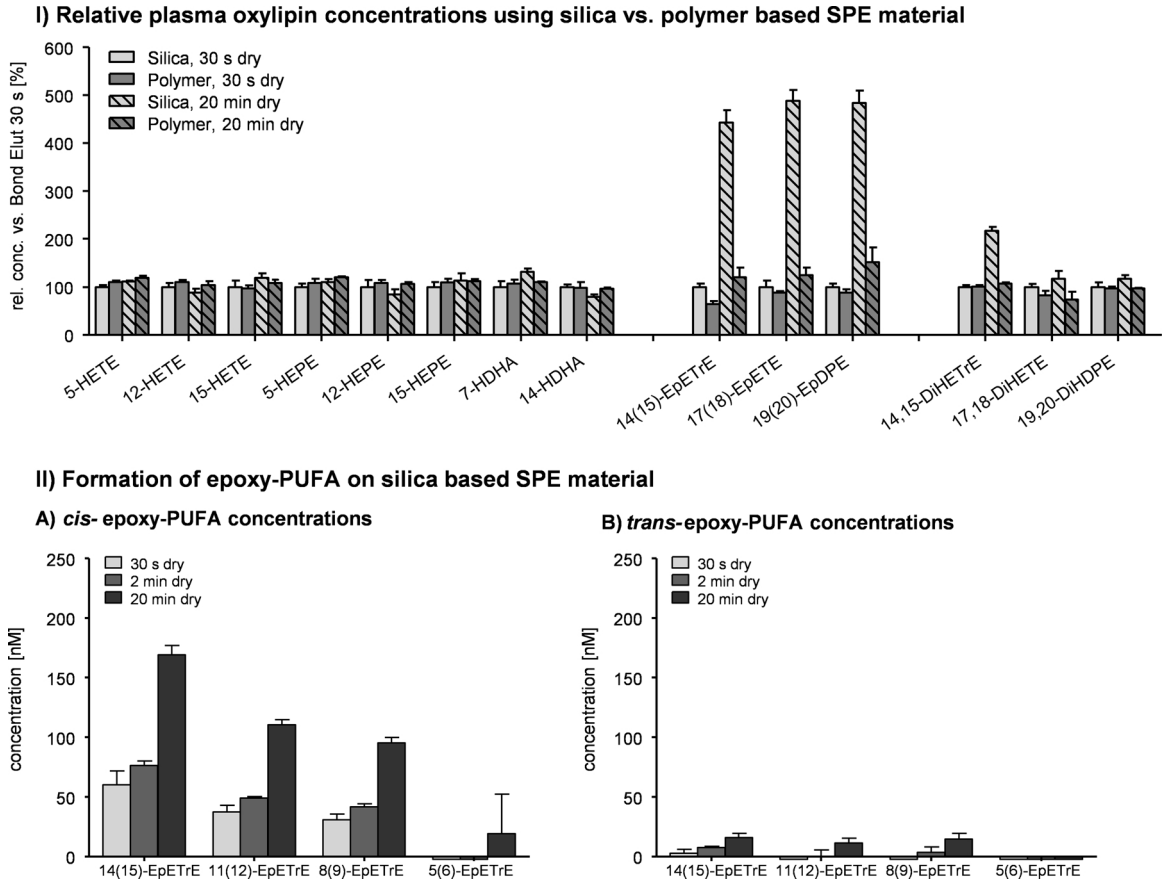

Fig. 4. Impact of sample drying on **(I)** plasma oxylipin concentrations using different cartridge materials and **(II)** the formation of epoxy-PUFA on silica-based cartridge material. **(I)** Samples were extracted either with silica (Bond Elut Certify II) or polymer (Oasis MAX) based cartridge material and dried with vacuum (mean ± SD; n = 3). Relative concentrations were calculated against the mean of the concentration following 30 s of drying on silica-based cartridges. Individual concentrations of *cis*- vs. *trans*-epoxy-PUFA in plasma can be found in the SI Fig. S6. **(II)** Effect of sample drying on epoxy-PUFA concentrations formed on silica based cartridge material (Bond Elut Certify II) using a neat ARA standard (0.7 mM) (mean ± SD, n = 3).

4.2. Hydrolysis conditions

Due to limited solubility in the predominantly organic sample after protein precipitation (*iso*-propanol/water (4/1, *v/v*)), base (sodium hydroxide, potassium hydroxide and sodium carbonate), base concentration as well as dilution solvent were critical parameters, since non-optimal set-ups resulted in two phases during hydrolysis or precipitation of the alkali salt. In this context, methanol in the solvent for the base (75/25 MeOH/H_2O, *v/v*) works as a bridging agent and enables a better solubility of potassium hydroxide in the aqueous, *iso*-propanol containing sample.

Different sample volumes of ~600 µL with ~0.1 M KOH and ~800 µL with ~0.56 M KOH led to comparable concentrations of oxylipins. Because of the elution power of higher portion of organic solvent on the SPE cartridge the low sample volume setup (i.e. hydrolysis following addition of 100 µL of 0.6 M KOH resulting in ~600 µL sample with ~0.1 M KOH) was chosen for the final protocol. Hydrolysis time was set to 30 min and longer times (45 min) led to similar oxylipin concentrations suggesting that the analytes are stable and do not degrade during the chosen hydrolysis conditions. Interestingly, in a recent report it was discussed, that longer hydrolysis time and higher temperature (37 °C for 60 min or 60 °C for 30 min; 0.2 M KOH, ~43% MeOH during hydrolysis) led to degradation of 7-HDHA in the sample [21], while this analyte is more stable in our hands (60 °C for 30 min, ~0.1-0.56 M KOH, 63% *iso*-propanol and 17% MeOH during hydrolysis, Fig. 3).

For thromboxanes, degradation under alkaline conditions has been described [17] which was also observed with our protocol (recovery of the $^2H_4$-TxB_2 < 5%, data not shown). Also, some classes of prostanoids, e.g. β-hydroxy-keto-prostanoids such as PGE_2 and PGD_2 degrade under alkaline conditions [17,37]. In this process PGE_2 dehydrates to PGA_2, which subsequently isomerizes to the more stable PGB_2 [37]. Using the presented hydrolysis conditions, the conversion rate of PGE_2 to PGB_2 was about 70% (SI Fig. S9) and no residual PGE_2 was observed in the chromatograms. Thus, the present protocol is suitable to use PGB_2 as a surrogate for estimating the concentration of PGE_2 in plasma.

4.3. Solid phase extraction of cleaved oxylipins

Oxylipins are extracted from the hydrolyzed samples using SPE on silica modified with C8 and anion exchange moieties (Bond Elut Certify II, [14,28,38,39]). While for free oxylipins this SPE mode results in good removal of potential matrix interferences [38] and reproducible oxylipin concentrations [40], drying of the hydrolyzed sample (both, by positive and negative pressure) was a critical parameter and unsuitable drying resulted in the artificial formation of *cis*-epoxy-PUFA (Fig. 4, SI Fig. S6). It has been described that unsaturated fatty acids adsorbed as a monolayer on silica are oxidized by atmospheric oxygen to – mainly – epoxy-PUFA (while unsaturated fatty acids adsorbed in a bulk phase are predominantly converted to hydroperoxides) [41,42]. Due to their orientation as a monolayer, *cis*-epoxy-PUFA are the main products of this reaction [41,42]. These results indicate that autoxidation following the

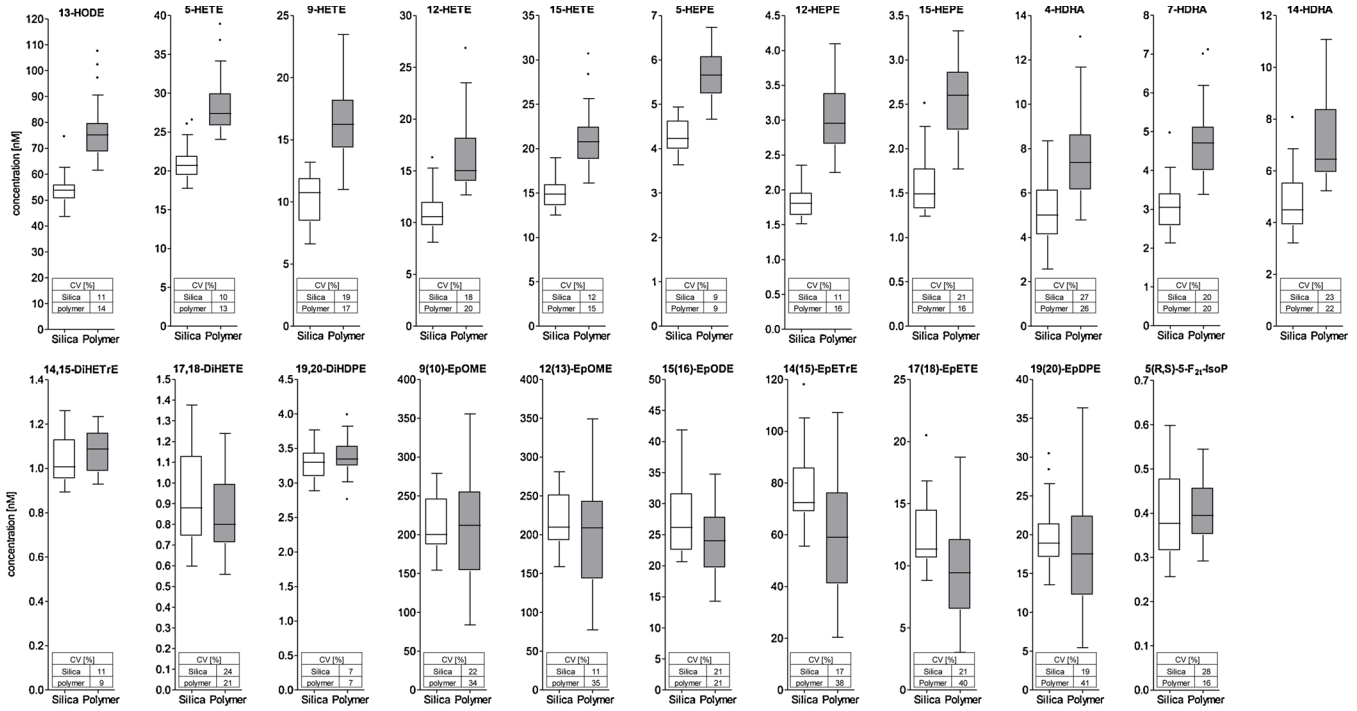

Fig. 5. Box-Whisker-plots and coefficients of variation of plasma oxylipin concentrations following SPE of the hydrolyzed plasma sample on Bond Elut Certify II (left, white) and Oasis MAX (right, grey) cartridges. Four different operators prepared and analyzed a triplicate set of samples on three consecutive days. Individual sample concentrations are shown in the SI Fig. S7.

Table 1
Plasma concentrations of total oxylipins following feeding of an EPA + DHA enriched diet to rats. The EPA + DHA group received a diet enriched with 1% EPA and DHA ethyl esters, while the control group was fed with a diet containing sunflower oil. Shown are concentrations of a representative set of analytes derived from EPA, DHA and ARA [a] Shown are mean ± SEM (n = 4–6). [b]t-test, non-paired, α = 0.05; [c]t-test, Welch correction, α = 0.05. Outlier excluded based on ROUT outlier test, Q = 1%.

Analyte	Control Group[a], [nM]	EPA + DHA Group[a], [nM]	Fold Change	p-value
Hydroxy-PUFA				
5-HETE	111 ± 5	39 ± 3	0.3	< 0.0001[b]
9-HETE	65 ± 4	19 ± 2	0.3	< 0.0001[b]
12-HETE	189 ± 4	130 ± 40	0.7	n.s.[c]
15-HETE	90 ± 4	27 ± 2	0.3	< 0.0001[b]
20-HETE	6.8 ± 0.6	28 ± 5	4.1	< 0.05[c]
5-HEPE	1.2 ± 0.1	47 ± 6	38	< 0.01[c]
12-HEPE	1.3 ± 0.2	190 ± 80	146	n.s.[c]
15-HEPE	< LLOQ	35 ± 4	–	–
18-HEPE	0.61 ± 0.09	44 ± 6	72	< 0.01[c]
20-HEPE	3.1 ± 0.2	110 ± 40	34	n.s.[c]
4-HDHA	5.5 ± 0.4	35 ± 5	6.3	< 0.01[c]
7-HDHA	3.2 ± 0.2	24 ± 4	7.5	< 0.01[c]
14-HDHA	9 ± 3	60 ± 10	6.8	< 0.05[c]
17-HDHA	18 ± 1	80 ± 10	4.5	< 0.05[c]
22-HDHA	3.2 ± 0.3	110 ± 20	34	< 0.05[c]
Epoxy-PUFA				
14(15)-EpETrE	260 ± 20	100 ± 8	0.4	< 0.0001[b]
17(18)-EpETE	1.3 ± 0.1	105 ± 9	82	< 0.001[c]
19(20)-EpDPE	12 ± 1	80 ± 10	6.6	< 0.01[c]
Dihydroxy-PUFA				
14,15-DiHETrE	3.7 ± 0.4	2.7 ± 0.6	0.7	n.s.[b]
17,18-DiHETE	0.15 ± 0.03	9.4 ± 1.0	63	< 0.001[c]
19,20-DiHDPE	1.5 ± 0.1	9.7 ± 0.5	6.4	< 0.00 1[c]

reaction described happens during the drying process of the hydrolyzed sample [43], presumably on non-endcapped residues of the cartridge material. In contrast to the extraction of free oxylipins – where drying is not such a crucial parameter – the high concentration of free PUFAs in the hydrolyzed sample (e.g. 0.7 mM ARA in the plasma used in this study determined by GC-FID) can be readily oxidized on the silica material. Consistently, SPE on silica-free cartridge material with a polymer backbone and similar hydrophilic binding sites and strong anion exchange moieties (Oasis MAX) did not result in an increase of epoxy-PUFA, even at long drying times (Fig. 4 I). Interestingly, drying of samples on silica-based SPE material has not been discussed before in the context of artifact formation, although it has been used to extract free oxylipins from cleaved samples [14,39].

For reliable quantification of oxylipins in large sample sets such as cohort studies, the analytical protocol needs to be highly reproducible. A direct comparison of SPE on silica based C8/anion exchange mixed mode and polymer based material showed that the SPE on silica-based material (with exact 30 s of sample drying) resulted in notably lower inter-day, intra-day and inter-operator variations in the reported oxylipin concentrations compared to the polymer-based material (Fig. 5). Thus, silica-based cartridge material is in our hands most suitable for the extraction of oxylipins from the hydrolyzed sample if the operator tightly abides by the optimized drying time. Using this overall optimized procedure, variations of most oxylipins were ≤21%. Taking international guidelines on method validation (e.g. European Medicines Agency [44] or Food and Drug Administration [45]) into account which define a precision of < 15% for analytes well above the LLOQ, variations of most analytes pass these criteria or are only slightly higher (≤21%) with our method. Also, this is in the same range as for free oxylipins [3]. Coefficients of variation described by Quehenberger et al. are overall lower (direct hydrolysis, SPE on Strata-X polymeric RP-columns) [21], however, they are based on the analysis of standards and no inter-operator variance is considered. In combination with the validation of the calibration, including definition of linear range, limit of detection etc. described in [28,30], alkaline hydrolysis using the presented procedure followed by LC–MS analysis of the free oxylipins is with this precision well suited for the analysis of total oxylipins in biological samples. However, because no reference material of esterified oxylipins in lipids is available it is actually impossible to validate the accuracy of the method.

4.4. Impact of n3-PUFA supplementation on total oxylipin concentrations in rat plasma

The optimized method was finally applied to investigate the effects of n3-PUFA feeding on the oxylipin pattern. Rat plasma was analyzed following feeding with 1% of EPA and 1% of DHA in the chow for three weeks. The recoveries of internal standards were above 55% and the variation of the determined concentration per group (n = 5–6 animals) were for all analytes acceptable with a standard deviation < 30% (except for 12-LOX metabolites) which supports the broad applicability of the method developed here for the quantification of total oxylipins. In line with previous reports for free oxylipins in rodents [14,32] and in humans [46,47], concentrations of EPA and DHA derived oxylipins were massively increased after feeding the n3-PUFA rich diet compared to the standard diet. While absolute changes of EPA and DHA metabolites were in the same range, relative changes in EPA metabolites were unequally higher. This is probably caused by lower baseline EPA compared to DHA plasma level in rats [14,48] and has been discussed earlier in a murine supplementation study [32].

5. Conclusion

A detailed procedure covering sample preparation steps for the quantification of esterified oxylipins in biological samples is presented. It is highlighted that different steps during sample preparation have a direct impact on the determined oxylipin concentration.

Both, LLE with acidified solvents and protein precipitation yielded oxylipin concentrations in the same range, however, protein precipitation is the preferred method because of its simplicity. Best conditions for hydrolysis are 30 min at 60 °C after addition of 100 µL 0.6 M KOH (in 75/25 MeOH/water). Our results indicate that these conditions lead to efficient liberation of oxylipins from the lipids and no degradation during hydrolysis. The SPE procedure, however, has to be strictly controlled if extraction of cleaved oxylipins is carried out on silica-based material since artificial formation of epoxy-PUFA can occur.

With the methodology described herein, precise quantification of oxylipins in biological matrices is achieved. Based on this the biological role of esterified oxylipins can be further investigated and the analytical strategy could pave the route for their use as biomarkers for diseases.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prostaglandins.2019.106384.

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Supplementary Information

Targeting esterified oxylipins by LC-MS – Effect of sample preparation on oxylipin pattern

Annika I. Ostermann[1], Elisabeth Koch[1], Katharina M. Rund, Laura Kutzner, Malwina Mainka, Nils Helge Schebb*

Chair of Food Chemistry, Faculty of Mathematics and Natural Sciences, University of Wuppertal, Gaußstraße 20, Wuppertal, Germany

*Corresponding author: nils@schebb-web.de

[1]Authors contributed equally

Detailed information on the characterization of sample preparation for the determination of total oxylipins

1 Lipid and oxylipin extraction from plasma (prior hydrolysis)

Extraction efficiencies using LLE and protein precipitation procedures were evaluated using aqueous bovine serum albumin (BSA) solution (50 mg/mL in phosphate buffered saline (PBS)) spiked with either alkali-stable deuterated oxylipins standards (internal standards (IS) for oxylipin quantification) or fatty acid lipids. Moreover, oxylipin profiles in 100 µL of human plasma with selected LLE and protein precipitation protocols were evaluated.

Three LLE procedures were tested: Extraction with i) ethyl acetate and *n*-hexane (EA/*n*Hex, 75/25, *v/v*), ii) chloroform and methanol ($CHCl_3$/MeOH, 2/1, *v/v*) [1] and iii) methyl *tert*-butyl ether and methanol (MTBE/MeOH, 3/1, *v/v*) [2] (see SI Table S1 for detailed procedures). Lipid extracts were evaporated in a vacuum concentrator (1 mbar, 30 °C; Christ, Osterode am Harz, Germany). For protein precipitation, plasma was extracted with a fourfold excess (100 µL sample + 400 µL organic solvent) of methanol (MeOH), acetonitrile (ACN), ethanol (EtOH), methanol/acetonitrile (MeOH/ACN, 3/2, *v/v*), or *iso*-propanol (iProp). Samples were frozen at -80 °C for at least 30 min and centrifuged (4 °C, 10 min, 20 000 x *g*).

Recovery of oxylipins was calculated via external calibration. Lipid samples were evaporated, derivatized to fatty acid methyl esters and analyzed via GC-FID [2]. For LLE, recovery of fatty acids was calculated against the directly derivatized lipid standard and for protein precipitation

against a lipid standard which was diluted with *iso*-propanol and PBS before evaporation and derivatization.

2 Hydrolysis conditions

Experiments were carried out using lipid extracts obtained following LLE or protein precipitation with *iso*-propanol (see above). Dried lipid extracts obtained from LLE were dissolved in 400 µL *iso*-propanol. For hydrolysis, 100 µL water and 300 µL 1.5 M potassium hydroxide (KOH) in MeOH/water (75/25, *v/v*) were added and hydrolyzed for 30 min at 60 °C

Supernatants from protein precipitation were hydrolyzed at 60 °C using KOH in methanol/water (75/25; *v/v*) and optimization steps comprised sample volume and base concentration during hydrolysis as well as hydrolysis time.

After hydrolysis samples were put on ice and neutralized (pH = 6) with diluted acetic acid (HOAc) prior further extraction.

3 Solid phase extraction (SPE) protocol for free oxylipins from hydrolyzed samples

After neutralization, hydrolyzed samples were diluted with 2000 µL 0.1 M aqueous disodium hydrogen phosphate buffer (Na_2HPO_4) adjusted to pH 6.0 with HOAc. pH in the samples was checked and adjusted to 6 before extraction of oxylipins by SPE on Bond Elut Certify II columns (200 mg, 3 mL, 47-60 µm particles, Agilent Technologies, Waldbronn, Germany) as described [3] or with a modified protocol using same solvents and buffers on Oasis MAX cartridges (60 mg, 3 mL, 30 µm particles, Waters, Eschborn, Germany).

Epoxidation of PUFA using silica based cartridges was investigated by extracting 100 µL of a methanolic standard solution containing IS (20 nM) and ARA (0.7 mM) with the final protocol (see below). *Trans*- and *cis*-epoxy-PUFA concentrations were determined [4] and compared to a diluted standard which was analyzed directly.

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Table S1: Overview over the liquid-liquid extraction procedures.

$CHCl_3$/MeOH [1] and $CHCl_3$/MeOH + HOAc	**MTBE/MeOH** [2] and MTBE/(MeOH+HOAc) [#]	**EA/*n*Hex** and EA/*n*Hex + HOAc
Sample 100 µL 50 mg/mL BSA in PBS + 10 µL deuterated oxylipin standard (1 pmol) or + 10 µL lipid standard (10 nmol) + *Antiox**	Sample 100 µL 50 mg/mL BSA in PBS + 10 µL deuterated oxylipin standard (1 pmol) or + 10 µL lipid standard (10 nmol) + *Antiox**	Sample 100 µL 50 mg/mL BSA in PBS + 10 µL deuterated oxylipin standard (1 pmol) or + 10 µL lipid standard (10 nmol) + *Antiox**
Vortex sample	Vortex sample	Vortex sample
+ $CHCl_3$/MeOH (2/1, *v/v*) [+ HOAc] to a final sample dilution of 20:1	+ 300 µL MeOH [+ HOAc]	+ 750 µL EA/*n*Hex (75/25, *v/v*) [+ HOAc]
Vortex sample	Vortex sample	Vortex sample for 5 min
Centrifugation (room temp., 10 min, 4000 x *g*)	+ 600 µL MTBE	Centrifugation (4 °C, 2 min, 20 000 x *g*)
+ 0.2 parts MeOH if phases are not separated and repeat centrifugation	Vortex Sample for 1.5 min	Collect organic phase
Transfer supernatant to new glass tube	Add 300 µL 0.15 M NH_4Ac	Repeat extraction with another 750 µL extraction solvent
+ $CHCl_3$ to $CHCl_3$:MeOH:water 8:4:3	Vortex sample	
+ 0.2 fold vol. 0.73% NaCl of crude extract	Centrifugation (4 °C, 5 min, 3500 x*g*)	
Vortex sample	Collect upper organic phase	
Centrifugation (room temp., 5 min, 4000 x *g*)	Rewash lower aqueous phase with 300 µL MTBE and combine organic phases	
Remove upper aqueous phase		
Wash lower organic phase two times with low volumes of upper phase pure solvent ($CHCl_3$:MeOH:0.58% NaCl 3:48:47)		

$CHCl_3$ = chloroform; MeOH = methanol; MTBE = methyl *tert* butyl ether; EA = ethyl acetate, *n*Hex = *n*-hexane; NaCl = sodium chloride, NH_4Ac = ammonium acetate

* BHT and EDTA (0.2 mg/mL each) with sEHi (*t*-AUCB) and indomethacin (100 µM each) in MeOH/water (50/50, *v/v*))

[#] While for $CHCl_3$/MeOH and EA/*n*Hex the total extraction solvent was acidified, for MTBE/MEOH only the methanol was acidified.

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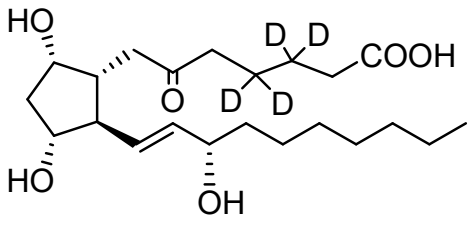
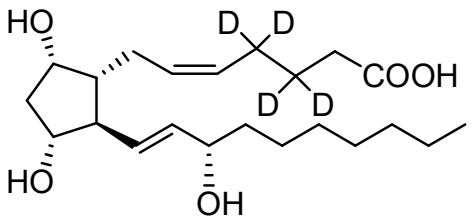
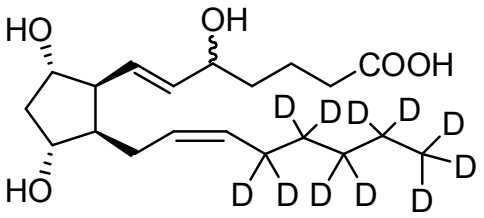


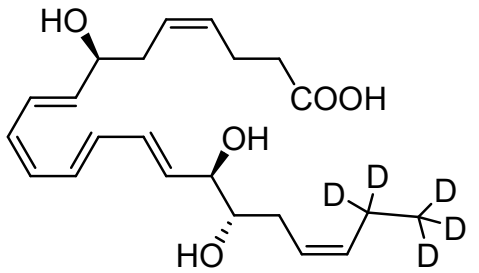
$^{2}H_4$-6-keto-$PGF_{1\alpha}$



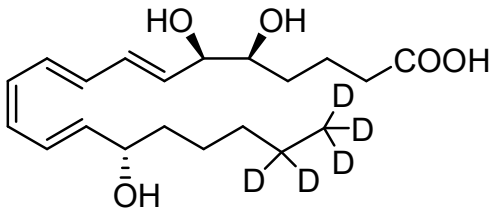
$^{2}H_4$-15-F_{2t}-IsoP



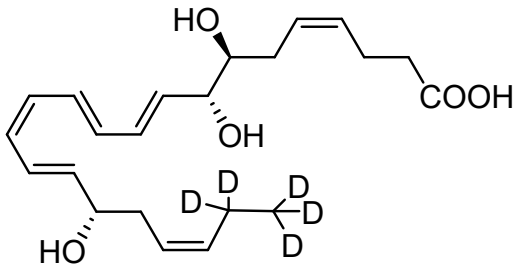
$^{2}H_{11}$-5(*R,S*)-F_{2t}-IsoP



$^{2}H_5$-RvD2



$^{2}H_5$-LxA_4



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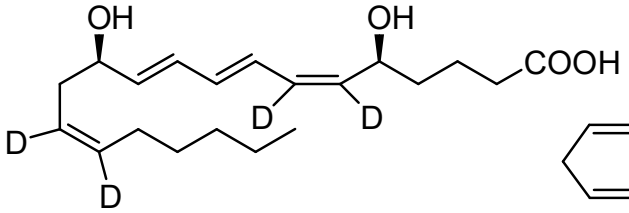
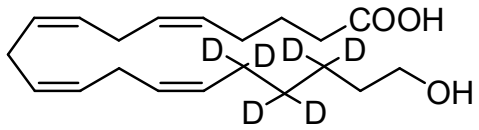
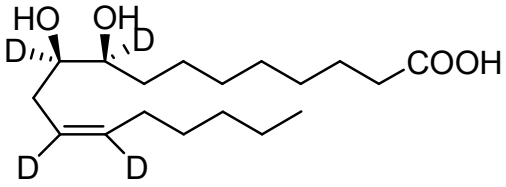


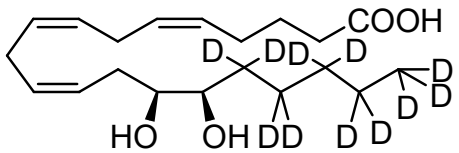
$^{2}H_4$-LTB_4



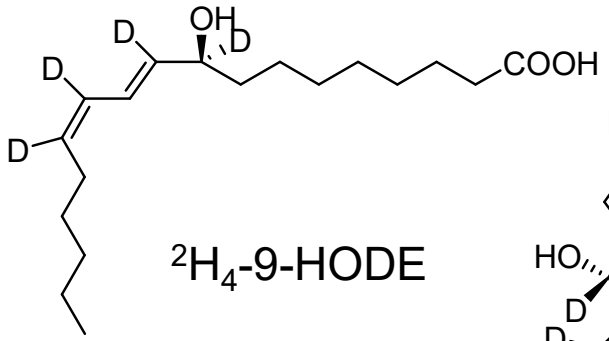
$^{2}H_6$-20-HETE



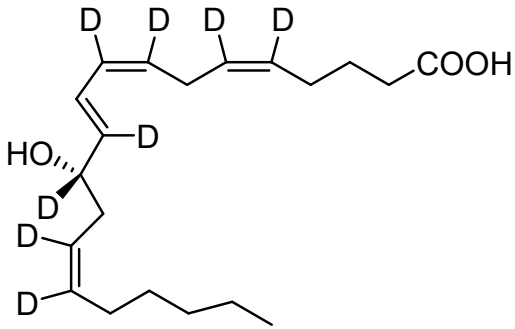
$^{2}H_4$-9,10-DiHOME



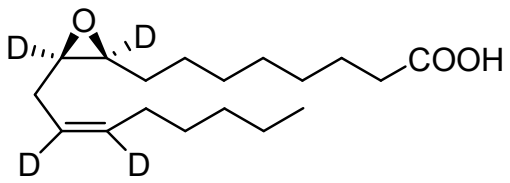
$^{2}H_{11}$-14,15-DiHETrE



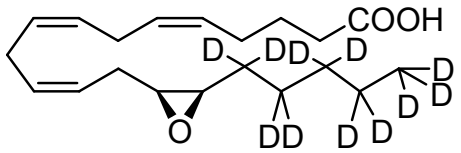
$^{2}H_4$-9-HODE



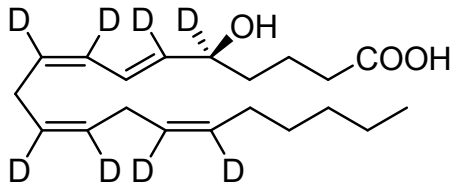
$^{2}H_8$-12-HETE



$^{2}H_4$-9(10)-EpOME



$^{2}H_{11}$-14(15)-EpETrE



$^{2}H_8$-5-HETE

Fig. S1 **Structures of internal standards used for quantification of oxylipins.** D indicates position of deuterium.

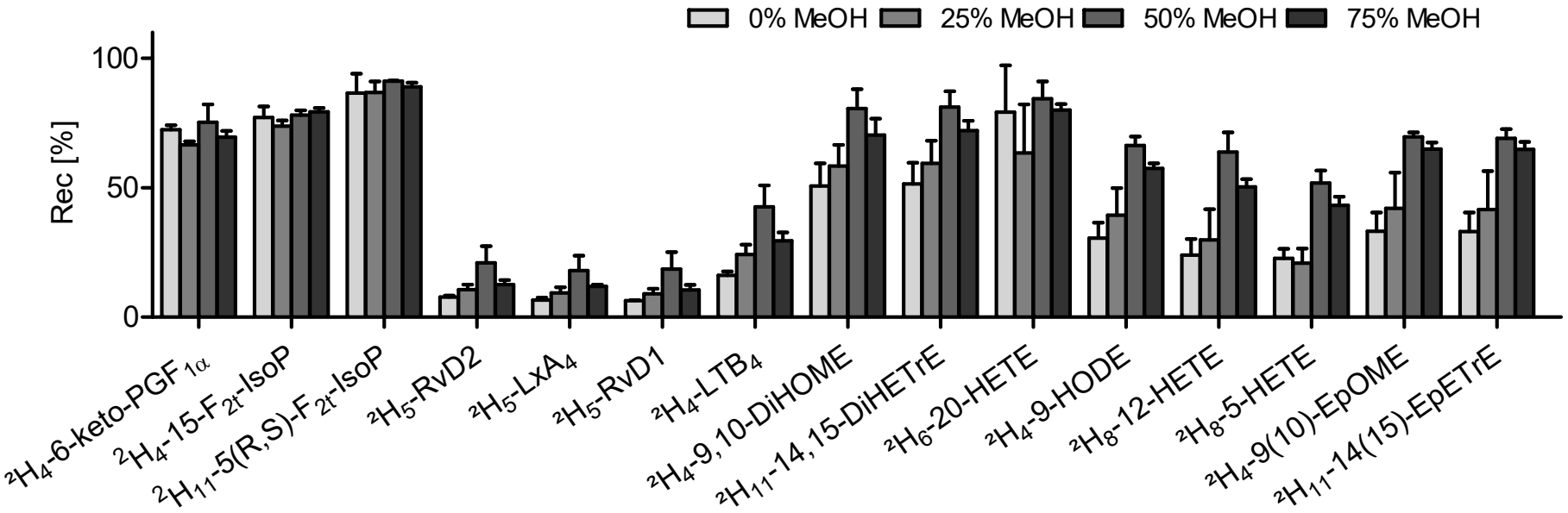


Fig. S2 Recovery rates of internal standards following direct hydrolysis of plasma. Shown are recoveries of deuterated oxylipin standards following direct hydrolysis of plasma without prior extraction of lipids (mean ± SD; n=3). Plasma samples (100 µL) were hydrolyzed using 700 µL 2 M aqueous sodium hydroxide solution with 0%, 29%, 57% and 86% methanol, resulting in 1.5 M sodium hydroxide during hydrolysis with an organic content of 0%, 25%, 50% and 75% (methanol). After neutralization, free oxylipins were extracted via SPE (on Bond Elut Certify II material) from the hydrolyzed sample and analyzed with LC-MS.

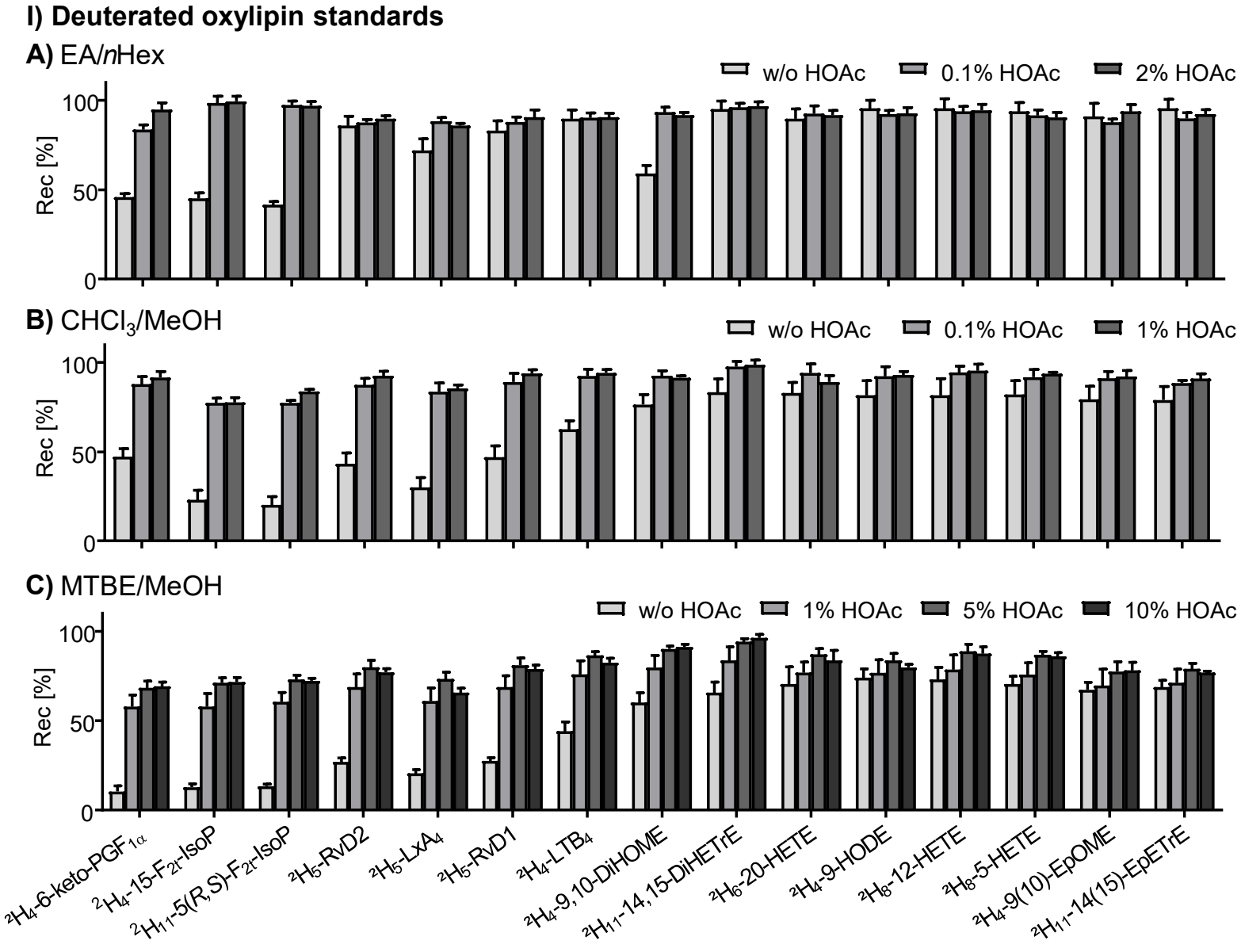


Fig. S3 **Optimization of LLE procedures for free oxylipins and lipids.** Shown are recoveries of **I)** deuterated oxylipin standards and **II)** fatty acid lipids from BSA solution (100 µL, 50 mg/mL in PBS) following LLE with **(A)** EA/*n*Hex (75/25, *v/v*), **(B)** $CHCl_3$/MeOH (2/1, *v/v*) and **(C)** MTBE/MeOH (2/1, *v/v*) (SI Table S1) with varying amount of HOAc in the extraction solvent (mean ± SD; n=4). For oxylipin analysis, samples were evaporated, reconstituted and analyzed with LC-MS. Lipid extracts were evaporated, derivatized to fatty acid methyl esters and analyzed via GC-FID [2]. Recovery of fatty acids was calculated against a directly derivatized lipid standard. Concentration of acid was selected based on the results for extraction of deuterated oxylipin standards.

[2] Ostermann AI, Muller M, Willenberg I, Schebb NH, Determining the fatty acid composition in plasma and tissues as fatty acid methyl esters using gas chromatography - a comparison of different derivatization and extraction procedures, Prostaglandins Leukot. Essent. Fatty Acids 91 (6) (2014) 235-241.

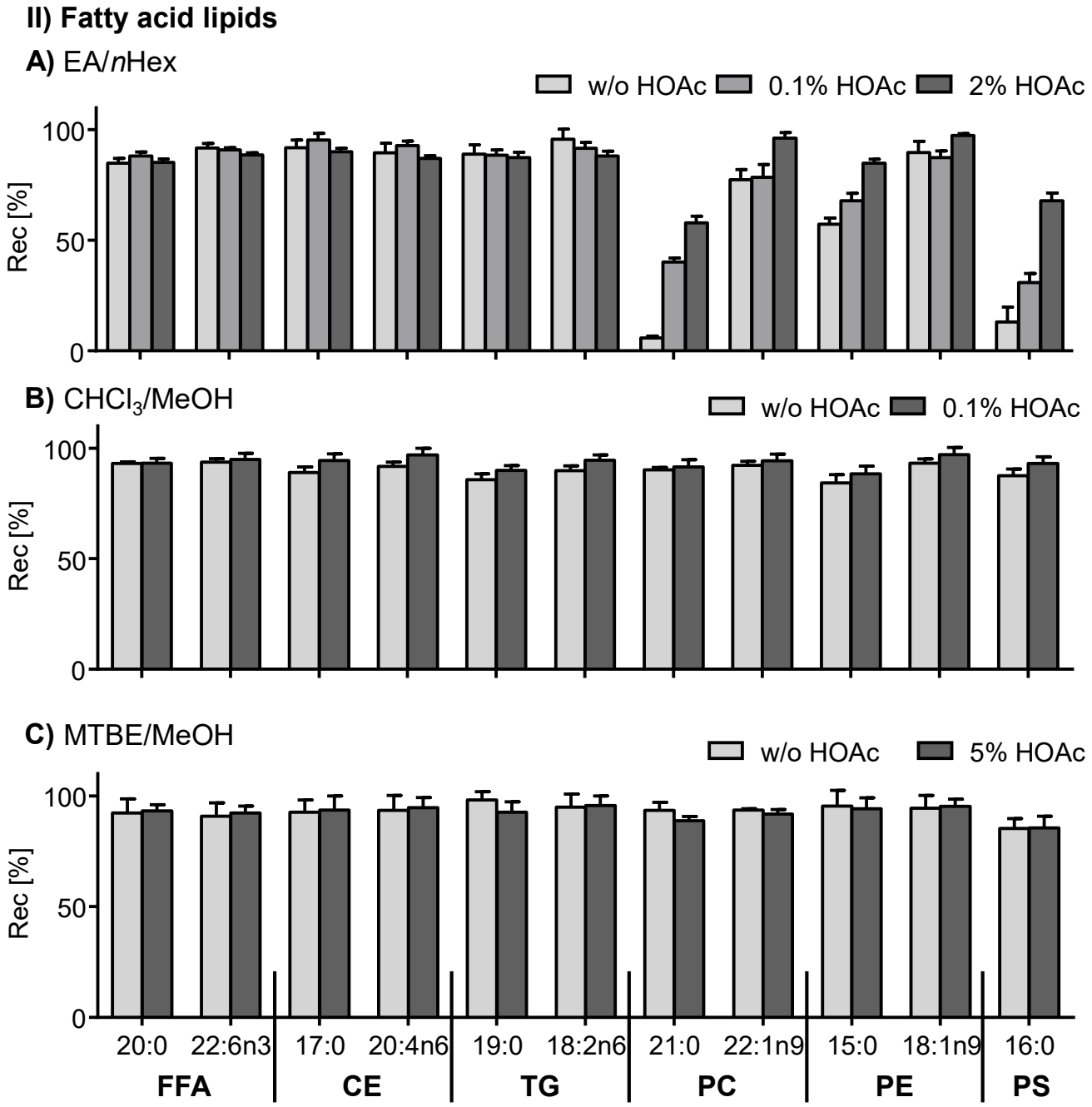


Fig. S3 **Continued.**

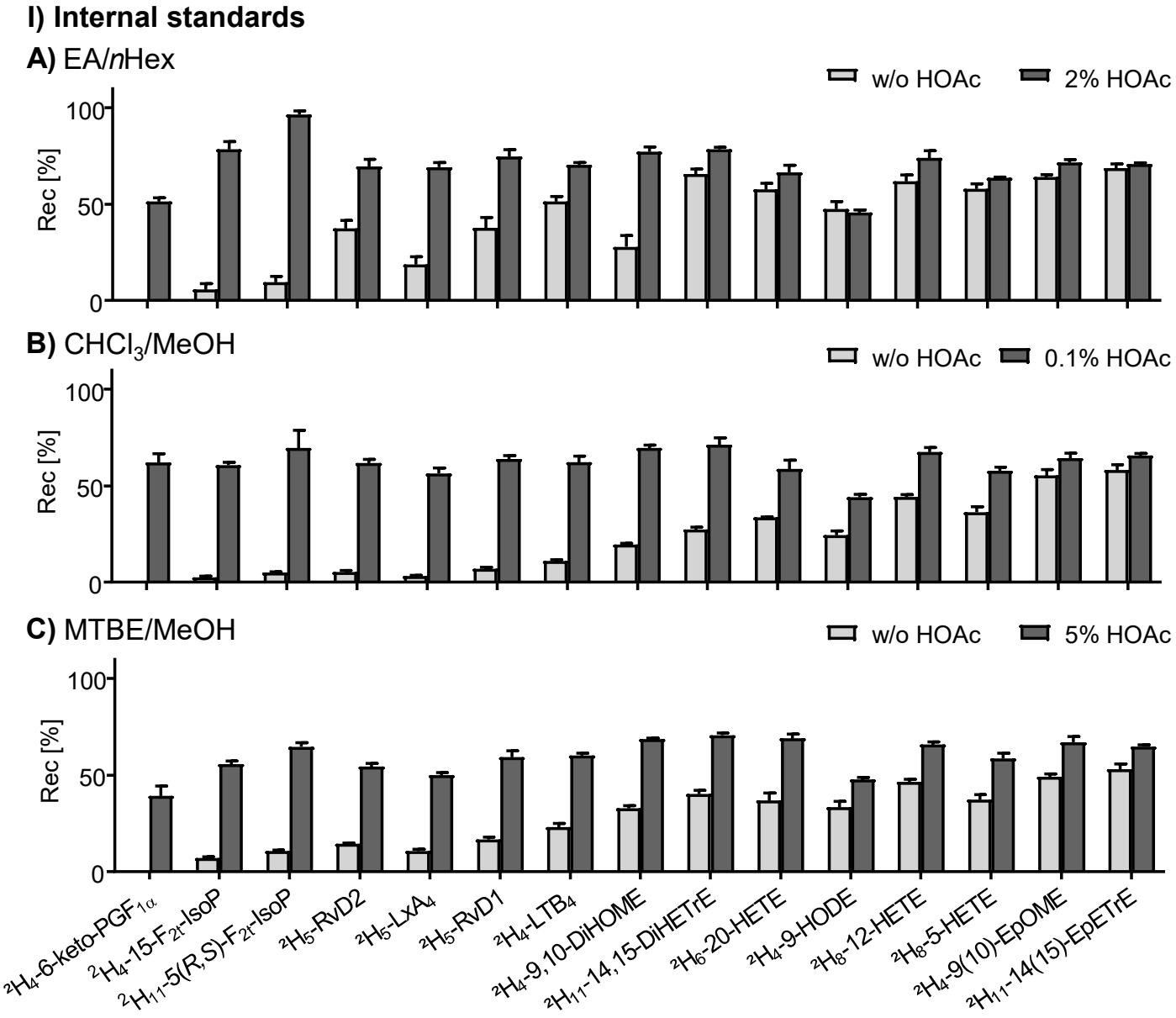
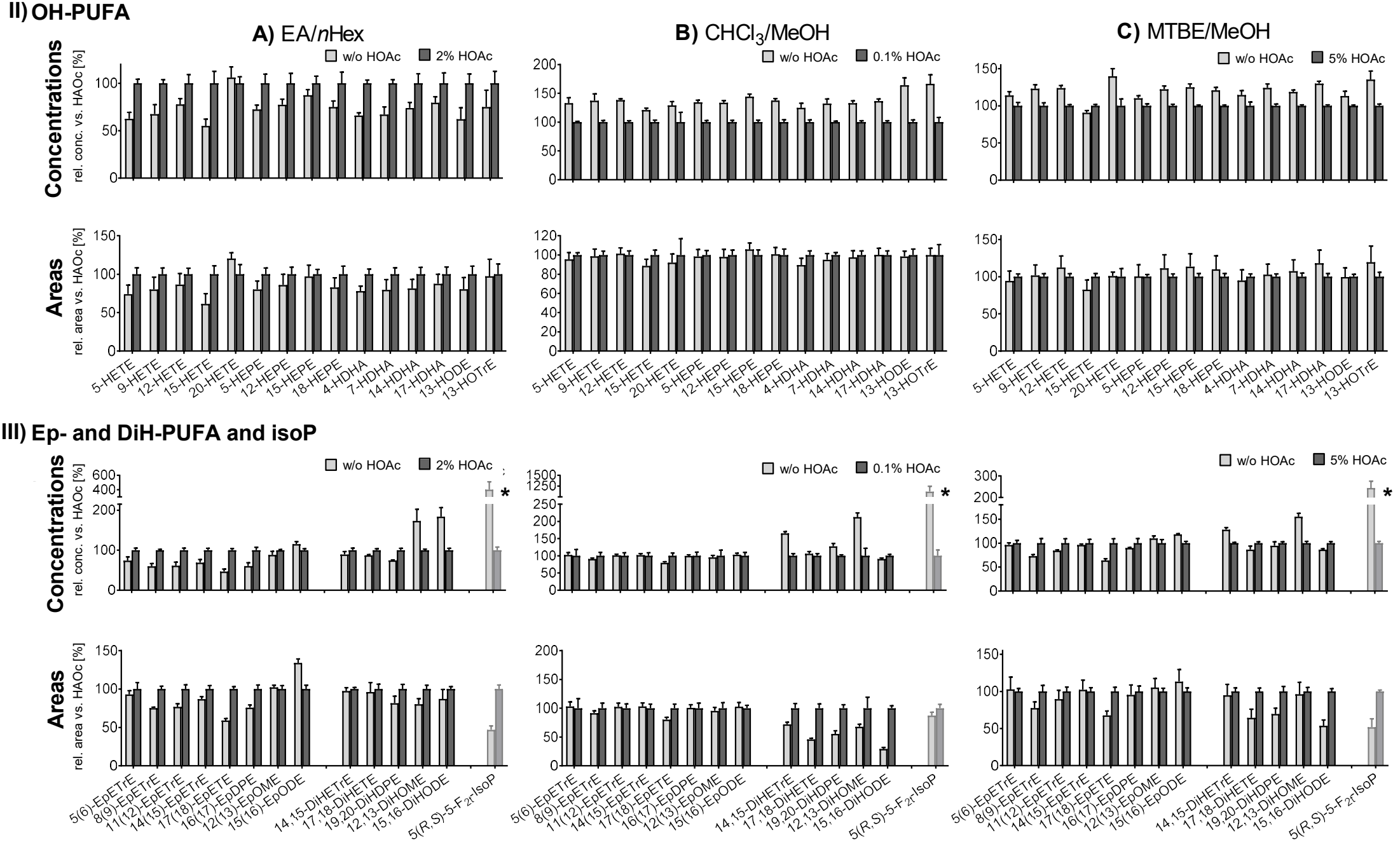


Fig. S4 LLE of plasma using acidified and non-acidified extraction solvents. Shown are (**I**) recoveries of deuterated oxylipin standards from plasma (100 µL) and relative concentrations and relative areas of representative (**II**) OH-PUFA and (**III**) Ep- and DiH-PUFA as well as isoP following LLE with **(A)** EA/*n*Hex (75/25, *v/v*), **(B)** $CHCl_3$/MeOH (2/1, *v/v*) and **(C)** MTBE/MeOH (2/1, *v/v*) (mean ± SD; n=3). Lipid extracts after LLE were evaporated to dryness and dissolved in 400 µL *iso*-propanol. Hydrolysis was carried out following addition of 100 µL water and 300 µL 1.5 M KOH in methanol/water (75/25, *v/v*) for 30 min at 60 °C. After neutralization, free oxylipins were extracted by SPE (on Bond Elut Certify II material) from the hydrolyzed sample and analyzed with LC-MS. Relative concentrations and areas in (**II**) and (**III**) were calculated against extraction with acidified solvents.



**Due to insufficient recovery of internal standard, 5(R,S)-5-F_{2t}-isoP should not be quantified and is only shown for demonstration.*

Fig. S4 Continued.

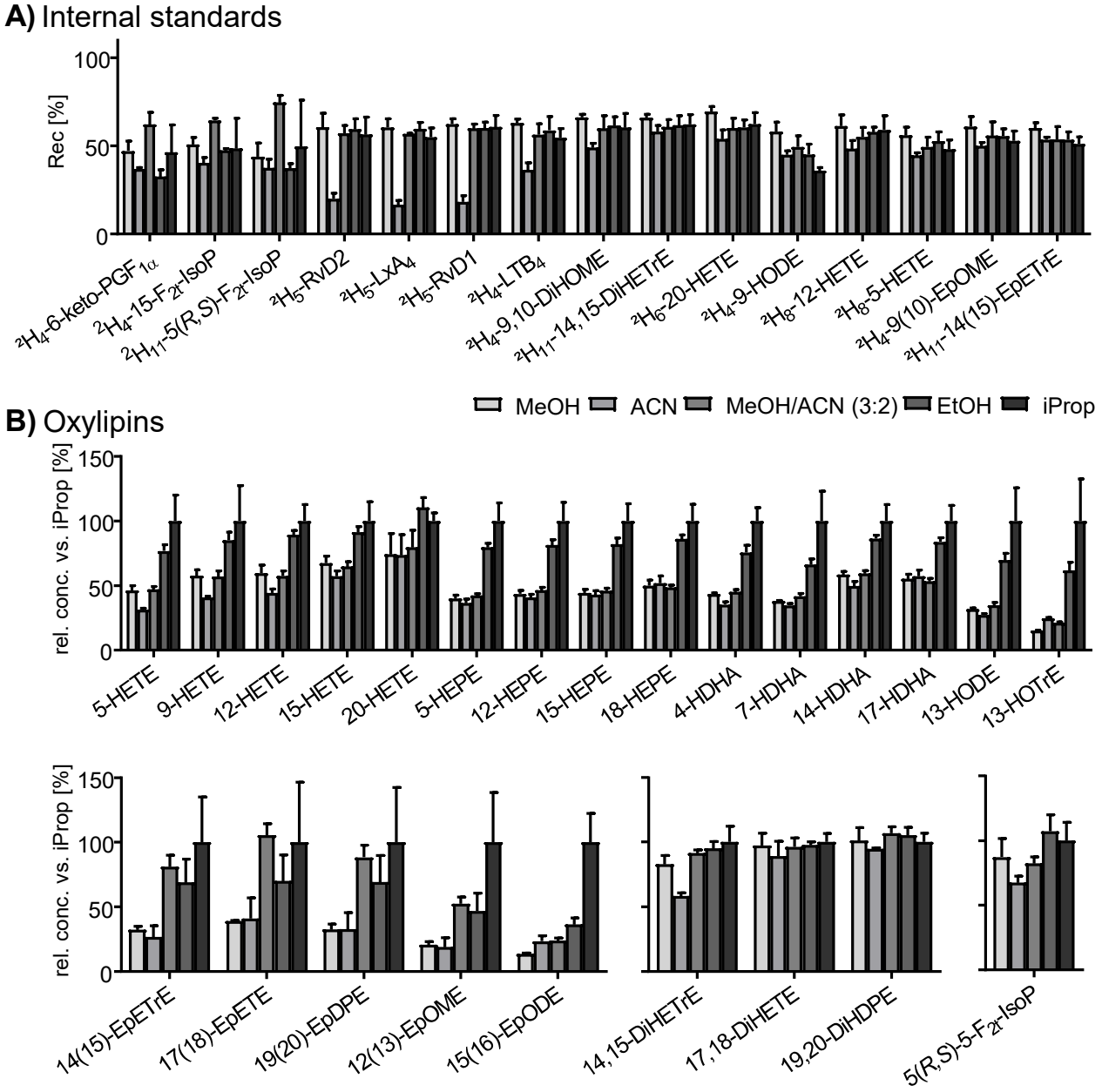


Fig. S5 **Extraction of lipids from plasma using protein precipitation.** Shown are **(A)** recoveries of deuterated oxylipin standards and **(B)** relative concentrations of a representative set of analytes in plasma (100 µL) following protein precipitation with 400 µL methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), methanol/ acetonitrile (3/2, *v/v*, MeOH/ACN) or *iso*-propanol (iProp) (mean ± SD; n=4) and base hydrolysis (300 µL 5.5 M NaOH (aq); 30 min, 60°C). Free oxylipins were extracted by SPE (on Bond Elut Certify II material) from the hydrolyzed sample and analyzed with LC-MS. Relative plasma oxylipin concentrations were calculated against iProp.

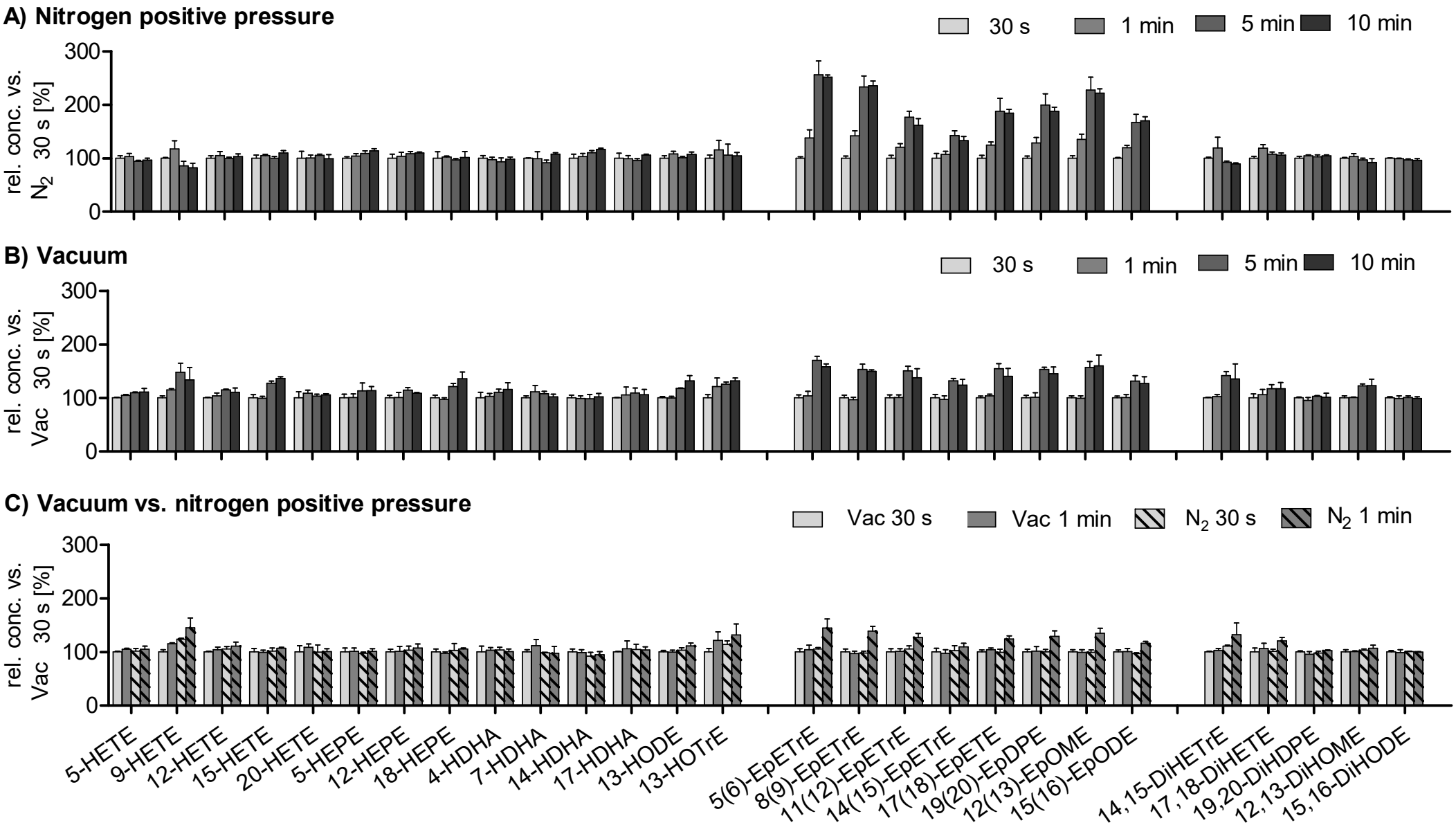


Fig. S6 **Effect of sample drying techniques during SPE (before elution) on the determined plasma oxylipin profile: Bond Elut Certify II cartridges.** Shown are relative plasma concentrations of a representative set of analytes following drying of samples with **(A)** nitrogen positive pressure and **(B)** vacuum (-200 mbar) for different time intervals (mean ± SD; n=3). Relative concentrations were calculated against the mean of the concentration following 30 s of drying. In **(C)** a comparison of drying with vacuum and nitrogen positive pressure is shown. Relative concentrations were calculated against drying with vacuum for 30 s. **(D)** shows the concentrations of *cis* and *trans* epoxy-PUFA in human plasma dried for either 30 s or 20 min prior to elution. For sample preparation, lipids were extracted from 100 µL plasma using 400 µL *iso*-propanol. Hydrolysis was carried out following addition of 300 µL 1.5 M KOH in methanol/water (75/25, *v/v*) for 30 min at 60 °C. After neutralization, free oxylipins were extracted via SPE from the hydrolyzed sample and analyzed with LC-MS.

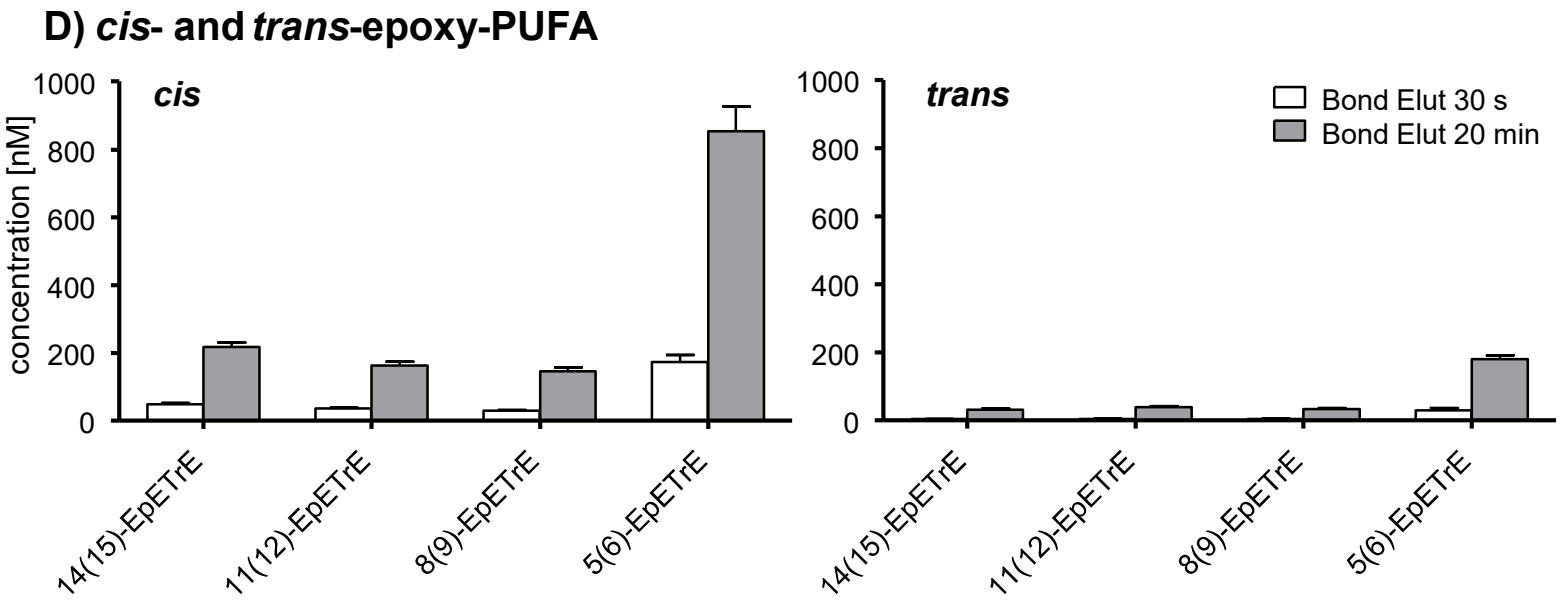


Fig. S6 **Continued.**

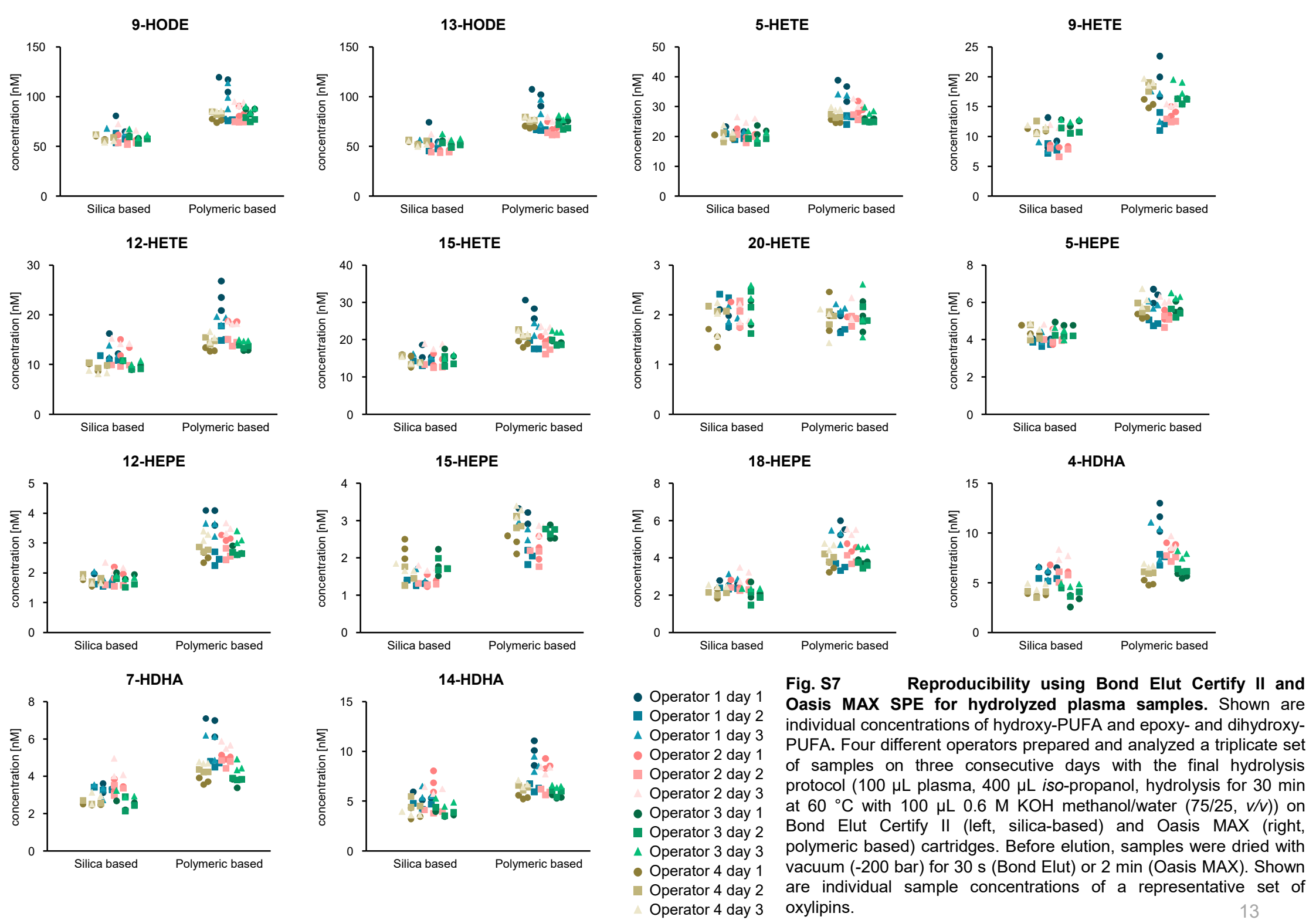


Fig. S7 Reproducibility using Bond Elut Certify II and Oasis MAX SPE for hydrolyzed plasma samples. Shown are individual concentrations of hydroxy-PUFA and epoxy- and dihydroxy-PUFA. Four different operators prepared and analyzed a triplicate set of samples on three consecutive days with the final hydrolysis protocol (100 µL plasma, 400 µL *iso*-propanol, hydrolysis for 30 min at 60 °C with 100 µL 0.6 M KOH methanol/water (75/25, *v/v*)) on Bond Elut Certify II (left, silica-based) and Oasis MAX (right, polymeric based) cartridges. Before elution, samples were dried with vacuum (-200 bar) for 30 s (Bond Elut) or 2 min (Oasis MAX). Shown are individual sample concentrations of a representative set of oxylipins.

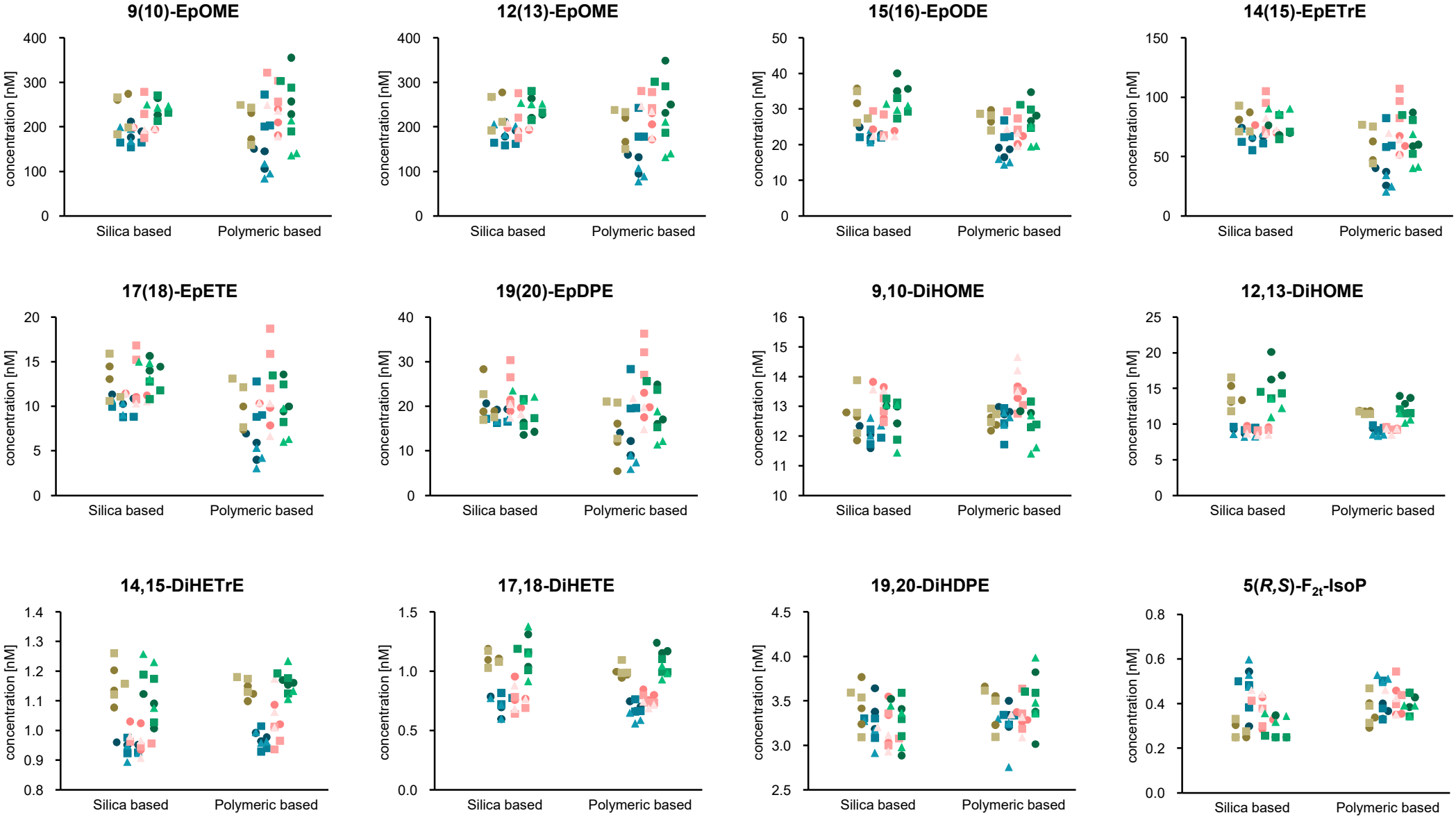


Fig. S7 Continued.

	100/400	200/400	200/600	200/800
Volume plasma [µL]	100	200	200	200
Volume *iso*-propanol [µL]	400	400	600	800
Ratio sample aq/org	1+4	1+2	1+3	1+4
Base concentration [M] (100 µL)	0.6	0.7	0.9	1.1
Dilute with buffer [µL]	2000	2000	2000	2000
Content organic on cartridge [%]	20	20	25	30

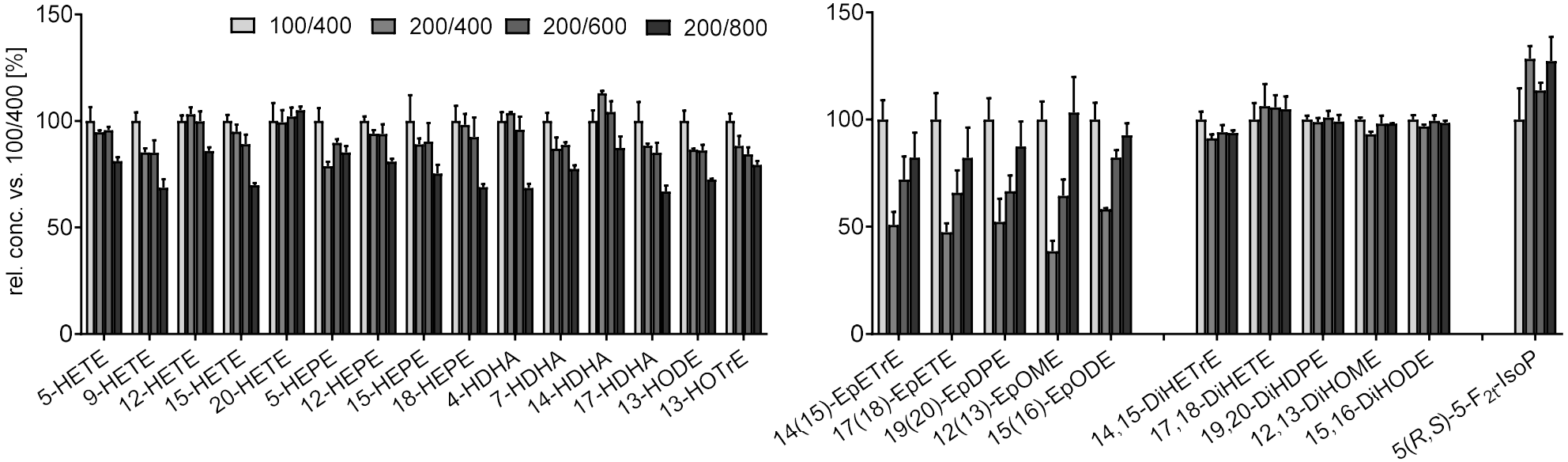


Fig. S8 **Upscaling of plasma volume.** For sample preparation, lipids were extracted from 100 or 200 µL plasma with *iso*-propanol (ratio sample+*iso*-propanol of 1+2 to 1+4). Hydrolysis was carried out following addition of KOH in methanol/water (75/25, *v/v*; final KOH concentration in sample 0.1 M) for 30 min at 60 °C. After neutralization, free oxylipins were extracted via SPE (on Bond Elut Certify II material) from the hydrolyzed sample and analyzed with LC-MS. Shown are relative concentrations of a representative set of analytes against sample preparation with 100 µL plasma and 400 µL *iso*-propanol (mean ± SD; n=3).

Using 200 µL plasma and 800 µL *iso*-propanol (200/800), recovery rate of $^2H_4$-6-keto-$PGF_{1\alpha}$ was insufficient (< 40%). For the other setups, recovery rates of internal standards were acceptable and no differences were observed between the different experimental protocols.

While 100 µL plasma were sufficient to cover a broad spectrum of hydroxy-, epoxy- and dihydroxy-PUFA, only one isoP (5(*R,S*)-5-F_{2t}-IsoP) could routinely be quantified. Only an increase of the sample volume to 200 µL in combination with a higher injection volume (10 µL instead of 5 µL) resulted in the quantification of one additional isoP (8-*iso*-$PGF_{2\alpha}$) which, however, was concomitant with reduced concentrations of other oxylipin classes (hydroxy- or epoxy-PUFA).

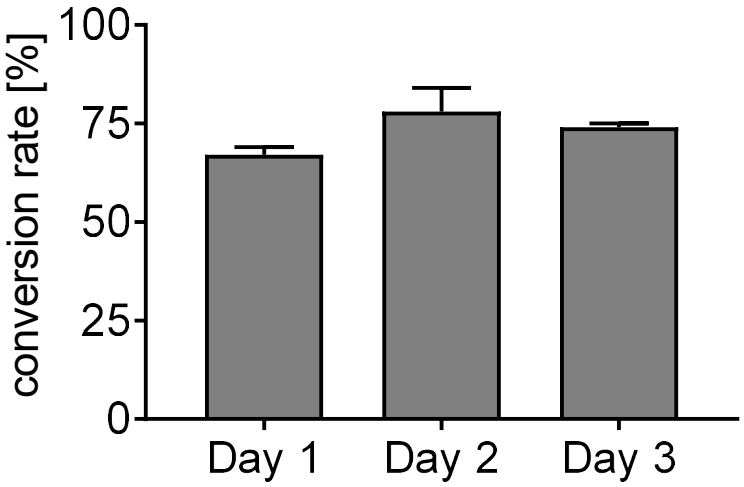


Fig. S9 Conversion of PGE_2 to PGB_2 during hydrolysis. PGE_2 was spiked to 100 µL plasma (final concentration in sample 50 nM). Samples were prepared according to the final protocol. Recoveries were calculated against a diluted PGE_2 standard and eqimolar conversion of PGE_2 to PGB_2 was assumed. Shown is mean conversion ± SD (n=3) on three days. Note: Following hydrolysis of a PGD_2 standard (spiked to plasma), no PGJ_2 formation was observed.