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Development of an LC-ESI(−)-MS/MS method for the simultaneous quantification of 35 isoprostanes and isofurans derived from the major n3- and n6-PUFAs

Katharina M. Rund, Annika I. Ostermann, Laura Kutzner, Jean-Marie Galano, Camille Oger, Claire Vigor, Sabine Wecklein, Nina Seiwert, Thierry Durand, Nils Helge Schebb

* Institute for Food Toxicology, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany
b Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 CNRS, Université de Montpellier, ENSCM, France
c Department of Toxicology, University Medical Center Mainz, Obere Zahlbacher Str. 67, 55131 Mainz, Germany
d Chair of Food Chemistry, Faculty of Mathematics and Natural Sciences, University of Wuppertal, Gaußstraße 20, 42119 Wuppertal, Germany

ABSTRACT

Misregulation of oxidative and antioxidative processes in the organism — oxidative stress — contributes to the pathogenesis of different diseases, e.g. inflammatory or neurodegenerative diseases. Oxidative stress leads to autoxidation of polyunsaturated fatty acids giving rise to prostaglandin-like isoprostanes (IsoP) and isofurans (IsoF). On the one hand they could serve as biomarker of oxidative stress and on the other hand may act as lipid mediators, similarly as the enzymatically formed oxylipins. In the present paper we describe the development of an LC-ESI(−)-MS/MS method allowing the parallel quantification of 27 IsoP and 8 IsoF derived from 6 different PLFA (ALA, ARA, EPA, AdA, n6-DPA, DHA) within 12 min. The chromatographic separation was carried out on an RP-C18 column (2.1 × 150 mm, 1.8 μm) yielding narrow peaks with an average width at half maximum of 3.3−4.2 s. Detection was carried out on a triple quadrupole mass spectrometer operating in selected reaction monitoring mode allowing the selective detection of regioisomers. The limit of detection ranged between 0.1 and 1 nM allowing in combination with solid phase extraction the detection of IsoP and IsoF at subnanomolar concentrations in biological samples. The method was validated for human plasma showing high accuracy and precision. Application of the approach on the investigation of oxidative stress in cultured cells indicated a distinct pattern of IsoP and IsoF in response to reactive oxygen species which warrants further investigation.

* Corresponding author. Chair of Food Chemistry, Faculty of Mathematics and Natural Sciences, University of Wuppertal, Gaußstraße 20, 42119 Wuppertal, Germany.
E-mail address: nils@schebb-web.de (N.H. Schebb).
1. Introduction

Oxidative stress results from misregulation of oxidative and antioxidative mechanisms in the organism and is characterized by increased formation of reactive oxygen and nitrogen species [1–3]. These highly reactive molecules attack biomolecules, thereby e.g. modifying proteins as well as DNA and causing mutations [4]. Oxidative degradation of membrane lipids alters the function of membranes and gives rise to a multitude of reactive and stable products [5,6]. Oxidative stress, and thus autoxidation, is associated with the pathophysiology of several diseases including inflammatory, cardiovascular, respiratory and neurodegenerative diseases.

In order to evaluate oxidative stress in health and disease, several biomolecules affected by autoxidation (i.e. proteins, DNA, lipids), their degradation products (e.g. malondialdehyde (MDA)) or endogenous antioxidants (e.g. glutathione (GSH)/glutathione di-sulfide) are used as common biomarkers [7].

In a multi-laboratory comparison of different oxidative stress markers the measurement of 15-F2t-IsoP, an isoprostane derived from arachidonic acid (C20:4 n6, ARA) was found to be promising for the evaluation of the oxidative stress status in vivo [8]. Iso-prostanes (IsoPs) are prostaglandin-like autoxidation products formed from free PUFA and under physiological conditions dominantly from PUFA esterified in phospholipids [9]. During non-enzymatic, free radical mediated autoxidation, PUFA are initially converted via radical abstraction of a bisallylic hydrogen and subsequent addition of molecular oxygen to hydroperoxy fatty acid radicals (Fig. 1). Depending on the position of the initial radical abstraction, different regioisomeric hydroperoxy radicals are formed. The primary formed hydroperoxy intermediates further undergo secondary reactions leading to a diverse product spectrum. For instance, a sequence of two 5-endo-cyclization steps of the hydroperoxy radicals followed by addition of molecular oxygen and reduction of the side chain hydroperoxide leads to prostaglandin-H-like bicyclic endoperoxide intermediates. These are under physiological conditions unstable and react in the presence of reducing cellular agents like GSH to stable F-ring isoprostanes or, when physiological conditions unstable and react in the presence of H-like bicyclic endoperoxide intermediates. These are under reduction of the side chain hydroperoxide leads to prostaglandin- hydroperoxy radicals followed by addition of molecular oxygen and trum. For instance, a sequence of two 5-

2. Materials and methods

2.1. Chemicals

The isoprostane standard 15-F2t-IsoP as well as the deuterated formation mechanisms – the cyclic peroxide cleavage pathway and the epoxide hydrolysis pathway – 8 regioisomeric isofurans comprising 32 stereoisomers are formed from ARA resulting in a total of 256 possible isomers [13]. Conversion of other PUFA by an analogous mechanism leads to PhytO [14,15], dihomo-IsoP and NeuroP from ALA, AdA and DHA, respectively.

Moreover, depending on the tissue fatty acid composition a characteristic pattern of the formed isoprostanes results. For example, DHA and AdA are both found in the brain, however AdA is predominantly enriched in myelin which is part of the white matter, whereas DHA is enriched in neurons present in the grey matter [16]. Consequently, F2-dihomo-IsoP are associated with oxidative stress of the white matter, whereas F2-NeuroP are related to oxidative damage of the grey matter in vitro [17].

The analysis of isoprostanes in biological matrices is challenging because of their low concentration and the multitude of possible isomers formed from different PUFA. Several methods using gas chromatography (GC) and liquid chromatography (LC) coupled to mass-spectrometry (MS) as well as immunoassays are described for the analysis [18–38]. Immunoassays like ELISA which only allow the analysis of single isomers are easy to handle and enable high sample throughput. However, due to the multitude of structurally similar isoprostanes, ELISAs are susceptible to cross-reactivity leading to an overestimation compared to LC- and GC-MS methods [38,39]. In contrast, chromatographic methods coupled to MS allow the selective and sensitive parallel quantification of multiple isomers derived from a single as well as different PUFA. In addition to sample preparation steps, including extraction and purification, GC-MS requires derivatization of the analytes to volatile derivatives. Since this is more laborious, LC-MS has been predominantly used in the past years [26–34,40,41]. Most LC-MS methods cover only ARA derived F2-IsoP, especially the prominent 15-F2t-IsoP isomer [26–29,32,41] or are focused on IsoP derived from a single PUFA [31,34,40]. Only few methods have been described enabling the parallel quantification of IsoP derived from different PUFA [30,33,42].

However, keeping the tissue specific PUFA pattern in mind, the simultaneous analysis of a comprehensive set of IsoP and IsoF from all biologically relevant PUFA is crucial for the evaluation of oxidative stress in vivo independent of the origin of the analyzed biological specimen. Therefore, we developed in the present study a new, sensitive LC-ESI(−)-MS/MS method covering a comprehensive set of IsoP and IsoF from 6 biologically relevant PUFA that was integrated in an established method covering enzymatically formed oxylipins. The mass-spectrometric and chromatographic parameters were optimized and the method performance was thoroughly characterized. Finally, the method was applied on the investigation of the formation of IsoP and IsoF in response to tert-butyl hydroperoxide (t-BOOH) induced oxidative stress in HCT116 cells.
internal standards (IS) $^2$H$_4$-15-F$_2$-IsoP ($^2$H$_4$-8-iso-PGF$_2$) and $^2$H$_2$-5(RS)-5-F$_2$-IsoP ($^2$H$_{11}$-5-IF$_{25}$-$\beta$VI) were purchased from Cayman Chemicals (local distributor: Biomol, Hamburg, Germany). All other IsoP and IsoF standards, i.e. ARA derived 5-F$_2$-IsoP, 5-epi-5-F$_2$-IsoP, 2,3-dinor-15-F$_2$-IsoP, 15-epi-2,3-dinor-15-F$_2$-IsoP, EPA derived 5-F$_3$-IsoP, 5-epi-5-F$_3$-IsoP, 8-F$_3$-IsoP, 8-epi-8-F$_3$-IsoP, DHA derived 4(R,S)-4-F$_4$-NeuroP, 10-F$_4$-NeuroP, 10-epi-10-F$_4$-NeuroP, 14(R,S)-14-F$_4$-NeuroP, 4(R,S)-ST-$\Delta^5$-8-NeuroF, AdA derived 17(R,S)-17-F$_3$-dihomo-IsoP, ent-7(R,S)-7-F$_3$-dihomo-IsoP, 17(R,S)-10-epi-SC-$\Delta^{15}$-11-dihomo-IsoF, 7(R,S)-ST-$\Delta^{5}$-11-dihomo-IsoF, n6-DPA derived 4-F$_3$-NeuroP$_{n6}$, 4-epi-4-F$_3$-NeuroP$_{n6}$, and ALA derived ent-9-F$_{14}$-PhytoP, ent-9-epi-9-F$_{14}$-PhytoP, ent-16-F$_{14}$-PhytoP, 16-B$_{1}$-PhytoP, 9-L$_{1}$-PhytoP, ent-16(R,S)-13-epi-ST-$\Delta^{14}$-9-PhytoF as well as the unlabeled odd-chain IS C19-17-epi-17-F$_1$-PhytoP and C21-15-F$_2$-IsoP were synthesized, using our last strategy, as described [14,43,51]. 4(R,S)-4-F$_{14}$-NeuroP, 14(R,S)-14-F$_{14}$-NeuroP, 4(R,S)-ST-$\Delta^5$-8-NeuroF, 17(R,S)-17-F$_2$-dihomo-IsoP, ent-7(R,S)-7-F$_2$-dihomo-IsoP, 17(R,S)-10-epi-SC-$\Delta^{15}$-11-dihomo-IsoF, 7(R,S)-ST-$\Delta^5$-11-dihomo-IsoF, ent-16(R,S)-13-epi-SC-$\Delta^{14}$-9-PhytoF were only available as epimeric mixtures. LC-MS-grade methanol (MeOH), acetonitrile (ACN) and acetic acid were purchased from Fisher Scientific (Schwerte, Germany). n-Hexane (HPLC Grade) was obtained from Carl Roth (Karlsruhe, Germany). All other chemicals were purchased from Sigma Aldrich (Schnell- dorf, Germany). Pooled human EDTA plasma was generated by centrifugation (10 min, 4 °C, 20,000 x g) of EDTA blood, followed by mixing plasma from six healthy male and female volunteers aged between 25 and 38 years and immediately stored at −80 °C until analysis. All volunteers gave their written informed consent and all procedures were conducted according to the guidelines laid down in the Declaration of Helsinki and approved by the ethic committee at the medical chamber of Lower Saxony (Hannover, Germany).

2.2. Calibration and quantification of IsoP

For calibration, stock solutions of the individual analytes (in MeOH) were mixed and diluted in glass volumetric flasks (5–100 mL) with MeOH at 10 concentration levels (0.1, 0.25, 0.5, 1, 2, 5, 10, 20, 100 and 500 nM), each with 20 nM of the four internal standards. Calibration curves were calculated using linear least square regression (weighting: $1/x^2$). Analyte quantification was carried out based on the analyte to corresponding IS (Table 1) area...
The proteins were precipitated by addition of 1400 mL of IS solution (containing 100 nM of $^{2}$H$_{4}$-15-F$_{2}$t-IsoP, C$_{19}$-17-$^{17}$-F$_{2}$t-IsoP, $^{2}$H$_{4}$-15-F$_{2}$t-IsoP, C$_{19}$-17-$^{17}$-F$_{2}$t-IsoP, and 13 isotope labeled internal standards used in the targeted metabolomics method [45], Table S2) and 10 mL of oxidant solution (0.2 mg/mL BHT and EDTA, 100 mM) and storage at $-80^\circ$C for at least 30 min. After centrifugation ($5,000$ × $g$ for 30 min and an accuracy in the calibration curve within ± 20%.

The collision cell exit potential (CXP) was for all analytes 33 V.

$^a$ The LOD was set to the lowest concentration standard injected yielding a signal to noise ratio $\geq 3$.

$^b$ LLOQ was set to the lowest calibration standard injected yielding a signal to noise ratio $\geq 5$ and an accuracy in the calibration curve within ± 20%.

$^c$ Calibration range was determined for all analytes to 15-F$_{2}$t-IsoP $\times 10$.
0.1 M disodium hydrogen phosphate buffer adjusted to pH 5.5 with acetic acid, thus yielding a MeOH content of <17% (pH = 6).

The SPE cartridge was preconditioned with one column volume of each, ethyl acetate/n-hexane (75/25, v/v) containing 1% acetic acid, MeOH and 0.1 M disodium hydrogen phosphate adjusted to pH 6.0 with acetic acid in water/MeOH (95/5, v/v). After loading onto the preconditioned cartridge, the sample was washed with 3 mL water and 3 mL water/MeOH (50/50, v/v) and dried with excess pressure of N2 for 1 min. The analytes were eluted with 2 mL 3 mL water and 3 mL water/MeOH (50/50, v/v) onto the preconditioned cartridge, the sample was washed with 3 mL water and 3 mL water/MeOH (50/50, v/v) and dried with excess pressure of N2 for 1 min. The analytes were eluted with 2 mL 3 mL water and 3 mL water/MeOH (50/50, v/v) and dried.

For the analysis of isoprostanes formed in cell incubations, 10 µL of antioxidant solution, 50 µL water and 300 µL MeOH were added to the cell pellets which contained about 1 × 10⁶ cells. After homogenization in a ball mill (MM 400, Retsch, Haan, Germany) using two 3 mm stainless steel beads (10 min, 25 Hz), 600 µL MTBE was added and the homogenate was vigorously mixed. For separation of the aqueous and the organic layers 300 µL 0.15 M ammonium acetate solution was added. Following mixing and centrifugation (5 min, 4 °C, 3500 × g), the upper organic layer was transferred to another tube and the aqueous phase was washed with 300 µL MTBE. The combined organic layers were evaporated using a vacuum concentrator (1 mbar, 30 °C, 45–60 min; Christ, Osterode am Harz, Germany). The residue was resuspended in 50 µL MeOH containing 40 nM of 1-(1-(ethylsulfonyl)piperidin-4-yl)-3-(4-(trifluoromethoxy)phenyl)urea as IS2 for the calculation of the extraction efficiency of the IS, centrifuged (10 min, 4 °C, 20,000 × g) and analyzed by LC-MS.

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The method was characterized and validated regarding linearity, sensitivity (limit of detection (LOD) and lower limit of quantification (LOQ)), extraction efficiency, intra- and interday accuracy and precision based on criteria of the guideline on bioanalytical method validation of the European Medicines Agency [47]. Linearity was assessed using standard solutions covering a concentration range from 0.1 to 500 nM. The LOD was determined as the concentration yielding a signal to noise ratio (S/N) ≥ 3. The concentration with a S/N ≥ 5 and an accuracy and precision within the calibration curve of ±20% was defined as LOQ and set as the lowest concentration of the calibration curve.

The extraction efficiency at three different level of plasma volumes (200, 500 and 1000 µL human plasma) was determined as the recovery of the IS spiked to the sample prior extraction calculated using a calibration curve normalized to IS2 which is added in the last step of the analysis.

Intraday accuracy and precision (relative standard deviation, RSD) were evaluated in plasma samples spiked at four concentration levels (3 nM, 10 nM, 30 nM, 100 nM in vial) with IsoP and IsoF prior extraction. Four replicates of each level as well as unspiked plasma were analyzed. For intraday accuracy and precision the samples were analyzed on 3 days. The accuracy was calculated by comparing the determined concentration with the added concentration.

Isoprostanes are autoxidative derivatives of fatty acids containing a carboxylic moiety, thus ionization was carried out in ESI(-) mode leading to the formation of [M-H]⁻ ions, whose dominating formation was confirmed in MS full scan experiments. For each compound the declustering potential (DP) was optimized in single ion monitoring mode of the [M-H]⁻ ion to achieve an effective transmission of the ionized analytes from the source to the 2.5. Method characterization

For selective and sensitive LC-MS analysis of the isoprostanes and related analytes the mass spectrometric parameters were optimized for each analyte and are summarized in Table 1. The LC-MS analysis of isoprostanes and related analytes from other PUFA was combined with an established multimethod for oxylipins [45,48,49]. A list of all covered analytes and their mass spectrometric parameters can be found in the supplementary information (SI, Table S1). For data acquisition and instrument control Analyst Software (version 1.6.2, Sciex) and for integration and quantification Multiquant (version 2.1.1, Sciex) was used.

3. Results and discussion

For selective and sensitive LC-MS analysis of the isoprostanes and related analytes the mass spectrometric parameters were optimized for each compound individually and the chromatographic conditions were adjusted in order to yield good and rapid separation of the isobaric isomers.

3.1. Mass spectrometric detection

Isoprostanes are autoxidative derivatives of fatty acids containing a carboxylic moiety, thus ionization was carried out in ESI(-) mode leading to the formation of [M-H]⁻ ions, whose dominating formation was confirmed in MS full scan experiments. For each compound the declustering potential (DP) was optimized in single ion monitoring mode of the [M-H]⁻ ion to achieve an effective transmission of the ionized analytes from the source to the
entrance of the vacuum chamber with minimal in-source fragmentation. For selective detection of regioisomers specific fragment ions for selected reaction monitoring (SRM) were chosen from the product ion spectra (SI, Fig. S1) and the collision energy (CE) was optimized. The optimized MS parameters are shown in Table 1. The structure of F-IsoP is characterized by a prostanoid-like F-ring with two hydroxy groups and one further hydroxy group in one of the two side chains, either the α-chain (carboxy terminus) or the ω-chain (methyl terminus) (Fig. 1). Accordingly, for different regioisomers identical abundant fragments resulting from unspecific loss of H2O or CO2 as well as combinations of CO2 and up to 3 fold H2O loss were observed (Fig. 2A and B). Thus, for selective MS detection of different regioisomers the choice of specific fragment ions is crucial. Specific results from backbone fragmentation and can be attributed to α-cleavage adjacent to the hydroxy group in the side chain which is referred to as “α-hydroxy-β-ene-rearrangement”, a common fragmentation for unsaturated hydroxy-fatty acids [50]. For the investigated F-IsoP the observed α-fragments result from backbone fragmentation and can be attributed to α-cleavage adjacent to the hydroxy group in the side chain which is referred to as “α-hydroxy-β-ene-rearrangement”, a common fragmentation for unsaturated hydroxy-fatty acids [50]. For the investigated F-IsoP the observed α-fragments result from backbone fragmentation and can be attributed to α-cleavage adjacent to the hydroxy group in the side chain which is referred to as “α-hydroxy-β-ene-rearrangement”, a common fragmentation for unsaturated hydroxy-fatty acids [50]. For the investigated F-IsoP the observed α-fragments result from backbone fragmentation and can be attributed to α-cleavage adjacent to the hydroxy group in the side chain which is referred to as “α-hydroxy-β-ene-rearrangement”, a common fragmentation for unsaturated hydroxy-fatty acids [50]. For the investigated F-IsoP the observed α-fragments result from backbone fragmentation and can be attributed to α-cleavage adjacent to the hydroxy group in the side chain which is referred to as “α-hydroxy-β-ene-rearrangement”, a common fragmentation for unsaturated hydroxy-fatty acids [50]. For the investigated F-IsoP the observed α-fragments result from backbone fragmentation and can be attributed to α-cleavage adjacent to the hydroxy group in the side chain which is referred to as “α-hydroxy-β-ene-rearrangement”, a common fragmentation for unsaturated hydroxy-fatty acids [50]. For the investigated F-IsoP the observed α-fragments result from backbone fragmentation and can be attributed to α-cleavage adjacent to the hydroxy group in the side chain which is referred to as “α-hydroxy-β-ene-rearrangement”, a common fragmentation for unsaturated hydroxy-fatty acids [50]. For the investigated F-IsoP the observed α-fragments result from backbone fragmentation and can be attributed to α-cleavage adjacent to the hydroxy group in the side chain which is referred to as “α-hydroxy-β-ene-rearrangement”, a common fragmentation for unsaturated hydroxy-fatty acids [50]. For the investigated F-IsoP the observed α-fragments result from backbone fragmentation and can be attributed to α-cleavage adjacent to the hydroxy group in the side chain which is referred to as “α-hydroxy-β-ene-rearrangement”, a common fragmentation for unsaturated hydroxy-fatty acids [50].

![Fig. 2. Collision induced dissociation (CID) product ion spectra of [M-H]- ions of α-linolenic acid derived PhytoP and PhytoF. (A) ent-9-F1t-PhytoP (F-ring-PhytoP carrying the hydroxy group in the α-chain) (B) ent-16-F1t-PhytoP (F-ring-PhytoP carrying the hydroxy group in the ω-chain) (C) 16-B1-PhytoP (cyclopentenone with B-ring structure) (D) ent-16-(R,S)-13-epi-ST-Δ14,9-PhytoF (enediol-furan).](image-url)
the transition reported previously corresponding to the fragment with the highest abundance was selected [51]. Regarding 4(R,S)-ST-D5-8-NeuroF the fragment used in previous methods (m/z 123) [52] showed poor intensity, thus the fragment (m/z 187) with the highest abundance was selected (SI, Fig. S1D(iv)).

3.2. Chromatographic separation

The chromatographic separation was optimized in order to obtain an efficient separation of the isobaric isomers in a reasonable time. The method covers 25 F-ring IsoP comprising 12 diastereomeric (epimeric) pairs and one single stereoisomer derived
from ALA, ARA, EPA, n6-DPA, DHA and AdA, one B- and one L-ring PhytoP and 4 epimeric pairs of IsoF derived from ALA, DHA and AdA. Using a C18 column with sub 2 μm particles and an optimized gradient of acidified water and ACN as well as MeOH, separation of the analytes could be achieved within 12 min (Fig. 3A). The ALA derived PhytoP and F1t-PhytoP elute first, followed by the polar β-oxidation metabolites 15(RS)-2,3-dinor-15-F2t-IsoP from ARA and the IsoP from EPA and ARA. B- and L-ring PhytoP and DHA derived F4t-NeuroP and NeuroP elute in the middle part of the chromatogram followed by AdA derived F3t-dihomo-IsoP and dihomo-IsoF as well as n6-DPA derived 4(RS)-4-F3t-NeuroPm (Fig. 3).

The initial gradient conditions were adjusted in order to achieve separation from the void volume (1.5 min), i.e. sufficient retention of the early eluting analytes, which is characterized by a retention factor k of at least 2. The first analytes (PhytoP) elute at 4.19 and 4.30 min (after more than two void volumes), which corresponds to a retention factor of k ≥ 1.8, showing a sufficient separation from the unretained void volume. The slope of the gradient was optimized in order to yield a high separation efficiency of most epimeric pairs.

A short isocratic step (1 min) with 25% organic at the beginning of the gradient was included to focus the analytes at the front end of the column. This led to narrow peaks over the whole separation time (exemplarily shown in SI, Fig. S2) characterized by a full width at half maximum (FWHM) of about 3.3–4.2 s for almost all analytes (Table 1). Some epimeric pairs, e.g. 14(RS)-14-F4t-NeuroP and ent-7(RS)-7-F2t-dihomo-IsoP (Table 1, Fig. 3B), showed a broader FWHM due to incomplete separation of the epimers.

The chromatographic separation showed a high stability regarding retention time with an interbatch RSD of ≤ 0.25%, i.e. < 0.02 min (n = 360) or < 0.01 min within one batch (n = 50).

The gradient allows an efficient separation of 7 critical epimeric pairs of IsoP and IsoF characterized by a resolution of R > 1.5. With exception of the ent-16(RS)-13-epi-ST-Δ14,9-PhytoF, which is the first eluting epimeric pair showing a resolution of R = 1.4, the resolution of the other separated epimers is R > 1.7. However, 3 epimeric pairs (14(RS)-14-F4t-NeuroP, ent-7(RS)-7-F2t-dihomo-IsoP and 7(RS)-ST-Δ5-11-dihomo-IsoF) show an incomplete separation and 6 epimeric pairs coelute (Fig. 3B). The use of a shallower gradient with an increase of 0.5% B/min instead of 2.7% B/min or isocratic separation (data not shown) only slightly improved the separation of the incompletely separated and coeluting stereoisomers while further prolonging the analysis time. Thus, only the use of a different column or mobile phase thereby changing the selectivity of the chromatographic system could solve this separation problem. However, we regarded these parameters as fixed during method development because the analytical procedure should be integrated in an existing method, already established in our lab for the analysis of enzymatically formed oxylipins [45,48,49]. This combination enables to determine 129 oxylipins in addition to the covered IsoP and IsoF, thus allowing the detection of a total of 164 oxylipins formed enzymatically and via autoxidation together with 17 IS within 30.5 min. The mass-spectrometric and chromatographic performance parameters for all analytes are summarized in the SI, Table S1.

3.3. **Method performance**

Quantification of the analytes was carried out based on the analyte to corresponding IS area ratio using the calibration curves. For the IsoP and IsoF two deuterated IsoP [(6H11-5(RS)-5-F2t-IsoP and 6H4-15-F2t-IsoP) and two odd-chain unlabeled IsoP (C19-17-epi-17-F1t-PhytoP and C21-15-F2t-IsoP)] that are eluted over the analytical time (Fig. 3A) were used as IS. For 5(RS)-5-F2t-IsoP and 15-F4t-Isop his respective isotopologs were used as IS. For the other analytes the assignment of the IS was based on retention time (Table 1).

Characterization of the method performance was oriented on the guideline on bioanalytical method validation of the European Medicines Agency [47]. The sensitivity of the method was assessed by determining the LOD and the LLOQ based on the signal to noise ratio (Table 1). The concentration leading to an S/N ≥ 3 was defined as LOD (exemplarily shown for 2,3-dinor-15-F2t-IsoP, Fig. S3). Almost all analytes showed a comparable LOD ranging from 0.15 to 2 pg on column. However 14(RS)-14-F4t-NeuroP and 4(RS)-ST-Δ3-8-NeuroF showed a considerably higher LOD with 19 pg and 39 pg on column, respectively. Since the same differences in the signal intensity were observed in SIM mode (data not shown), these higher LOD could either be explained by different ionization behavior of the compounds or by impure standard stock solutions of the two compounds. Similar to the LOD, the LLOQ (i.e. the concentration with a S/N ≥ 5 and an accuracy within 80–120% of the calibration curve) ranged from 0.25 to 2 nM for almost all IsoP and IsoF except 14(RS)-14-F4t-NeuroP and 4(RS)-ST-Δ3-8-NeuroF. The sensitivity was also reflected by the slope of the calibration curves. Comparing the slopes of all covered IsoP and IsoF normalized to the same IS revealed that 14(RS)-14-F4t-NeuroP and 4(RS)-ST-Δ3-8-NeuroF showed a remarkably lower slope compared to the other analytes (factor 10 and 50 lower, respectively, compared to the mean of normalized slopes) which is consistent with the observed higher LLOQ and LOD (Table 1). Interestingly this higher LLOQ for 14(RS)-14-F4t-NeuroP was also observed previously [33]. Remarkably, 5(RS)-5-F3t-IsoP with an LLOQ of 2 nM, showed a comparably low slope. Compared to other IsoP, a slightly higher LLOQ for 5(RS)-5-F3t-IsoP was also reported previously [33].

The obtained LOD and LLOQ are in a similar range as reported previously for other oxylipins [45] and in comparison with previous reports for isoprostanes the obtained sensitivity was comparable or slightly better [30,33]. Noteworthy, two methods which were focused only on F2t-IsoP [41] and PhytoP and PhytoF [34] reported LLOQs one to two orders of magnitude lower despite using a comparable triple quadrupole mass spectrometer. This may be in part explained by the high injection volume of 50 μl as well as the use of a micro-HPLC instrumentation [34,41]. In our method, using a higher injection volume led to peak splitting and unacceptable peak shape (SI, Fig. S4). This is caused by the high elution power of the methanol which is used to reconstitute the samples after SPE. Using a more polar solvent leads to an insufficient solution of less polar hydroxy- and epoxy fatty acids which are also covered by our method and thus the amount of sample injected cannot be further

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**Fig. 4.** Recovery of internal standards added to 200 μl, 500 μl and 1000 μl human plasma at the beginning of sample preparation followed by protein precipitation with MeOH and solid phase extraction. Shown are mean ± SD (n = 4).
both isomers in the mixture and individual spiking level were calculated based on the isomeric ratio of the substances. Precision was calculated as relative standard deviation of the extraction of all IsoP and IsoF comprised by the method from human plasma (500 μL). For IsoP and IsoF which were available as isomeric mixtures, the spiking level correspond to the sum concentration of both isomers in the mixture and individual spiking level were calculated based on the isomeric ratio of the substances.

Intra- and interday accuracy (acc.) as well as precision (prec.) of the extraction of all IsoP and IsoF comprised by the method from human plasma (500 μL). For IsoP and IsoF which were available as isomeric mixtures, the spiking level correspond to the sum concentration of both isomers in the mixture and individual spiking level were calculated based on the isomeric ratio of the substances.

Table 2

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increased. Nevertheless, our approach is still one of the most sensitive methods described so far for the detection of IsoP. Moreover, it is the most comprehensive approach for the simultaneous quantification of prostanoid-like autoxidation products and covers more analytes than all previous methods.

Matching between method sensitivity and expected concentration in biological samples one should take into account that the baseline levels of IsoP and IsoF are very low, e.g. the commonly analyzed 15(R,S)-15-F2t-IsoP is in the range of 40–170 pg/mL human plasma (total, i.e. free and esterified) of healthy subjects [53], corresponding to 0.1–0.5 nM. Sufficient sample preparation including a pre-concentration step as carried out in the presented method by an optimized SPE procedure is therefore needed to determine these compounds in biological samples.

For quantification, calibration curves at 10 concentration levels covering a concentration range from 0.1 to 500 nM for each analyte were prepared. With linear least square regression based on 1/x² weighting correlation coefficients ≥ 0.994 were achieved for all analytes indicating linearity for the covered concentration range (Table 1). The highest calibration level was set as upper limit of quantification (ULOQ), however does not represent the end of the linear detector response. Taking the low physiological
concentration of IsoP and IsoF into account the calibration of higher concentration as carried out in other methods [30–33] was for the intended application not necessary.

3.4. Extraction efficiency

The extraction efficiency from biological samples was characterized based on the recovery of IS spiked to the sample prior extraction. For the evaluation of the extraction efficiency of the used sample preparation procedure different volumes (200 μL, 500 μL, 1000 μL) of plasma from healthy volunteers were extracted by SPE. As shown in Fig. 4, recoveries in the range of 80–100% for all IS independent from the used plasma volume indicate a sufficient extraction for quantitative determination.

Regarding all IsoP and IsoF covered by the method, no IsoF and only ARA derived F2t-IsoP were detected in the analyzed human plasma from healthy subjects. The most abundant regioisomer was 5(R,S)-5-F2t-IsoP with comparable concentration for all investigated volumes (128 ± 13; 138 ± 6 and 132 ± 6 pM for 200 μL, 500 μL and 1000 μL respectively) indicating a good accuracy and precision regardless of the amount of sample/matrix injected. For 15-F2t-IsoP, which is the most studied regioisomer, lower levels were detected (48 ± 1 and 38 ± 1 pM for 500 μL and 1000 μL respectively). This is consistent with previous findings reporting concentrations for 15(R,S)-15-F2t-IsoP in the range of 16–48 pg/mL (45–135 pM) [19] and 3–25 pg/mL (8–71 pM) [54] in human plasma of healthy subjects.

3.5. Intra- and interday accuracy and precision

Intra- and interday accuracy and precision were characterized oriented on the guideline on bioanalytical method validation of the European Medicines Agency. In order to evaluate the accuracy and precision of the analytical procedure, human plasma samples spiked at four concentration levels prior extraction were analyzed in four replicates. The intraday accuracy ranged for all analytes from 77 to 121% indicating low interference of plasma matrix and good extraction efficiency of the analytes using the presented SPE procedure. Intraday precision calculated as RSD was < 15% for almost all analytes at all spiking levels with few exceptions being, however, below 22% (Table 2). Interday precision was better, below 11% for 10, 30 and 100 nM spiking levels, thus indicating a stable method which allows the analysis of large sample batches. However, at the low concentration level (3 nM), which is close to LLOQ, RSDs showed a trend towards higher values, but were still < 22% for almost all analytes, except 7(R,S)-ST-Δ11-dihomo-IsoF (LLOQ of 2 nM, RSD = 32%, Table 2).

3.6. Formation of IsoP in cell culture

The developed method was applied to investigate the formation of IsoP and IsoF in HCT116 cells during oxidative stress caused by t-BOOH. First, the viability of HCT116 cells treated with increasing doses of t-BOOH for 2 h was determined with the MTS assay, revealing no effects at a dose of 50 μM and a moderate reduction of...
the viability to 84% in cells incubated with 200 μM t-BOOH (SI, Fig S5). Following incubation a large number of IsoP were detected in the cells, i.e. 5(R,S)-5-F2t-IsoP, 15-F2t-IsoP, ent-7(R,S)-7-t-3homo-IsoP, 17(R,S)-17-t-3homo-dihomo-IsoP 2, 5(R,S)-5-F3t-IsoP, 4(R,S)-4-F4t-NeuroP, 10-F4t-NeuroP, 10-epi-10-F4t-IsoP (Fig 5, Table S3). In control incubations the levels of the detected IsoP were low or below the LLOQ (Table S3, Fig 5), with EPA derived 5(R,S)-5-F2t-IsoP being most abundant.

Regarding distribution of the different IsoP, a dominant formation of regioisomers carrying the side chain hydroxyl group in proximity to the carboxy terminus was observed for the IsoP formed from all different PUFA: 5(R,S)-5-F2t-IsoP, 5(R,S)-5-F3t-IsoP, ent-7(R,S)-7-t-3homo-IsoP and 4(R,S)-4-F4t-NeuroP. At high concentrations of t-BOOH also other regioisomers were formed and the IsoP concentration increased dose dependently with the t-BOOH concentration. IsoP levels after 30 min with 200 μM were generally higher than after 2 h of incubation with 50 μM t-BOOH. Moreover, with 200 μM t-BOOH, even if not statistically significant, a trend towards higher levels of IsoP with increasing incubation time was observed while in incubations with 50 μM t-BOOH similar concentrations of IsoP were found for the different time points. This may indicate a cellular defense mechanism against ROS and thus IsoP formation decreased. It is also remarkable that with higher t-BOOH concentration the regioisomers with the side chain hydroxyl group in proximity to the carboxy terminus were stronger elevated than other regioisomers (Fig 5), which may indicate not only differences in formation but also in the metabolism rates of the different regioisomers. This becomes particularly evident for the Ara derived IsoP. Here, 3–4 fold higher levels of 5(R,S)-5-F2t-IsoP were found compared to 15-F2t-IsoP while in livers of CCl4 exposed rats a ratio of 1.5:1 was found [12]. However, it should be noted that these differences might also indicate species specific formation and metabolism rates of different regioisomers. The metabolism of the F2-IsoPs, particularly 15-F2t-IsoP has been well described [55,56]. By contrast the metabolism of the NeuroPs is not yet well understood, except an earlier investigation reported by Lawson et al. who showed that 7-F4t-NeuroP is readily beta-oxidized to 5-F3t-IsoP (an isoprostanoic metabolite from EPA oxidation) and excreted [57] whereas certain NeuroPs like 4-F4t-NeuroP are probably more stable due to the presence of the side chain hydroxyl group at C4, which can limit access of the enzymes involved in beta-oxidation as it is the case for 5-hydroxyeicosanoids [58].

These results indicate that either the cellular formation is cell specific or the products are metabolized at different rates. The cellular formation of IsoP in response to oxidative stress and especially the resulting IsoP pattern warrants further investigation. In particular, application of different cell types and cell lines, e.g. deficient in different ROS defense mechanisms, should be used to understand the intracellular formation and the metabolic fate of IsoP. This investigation will allow gaining more insights into the role of IsoP in biology and help to identify specific biomarkers for distinct physiological states. With the developed method described herein, we provide the ideal tool to comprehensively quantify IsoP derived from all biologically relevant PUFA.

Acknowledgement

Our work is supported by a grant (01EA1702) of the Federal Ministry of Education and Research of Germany (BMBF) in the framework of the Joint Program Initiative ERA-HDIL to NHS, in part by the German Research Foundation (DFG, Grant SCHM 1801 to NHS) and by the Fonds der Chemischen Industrie in form of a PhD scholarship to KR. TD thanks his two colleagues, Dr Valérie Bultel-Poncé and Dr Alexandre Guy, for having synthesized the two unlabelled odd-chain IS.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jaca.2017.11.002.

References


