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Simultaneous determination of PUFA-derived pro-resolving metabolites and pathway markers using chiral chromatography and tandem mass spectrometry

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A B S T R A C T

Lipid mediators play an important role as biological messengers involved in inflammatory processes. Deriving from different polyunsaturated fatty acids, endogenously built mediators featuring both pro- and anti-inflammatory properties as well as pro-resolving lipid mediators and their biological precursors have been investigated. A newly developed method using chiral chromatography-tandem mass spectrometry on human plasma has demonstrated its suitability for the simultaneous determination of prostaglandins, lipoxins, D-series derived resolvins as well as protectins, maresin 1, leukotriene B₄ and several precursors of them in order to yield information about metabolic pathways. Due to the matrix complexity, a solid phase extraction method using an octadecyl-modified silica gel cartridge was carried out. The developed method allows the determination of 34 analytes in 25 min showing enough selectivity as well as precision and accuracy ($\leq 15\%$ relative standard deviation, $\leq 15\%$ relative error) in the calibration range of

0.1–10 ng mL⁻¹ or 0.2–20 ng mL⁻¹ depending on the analytes. Stability of the analytes in plasma has been demonstrated for at least 3 h at room temperature, 72 h in the autosampler and 60 days in the freezer at $-80\text{ }^{\circ}\text{C}$. This method has been validated and shown its suitability for the determination of all studied analytes in human plasma samples.

Keywords:

Specialized pro-resolving lipid mediators

Chiral chromatography

LC-MS/MS

Sensitivity

Resolution

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1. Introduction

Specialized pro-resolving mediators (SPM) are endogenously produced lipid mediators (LM) derived from different polyunsaturated fatty acids (PUFA), mainly the omega-6 fatty acid arachidonic acid (AA) and the omega-3 fatty acids docosahexaenoic (DHA) and eicosapentaenoic acid (EPA). Additionally, mediators may result from the conversion of docosapentaenoic acid, which has been found to have its double bonds at different positions and may thus be categorized as n-3 (DPA_{n-3}) and n-6 (DPA_{n-6}) derivatives, respectively. Through endogenous biological stimuli SPM are built at their intended site of action via cyclooxygenases (COX) 1 and 2 and lipoxygenases (LOX) 5, 12 and 15 [1–3]. As intracellular messengers, these mediators play an important role in inflammation and thus have a versatile use in pathophysiological processes and are highly interesting for the possible use for therapy.

On one hand, COX-2, besides being involved in the prostaglandin synthesis together with COX-1 [1,4], builds enzymatic products of PUFA with a stereochemical R-positioned hydroxyl group after treatment and acetylation by the non-steroidal anti-inflammatory drug Aspirin® [2]. On the other hand, LOXs oxidize PUFAs into cell signaling agents featuring mainly S-configured functional groups. Due to their molecular structure, SPM decompose when exposed to light. Furthermore, in part through the molecules central tri- and tetraene-system, respectively, they are also sensitive to acids as well as thermally unstable for extended periods of time [5–7]. Development of suitable new analytical detection methods is thus faced with the problems mentioned above. Furthermore, multi-component analytical methods for endogenous compounds in biological matrices, especially when the expected analyte concentrations are in the low range of the calibration curve, are very challenging due to the high number of interferences, such as isobaric and isomeric compounds. In these cases, direct analytical technologies such as mass spectrometry (MS) coupled with gas (GC) or liquid (LC) chromatography are essential. These techniques have both advantages as well as drawbacks though. Whereas GC-MS may have successful uses for some lipid mediators and metabolites, analytes have to be converted into volatile forms to elute with the gaseous phase, which is a handicap for thermally unstable components. To our knowledge, to date GC has been used only for the characterization of lipoxins and quantification of lipoxin A4 (LXA4) [8–10]. For further quantification of SPM, the technique of choice reflected in the bibliography is LC-MS or LC-MS/MS. Extracted samples can be directly injected into the system for analysis without prior derivatization.

Several approaches for the determination of pro-resolving lipid mediators in biological samples have been described in the bibliography: maresin 1 (MaR1) and LXA4 in human synovial fluid taken from rheumatoid arthritis patients [11], LXA4, 15-*epi*-LXA4 (ATL), MaR1, protectin DX (PDX) as well as D-series resolvins and 17-*epi*-resolvin D1 (ATR) in healthy human milk [12,13] or MaR and its precursor in human macrophages [14]. In those cases, the enantiomers were not measured separately and the results represent the values for possible racemic mixtures. In contrast, Mas et al. described a method in 2012 profiling DHA-derived mediators in both serum and plasma. The enantiomers resolvin D1 (Rv D1) and ATR as well as neuroprotection D1 (NPD1) and PDX could be distinguished and quantified separately. Concentrations for the resolvins were found to be similar for serum and plasma at average levels of around 30 pg mL⁻¹ for Rv D1 and 65 pg mL⁻¹ for ATR whereas the values of both PDX and NPD1 were below the lower limit of quantification (LLOQ) of 25 pg mL⁻¹ [15]. Also a chiral method employing a Chiralpak AD-RH stationary phase to separate maresins 1 and 2 as well as both 14(S)- and 14(R)-HDoHe has been described. Of the latter, only the derivative with the hydroxyl group in S-configuration is important for the raise of maresins and it could be confirmed by comparison with standards, that 14(S)-HDoHe is actually built as intermediary pathway marker. Using chiral chromatography, analytes differing in their configuration and, more

importantly, isomers can be easily distinguished through enhanced interactions between the stationary phase and the analytes [16,17].

To allow for the characterization of an inflammation as well as the potential inflammatory phase prevailing in a patient, a selective and sensitive quantification method for the determination of inflammatory lipid mediators in human plasma has been developed. Due to their important role during these processes and consequently in recovering tissue homeostasis [18], prostaglandins featuring both pro- and anti-inflammatory properties [1,19] and the pro-resolving lipoxins as well as resolvins, protectins and maresins, which are also considered potent regulators of pain, have been studied simultaneously. Furthermore, several pathway markers of SPMs have been included in the method [2,16,20–26]. In summary, in this study the AA derivatives LXA4, 6-*epi*-LXA4, ATL, lipoxin B4 (LXB4), prostaglandins E2 (PGE2), PGD2, PGJ2, 6-keto-PGF1 α , PGF2 α , thromboxane B2 (TXB2), 11-dh-TXB2, leukotriene B4 (LTB4) and 12-*epi*-LTB4, DHA derivatives Rv D1, ATR, Rv D2, MaR1, PDX, NPD1, dinor-NPD1 and tetranor-NPD1, the EPA derivative lipoxin A5 (LXA5), DPA derivatives 10(S),17(S)-DiHDPA_{n-3} and 10(S),17(R)-DiHDPA_{n-6} and the docosatetraenoic acid (AdA) derivative 10(S),17(R)-DiHAdA as well as the pathway markers 5(S)-hydroxyeicosatetraenoic acid (5(S)-HETE), 12(S)-HETE, 15(R)-HETE, 15(S)-HETE, 20-HETE, each deriving from AA, 17(R)-hydroxydocosahexaenoic acid (17(R)-HDoHe), 17(S)-HDoHe, 14(S)-HDoHe from DHA and (\pm)-18-hydroxyeicosapentaenoic acid ((\pm)-18-HEPE) from EPA have been investigated in a single run of 25 min. Additional data for all studied analytes is collected in [Suppl. Table 1](#). All analytes have been determined using gradient elution mode and advanced scheduled multiple reaction monitoring (asMRM) has been used to increase both the number of points per peak as well as the quality of data generated by the MS. The developed method has been validated according to the guidelines of the Food and Drug Administration (FDA) [27]. The required selectivity to avoid false positive results is in detriment to the sensitivity [28]. However, even using chiral chromatography, the developed method has achieved LLOQ values varying from 0.1 to 0.2 ng mL⁻¹, depending on the analytes. Some publications describe lower LLOQ values [12,13,15] but in these cases, potential interactions with isomers and isobaric compounds cannot be discharged. Chiral separation has already been described in the bibliography for the quantification of SPM [16,29]. However, to our knowledge and until date, no simultaneous quantification method for such a large number of compounds has been developed and fully validated.

2. Materials and methods

2.1. Chemicals and solvents

The standards lipoxin A4 (LXA4), 6-*epi*-lipoxin A4 (6-*epi*-LXA4), aspirin-triggered lipoxin A4 (15-*epi*-LXA4, ATL), lipoxin B4 (LXB4), lipoxin A5 (LXA5), resolvin D1 (Rv D1), resolvin D2 (Rv D2), aspirin-triggered resolvin D1 (17-*epi*-Rv D1, ATR), maresin 1 (MaR1), 10(S),17(S)-DiHDoHe (PDX), 14(S)-hydroxydocosahexaenoic acid (14(S)-HDoHe), 17(S)-hydroxydocosahexaenoic acid (17(S)-HDoHe), 17(R)-hydroxydocosahexaenoic acid (17(R)-HDoHe), (\pm)-18-hydroxyeicosapentaenoic acid ((\pm)-18-HEPE), prostaglandin D2 (PGD2), prostaglandin E2 (PGE2), prostaglandin J2 (PGJ2), thromboxane B2 (TXB2), 11-dehydro-thromboxane B2 (11-dh-TXB2), 6-keto-prostaglandin F1 α (6-keto-PGF1 α), prostaglandin F2 α (PGF2 α), leukotriene B4 (LTB4), 12-*epi*-leukotriene B4 (12-*epi*-LTB4), 5(S)-hydroxyeicosatetraenoic acid (5(S)-HETE), 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), 15(R)-hydroxyeicosatetraenoic acid (15(R)-HETE), 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE), 20-hydroxyeicosatetraenoic acid (20-HETE), lipoxin a4-d5 (LXA4-d5), resolvin D2-d5 (Rv D2-d5),

aspirin-triggered resolvin D1-d5 (17-*epi*-Rv D1-d5), 5(S)-hydroxyeicosatetraenoic acid-d8 (5(S)-HETE-d8), 12(S)-hydroxyeicosatetraenoic acid-d8 (12(S)-HETE-d8), 15(S)-hydroxyeicosatetraenoic acid-d8 (15(S)-HETE-d8), 20-hydroxyeicosatetraenoic acid-d6 (20-HETE-d6), leukotriene B4-d4 (LTB4-d4), prostaglandin E2-d4 (PGE2-d4), prostaglandin D2-d4 (PGD2-d4), thromboxane B2-d4 (TXB2-d4), 11-dehydro-thromboxane B2-d4 (11-dh-TXB2-d4), prostaglandin F2 α -d4 (PGF2 α -d4) and 6-keto-prostaglandin F1 α -d4 (6-keto-PGF1 α -d4) were purchased from Cayman Europe (Tallinn, Estonia). The analytes NPD1, dinor-NPD1, tetranor-NPD1, 10(S),17(S)-DiHDPAA_{EEZ,n-3}, 10(S),17(R)-DiHDPAA_{EEZ,n-6}, and 10(S),17(R)-DiHADAA_{EEZ} were in-house synthesized according to our published strategies [30,31]. Water (LC-MS grade) as well as acetonitrile (ACN), methanol (MeOH) and isopropyl alcohol (IPA) each $\geq 99,95\%$ were purchased from Carl Roth (Karlsruhe, Germany). Formic acid 99–100% (FA) was obtained from VWR Prolabo Chemicals (Darmstadt, Germany). Sodium acetate anhydrous 99–101% was purchased from Merck (Darmstadt, Germany). Dulbecco's Phosphate Buffered Saline (PBS) solution was obtained from Gibco life technologies (Dreieich, Germany).

2.2. Standards preparation

Stock solutions of all analytes and internal standards were each prepared at a concentration of 10 $\mu\text{g mL}^{-1}$ in methanol. Working solutions for the calibrators with concentrations of 1, 2, 5, 10, 20, 50, 85 and 100 ng mL^{-1} for all analytes of group A and 2, 4, 10, 40, 100, 170 and 200 ng mL^{-1} for group B, respectively, were prepared by pipetting adequate volumes of the stock solutions and diluting with methanol. Analog to the calibrators a second series of working solutions for quality control samples was built containing all analytes at concentration levels for the high concentrated quality control (HQC, 80% of upper limit of quantification (ULOQ)), middle concentrated quality control (MQC, 40% ULOQ), low concentrated quality control (LQC, 3 \times LLOQ) and the LLOQ. Calibration and quality control samples were freshly prepared before use.

A mixture of all deuterated standards was prepared to be used as internal standard. Stock solutions were pipetted and diluted with methanol to a final concentration of 200 ng mL^{-1} for LXA4-d5 and Rv D2-d5, 100 ng mL^{-1} of 17-*epi*-Rv D1-d5, 5(S)-HETE-d8, 12(S)-HETE-d8, 15(S)-HETE-d8, 20-HETE-d6 and LTB4-d4 and 50 ng mL^{-1} for PGE2-d4, PGD2-d4, TXB2-d4, 11-dh-TXB2-d4, PGF2 α -d4, 6-keto-PGF1 α -d4.

All stock and working solutions were stored in amber glass vials at -80°C .

2.3. Instrumentation

During sample pretreatment a vortexer (IKA[®]-Werke GmbH & Co. KG, Staufen, Germany), centrifuges (5424 and 5810R, both from Eppendorf, Hamburg, Germany), an Evaporator[®] (Liebsch Labor-technik, Bielefeld, Germany), a 16-fold vacuum manifold (Macherey-Nagel GmbH & Co. KG, Weilmünster, Germany) and manometer (Ashcroft Instruments, Baesweiler, Germany), glass pipettes for single use (Brand, Wertheim, Germany) and Chromabond C18 SPE-cartridges, 3 mL, 500 mg (Macherey-Nagel GmbH & Co. KG, Weilmünster, Germany) were used.

For the chromatographic separation, an Agilent 1200 LC system (Agilent Technologies, Waldbronn, Germany) consisting of a binary pump (G1312B), a thermostated column compartment (G1316B) and a CTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) were employed. Mass spectrometric detection was performed using a hybrid quadrupole-ion tandem mass spectrometer, 5500 QTrap (Sciex, Darmstadt, Germany) equipped with an electrospray ion-source (ESI). Nitrogen in the required purity for the mass

spectrometer was produced by the nitrogen generator NGM 11 S (cmc Instruments, Eschborn, Germany). Instrument was operated by the Analyst Software Version 1.6.2 (Sciex, Darmstadt, Germany).

Quantification of the analytes was carried out using MultiQuant software version 3.0 (Sciex, Darmstadt, Germany).

2.3.1. Chromatographic separation and mass spectrometry conditions

Determination of the analytes was based on both chiral chromatographic separation and mass spectrometric detection allowing for high selectivity quantification. Chromatographic separation was achieved using a Lux Amylose-1[®] column (250 \times 4.6 mm, 3 μm particle size and 1000 \AA pore size – Phenomenex, Aschaffenburg, Germany). Analytes were eluted from the column using water:FA (100:0.1, v/v) (phase A) and ACN:MeOH:FA (95:5:0.1, v/v/v) (phase B) in gradient elution mode. The separation was achieved with a flow rate of 700 $\mu\text{L min}^{-1}$ within 25 min. The elution gradient was as follows: from $t = 0$ –1.0 min 85% A, within 0.5 min the content of A was decreased to 65% and maintained constant from $t = 1.5$ –4.5 min. Within 1.5 min the content of A was decreased to 40% remaining for 2.5 min. Within 2.5 min the content of A was further decreased to 25% and stayed constant from $t = 11.0$ –13.5 min. Within 6.9 min the content of A ramped down to a low of 5% and stayed like that for 2.6 min. Within 0.1 min from $t = 20.4$ min the flow rate increased to 1000 $\mu\text{L min}^{-1}$ and stayed constant for $t = 20.5$ –22.5 min. Within 0.1 min the flow rate decreased to 700 $\mu\text{L min}^{-1}$ again. From $t = 23$ –23.5 min the content of A increased to 85%. For re-equilibration of the system, the initial conditions were maintained for 1.5 min. The column oven was set at 40°C and the temperature of the autosampler at 7°C . Composition of the solvents during the gradient as well as the flow rate are shown in Fig. 1.

The mass spectrometer was operated using a Turbo Ion Spray source in the negative ionization mode at 500°C with an electrospray voltage of -4500 V . Curtain gas was set at 35 psi and collision gas at 9 psi. Nebulizer gas and heater gas were set at 50 and 60 psi, respectively. Analysis was performed in asMRM mode. Therefore, the specific elution time and width of the peak window, in which the analyte is monitored, as well as the classification as primary and secondary transition was determined for each analyte. Primary transitions usually give the most intense peak and are monitored over the whole retention window whereas secondary transitions only appear when specified thresholds are reached. Primary transitions were used as quantifier ions. Suppl. Table 2 collects the chromatographic characteristics and the mass spectrometric information.

2.4. Sample preparation

For this study, human plasma was used as biological matrix. Due to its complexity, the efficiency of a suitable extraction procedure was studied by spiking several analytes (LXA4, ATL, LXB4, 12(S)-HETE, 6-keto-PGF1 α , ATR, MaR1 and 17(S)-HDoHe) in human plasma. To determine the optimal extraction conditions, several solid phase extraction (SPE) cartridges and procedures were investigated: Chromabond C18 500 mg (Macherey-Nagel GmbH & Co. KG, Weilmünster, Germany), Oasis[®] WCX 1 cc, 30 mg sorbent and MCX 1 cc, 30 mg sorbent (Waters, Milford, USA) and Strata-X-A & Strata-XL-A polymer-based sorbent 1 mL, 30 mg (Phenomenex, Aschaffenburg, Germany). The washing solutions examined were sodium acetate (NaAc) and ammonium acetate in different concentrations between 50 and 500 mM as well as a solution consisting of 3% phosphoric acid. For the elution from the cartridges, different concentrations of FA in MeOH were tested.

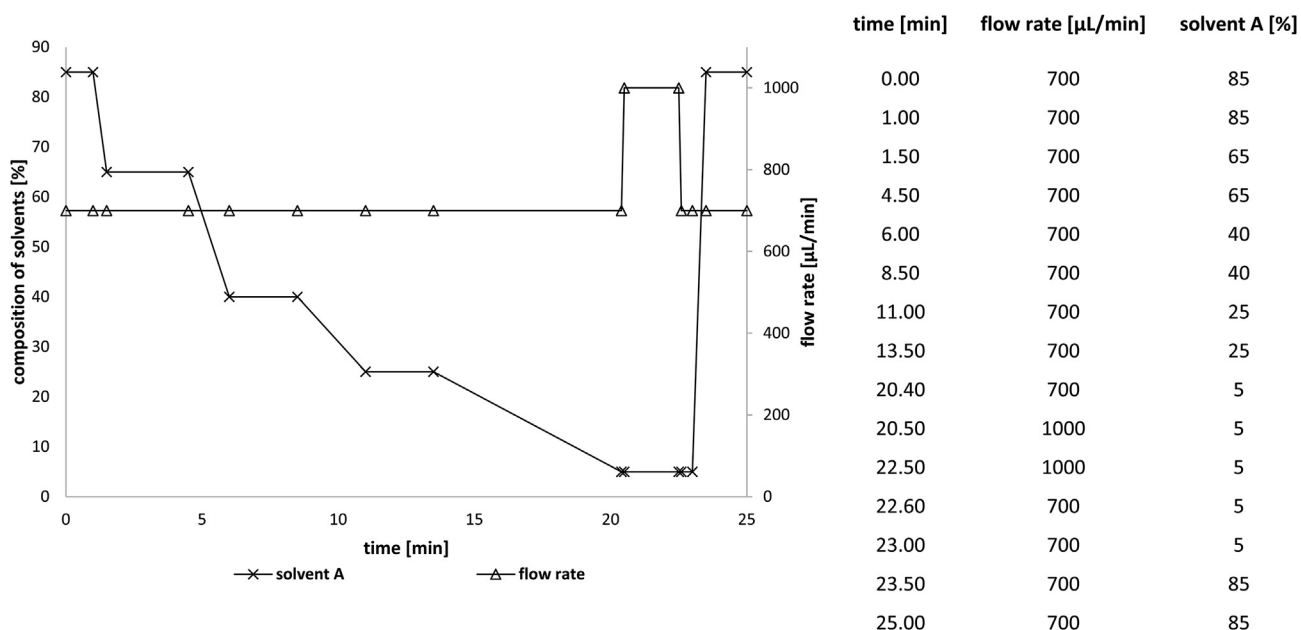


Fig. 1. Chromatographic gradient, solvent composition and flow rate during the chromatographic run.

2.4.1. SPE procedure for human plasma

For extraction purposes, human plasma was thawed, vortexed for 10 s and centrifuged at 15,000 g for 1 min. 200 µL of plasma were sampled and 20 µL of the IS-mixture were added, let sit for 30 s and vortexed. After addition of 300 µL MeOH the mixture was vortexed for 30 s and centrifuged at 15,000 g for 5 min. The supernatant was transferred into an amber glass vial (1.5 mL) and dried under a stream of nitrogen at 45 °C. After addition of 800 µL water and vortexing for 30 s the aqueous sample was transferred onto a vacuum-connected SPE cartridge which was pre-treated with 2 × 800 µL MeOH for conditioning and 2 × 1 mL of water for equilibration, subsequently. After sample loading, the cartridge was washed with 3 × 800 µL 50 mM NaAc:MeOH (95:5, v/v) and dried for 5 min. Finally, after addition of 500 µL 1% FA in MeOH and a duration of 30 s for the interaction between elution-solvent and cartridge-bound analytes, the liquid phase was eluted; the elution procedure was repeated twice. The three fractions were combined and again transferred into an amber glass vial and evaporated to dryness at 45 °C under a nitrogen stream. The samples were reconstituted in 50 µL water:ACN:MeOH (50:47.5:2.5, v/v/v) containing 0.1% FA, vortexed and transferred to a conical glass insert. 10 µL of this extract were injected into the LC-MS/MS system.

2.5. Method validation

According to the recommendation of the FDA, the developed method has been validated in terms of linearity, calibration range, carry-over effects, selectivity, precision and accuracy, matrix effects and recovery, and stability [27]. High selectivity due to the combination of chiral separation and tandem mass spectrometry together with the asMRM-mode to increase the number of points per peak for multi-analyte methods allowed the quantification of each analyte in biological samples with enough sensitivity and avoiding false positive results. Since not for all analytes stable isotopically labelled standards are commercially available, internal standards were chosen for criteria of structural molecular similarity, chromatographic behavior and elution time. Thus, different analyte/IS pairings were investigated and the most likely pair was chosen and used for validation. PGE₂, PGD₂, TXB₂, 11-dh-TXB₂, 6-

keto-PGF1 α and PGF2 α each had their own isotopically labelled d4-derivative; for PGJ₂, PGD2-d4 was employed. 17-*epi*-Rv D1-d5 was used as IS for dinor-NPD1, tetranor-NPD1, 15-*epi*-LXA4, PDX and 17-*epi*-Rv D1. For LTB₄, 12-*epi*-LTB₄ and 6-*epi*-LXA₄, LTB₄-d4 was used. 10(S),17(R)-DiHDPA_{n-6} and 12(S)-HETE shared 12(S)-HETE-d8 as internal standard. 5(S)-HETE-d8 was employed for 5(S)-HETE, 14(S)-HDoHe and 17(S)-HDoHe. 15(S)-HETE as well as 20-HETE each had their own d8-and d6-labelled derivatives, respectively. LXA₄-d5 was employed for 10(S),17(S)-DiHDPA_{n-3}, LXA₄ and 10(S),17(R)-DiHAdA. 15(R)-HETE, LXA₅, LXB₄, Rv D1, NPD1, MaR1, 17(R)-HDoHe, (\pm)18-HEPE and Rv D2 shared Rv D2-d5 as IS.

2.5.1. Lower limit of quantification

The LLOQ was defined as the lowest detectable amount of analyte that can be quantified according to the quantification criteria for precision and accuracy. As a measure of precision, the percentage relative standard deviation (RSD) was employed being defined as (standard deviation/mean concentration value) × 100. Accuracy was evaluated in terms of relative error (RE), being calculated as (calculated concentration – nominal concentration)/nominal concentration, expressed as a percentage value. For the determination of a suitable LLOQ, both values were set at 20%. Additionally, each peak should be calculated as minimum 10 times the signal to noise ratio of a blank sample [27]. Assessed in measurements previous to the validation, studied analytes were assayed into two groups with each a different LLOQ value and calibration range: group A featuring a LLOQ of 0.1 ng mL⁻¹ and group B with a LLOQ value of 0.2 ng mL⁻¹. Classification data are collected in [Suppl. Table 2](#).

2.5.2. Sample validation

Validation of analytical methods for endogenous compounds is challenging because no blank matrices (biological matrices without the analytes) are available. In this case phosphate-buffered saline solution (PBS) was used as substitute matrix for calibrators and quality control samples. 180 µL of PBS were mixed with 20 µL of any given working solution as well as 20 µL of IS. Concentrations ranged from 1.0 ng mL⁻¹ to 100 ng mL⁻¹ for Group A and 2.0 ng mL⁻¹ to

200 ng mL⁻¹ for Group B containing all studied analytes simultaneously giving a concentration range related to plasma samples of 0.1 to 10 ng mL⁻¹ and 0.2 to 20 ng mL⁻¹, respectively. All samples were extracted and prepared according to the outlined SPE method described in section 2.4.1.

2.5.3. Linearity and calibration range

Seven calibration curves each consisting of a blank sample (matrix sample without analytes or IS), a zero sample (matrix sample processed without analytes but internal standards) and 8 calibrators covering a range from LLOQ to 10 ng mL⁻¹ or 20 ng mL⁻¹ were prepared in PBS, extracted and analyzed in different analytical runs. Analyte concentrations were calculated by linear regression using a weighting factor of 1/x. Linearity of each standard line was confirmed by plotting the peak area ratios (analyte/IS) versus analyte concentrations. Absence of the analytes in blank and zero samples was evaluated visually.

2.5.4. Carry-over

For the determination of possible carry-over effects, after analysis of an ULOQ sample, a blank sample was injected into the system followed by a LLOQ measurement. By dividing the area detected in the blank through the corresponding area in the LLOQ sample, the percentage values were calculated (n = 3). The area determined in the blank sample must not exceed an intensity of 20% for any given analyte and 5% for the internal standard, respectively, in comparison to those found in the LLOQ sample. The arithmetic mean of all three measurements was determined.

2.5.5. Selectivity

Selectivity for the analytes and deuterated standards was determined in PBS. Three aliquots each of a blank sample, a zero sample and an analyte-containing sample at LLOQ-level were freshly prepared and analyzed. Calculated as the area found in the blank-aliquots divided by the corresponding area in the zero and analyte samples, selectivity was not to exceed an intensity of 20% for any given analyte and 5% for the internal standard, respectively. Additionally, selectivity for the internal standards was assessed likewise in six different plasma samples each obtained from a different donor.

2.5.6. Precision and accuracy

Within-run precision and accuracy was examined at four different concentrations (described in 2.2) corresponding to LLOQ, LQC, MQC and HQC levels by extraction and analysis of six aliquots each prepared in PBS on the same day. Arithmetic means of each level were composed and the RE and RSD values were calculated, acceptance criteria being set at a discrepancy tolerance of maximum of 15% each. This procedure was repeated on two more days in order to evaluate reproducibility and between-run precision and accuracy.

2.5.7. Matrix effects and recovery

Both matrix effects (ME) and recovery (RCV) were evaluated for the isotopically labelled internal standards in human plasma each obtained from a different donor (n = 6). For ME, two samples from each plasma were extracted as blanks and spiked with IS solutions to receive a concentration of either 0.3 ng mL⁻¹ (LQC) or 8 ng mL⁻¹ (HQC). The intensity of each of those samples was compared to a sample prepared in solvent by dividing the area found in plasma through the area found in solvent, given as percentage. Results with a percentage of lower than 100% suggest ion-suppression by the matrix whereas values above point to matrix enhancement. Recovery was determined likewise, however by spiking the blank samples before extraction. The absolute recovery was calculated as

the percentage of analyte that can be extracted from a spiked sample.

2.5.8. Stability

After determination of the endogenous concentrations present in the blank plasma, if necessary the samples were spiked to fetch concentrations at LQC and HQC levels and analyzed, subsequently. Stability of the analytes was assessed under different conditions for short-, mid- and long-term conditions with five aliquots of each concentration level in spiked human plasma. Samples were extracted after various stages of treatment and analytes were quantified against freshly prepared calibration curves in PBS. For the determination of the t₀-concentration, freshly spiked plasma samples were extracted and analyzed to give the actual concentration that is to be found in treated samples. To reflect actual sample treatment and analysis in the laboratory, short-term stability was determined for two different condition: spiking the samples and maintaining them for 3 h at room temperature before extraction and a second set of samples that was extracted directly after spiking and then let sit for 3 h at room temperature before analysis. An additional set of samples was extracted, dried under a continuous stream of nitrogen and frozen at -80 °C for 72 h before reconstitution and analysis. For mid-term stability, t₀-samples were reconstituted and analyzed again after storage for 72 h at 7 °C. Freeze/thaw stability was monitored for samples that were spiked and frozen after preparation at -80 °C for at least 12 h and thawed at room temperature; this procedure was repeated twice. To assess long-term stability, two sets of samples were stored at -80 °C and analyzed after a period of 60 and 90 days, respectively.

3. Results

3.1. Limit of quantification and calibration range

Arising from the criteria outlined in section 2.5.1, the analyte specific LLOQ values were determined due to the different chromatographic behavior and sensitivity of studied analytes. Values for each analyte were calculated based on the signal to noise ratio. The suitability of chosen concentrations as LLOQ values was tested in terms of precision and accuracy. In all cases, RSD values were lower than 17% with RE values lower than 18%. RSD and RE values for each analyte are collected in [Suppl. Table 3](#).

In accordance to instrumentation capability as well as the anticipated highest concentrations present in endogenous material, the calibration ranges were set as follows. Group A consisting of 5(S)-HETE, 12(S)-HETE, LTB4, 12-*epi*-LTB4, 15-*epi*-LXA4, 6-*epi*-LXA4, Rv D1, 17-*epi*-Rv D1, PDX and NPD1 with a LLOQ of 0.1 ng mL⁻¹ and an ULOQ of 10 ng mL⁻¹ (calibration range from 0.1 to 10 ng mL⁻¹) and group B consisting of PGE₂, PGD₂, PGJ₂, TXB2, 11-dh-TXB2, 6-keto-PGF1 α , PGF2 α , dinor-NPD1, tetranor-NPD1, 10(S),17(S)-DiHDPAN-3, 10(S),17(R)-DiHDPAN-6, 10(S),17(R)-DiHADA, 15(R)-HETE, 15(S)-HETE, 20-HETE, Lx A4, Lx A5, Lx B4, Rv D2, MaR1, 17(R)-HDoHe, 17(S)-HDoHe, (\pm)18-HEPE and 14(S)-HDoHe with a LLOQ of 0.2 ng mL⁻¹ and an ULOQ of 20 ng mL⁻¹ (calibration range from 0.2 to 20 ng mL⁻¹).

3.2. Sample preparation

For each cartridge tested, three plasma samples were spiked with the analytes mentioned in section 2.4 in a concentration of 20 ng mL⁻¹. After extraction and measurement, the calculated concentrations were compared with the same amount of analytes in solvent. Whereas some cartridges were better suited for single analytes and analyte-groups, respectively, the C18-cartridge showed the best recovery for all analytes in this series of

experiments. Consequently, plasma samples were spiked with all analytes simultaneously and extracted over the Chromabond-cartridges to prove suitability of chosen SPE cartridge and extraction procedure. Extracted ion chromatograms of all studied analytes after extraction are shown in Fig. 2.

3.3. Linearity and calibration range

All calibration curves ($n = 7$) showed adequate linearity for all analytes with correlation coefficient values (R^2) higher than 0.995 (Suppl. Table 4). Precision values of the standard line slopes, given as RSD, were less than 11% for all analytes. The average values of all slopes and corresponding precisions are collected in Table 1.

3.4. Carry-over

Analysis of possible carry-over for both the analytes and the internal standards were determined and in accordance to the measurement criteria described in section 2.5.4. mean values were calculated ($n = 3$). For the internal standards values lower than 0.3% and for the analytes values of less than 14.7% could be assessed, collected data can be found in Tables 1 and 2.

3.5. Selectivity

As alternative matrix for the calibrators and quality controls, PBS as aqueous salt solution was used. The study shows that the selectivity for the internal standards in PBS has values lower than 0.6% and for the analytes lower than 8.0%. Furthermore, selectivity of the IS in plasma from six different donors was assessed and values were calculated to be lower than 0.1%. Data for the analytes and deuterated standards are collected in Tables 1 and 2, respectively.

3.6. Precision and accuracy

Suitability of the developed method was demonstrated by investigation of both the within- and between-run precision and accuracy. For all analytes three different concentrations (LQC, MQC and HQC) were tested besides the LLOQ (described in 3.1) with six replicates each. The within-run precision, given in RSD, yielded values lower than 12% with an accuracy value (RE) ranging between -14.7% and 14.6% . For between-run precision, the RSD values obtained were lower than 10.5% with accuracy values ranging from -11.3 to 2.1% (data s. Suppl. Table 3).

3.7. Matrix effects and recovery

The values obtained for the ME in plasma of both low and high concentrations spiked after extraction ranged between 99% and 120% with exception of 133% for prostaglandin D2-d4 and 134% for 11-dehydro-thromboxane B2-d4. Analog to the matrix effects, for recovery studies analyte peak areas in a solvent were compared with those measured in a plasma sample, however spiked before extraction. All analytes showed values higher than 90%. The values obtained for the mean absolute recovery ranged from 93 to 120% with exception of 123% for prostaglandin D2-d4 and 125% for 11-dehydro-thromboxane B2-d4. Results for both studies are collected in Table 2.

3.8. Stability

Stability of the analytes in the matrix for short-term studies has been demonstrated without degradation. After extraction and reconstitution in solvent, the analytes proved to be stable in the autosampler at 7°C for at least 72 h with calculated values ranging from -14.1 to 7.4% RE (Suppl. Table 5). Values obtained after three freeze/thaw cycles ranged from -8.3 to 7.5% RE, with the exception of tetranor-NPD1 for which an accuracy of 12.7% RE was obtained

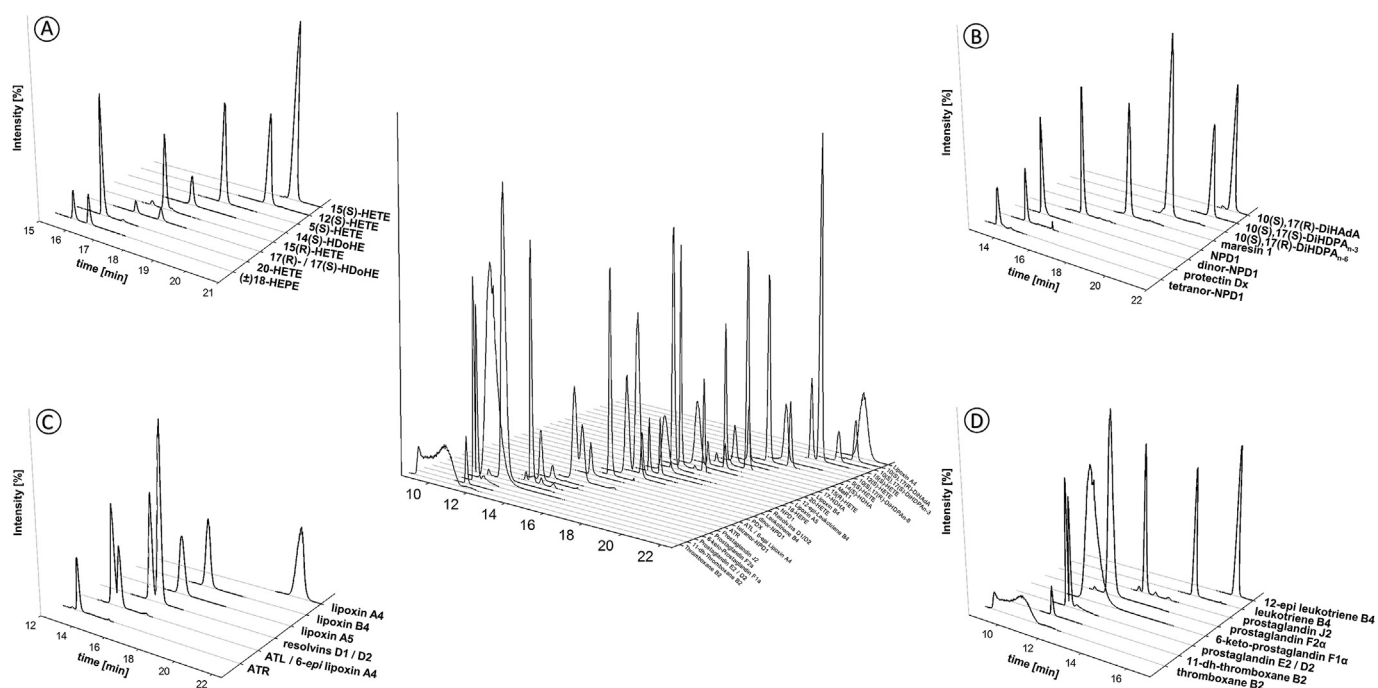


Fig. 2. Extracted ion chromatograms for all analytes at a concentration of 20 ng mL^{-1} each in a spiked plasma sample are displayed (center). Chromatograms showing the analytes classified by family are shown in figures A to D as follows: A) prostaglandins and isomers leukotriene B4, B) maresin 1, protectin DX and protectin-, DPA- and AdA-derived analogues, C) lipoxins and resolvins, D) metabolic precursors: (\pm)18-HEPE, HDoHEs and HETEs.

Table 1
Calibration range parameters, carry-over and selectivity for studied analytes.

	Calibration range parameters (n = 7)			Carry-Over (n = 3)	Selectivity (n = 3)
	Average slope	RSD [%]	Average R ²	[%]	[%]
TXB2	0.14	2.9	0.9988	0.0	0.0
PGE2	0.24	6.7	0.9982	0.0	0.0
11-dh-TXB2	0.08	5.6	0.9976	0.0	0.0
PGD2	0.18	4.5	0.9972	0.0	0.0
6-keto-PGF1 α	1.38	4.2	0.9988	3.5	0.0
PGF2 α	1.36	3.3	0.9990	3.9	0.0
PGJ2	0.23	4.8	0.9964	8.4	0.0
17- <i>epi</i> -Rv D1	0.10	2.2	0.9990	0.0	2.2
tetranor-NPD1	0.01	4.9	0.9976	0.0	0.0
15- <i>epi</i> -LXA4	0.25	3.5	0.9990	0.0	0.0
PDX	0.24	4.1	0.9988	6.7	2.3
dinor-NPD1	0.03	4.7	0.9990	0.0	0.0
6- <i>epi</i> -LXA4	0.11	5.8	0.9984	0.0	0.0
LTB4	0.23	5.7	0.9988	3.4	0.0
Rv D1	0.16	2.6	0.9980	3.2	0.0
NPD1	0.04	3.7	0.9980	0.0	0.0
Rv D2	0.18	3.7	0.9990	2.6	0.0
(\pm)18-HEPE	0.02	5.3	0.9984	2.6	0.0
20-HETE	0.32	3.7	0.9986	14.4	4.1
12- <i>epi</i> -LTB4	0.29	3.9	0.9990	1.3	0.9
LXA5	0.07	6.0	0.9990	0.0	0.0
LXB4	0.08	3.6	0.9988	0.0	0.0
17(R)-HDoHe	0.01	9.7	0.9976	3.8	0.0
15(R)-HETE	0.06	8.0	0.9978	6.8	2.5
MaR1	0.02	4.3	0.9984	0.0	0.0
14(S)-HDoHe	0.03	10.2	0.9988	11.6	5.3
17(S)-HDoHe	0.01	8.1	0.9986	5.4	5.6
5(S)-HETE	0.25	3.7	0.9986	14.7	4.0
10(S),17(R)-DiHDPAn-6	0.08	7.9	0.9982	3.4	0.0
12(S)-HETE	0.27	3.7	0.9990	12.5	8.0
15(S)-HETE	0.35	2.9	0.9990	9.4	5.8
10(S),17(S)-DiHDPAn-3	0.01	6.0	0.9984	0.0	0.0
LXA4	0.07	3.5	0.9988	0.0	0.0
10(S),17(R)-DiHADa	0.02	4.6	0.9984	0.0	0.0

Table 2
Carry-over, selectivity, matrix effects and recovery for internal standards.

	Carry-Over (n = 3)		Selectivity in PBS (n = 3)		Selectivity in plasma (n = 6)		Matrix effects (n = 6)		Recovery studies (n = 6)	
	[%]	[%]	[%]	[%]	[%]	Low ME [%]	High ME [%]	Low RCV [%]	High RCV [%]	
TXB2-d4	0.0	0.0	0.0	0.0	0.0	116.2	105.8	107.2	106.4	
PGE2-d4	0.0	0.6	0.1	0.1	0.1	119.6	107.4	112.6	111.7	
11-dh-TXB2-d4	0.0	0.0	0.0	0.0	0.0	134.5	101.3	125.4	104.4	
PGD2-d4	0.0	0.0	0.0	0.0	0.0	133.0	99.4	123.2	98.0	
6-keto-PGF1 α -d4	0.0	0.0	0.0	0.0	0.0	117.5	105.6	113.5	109.7	
PGF2 α -d4	0.0	0.0	0.0	0.0	0.0	113.5	114.1	116.2	111.0	
17- <i>epi</i> -Rv D1-d5	0.0	0.0	0.0	0.0	0.0	106.0	109.3	99.8	112.6	
LTB4-d4	0.0	0.0	0.0	0.0	0.0	107.5	109.4	103.6	114.6	
Rv D2-d5	0.0	0.0	0.0	0.0	0.0	107.2	114.5	103.0	112.5	
20-HETE-d6	0.1	0.0	0.0	0.0	0.0	106.6	118.0	104.7	119.4	
5(S)-HETE-d8	0.0	0.1	0.0	0.0	0.0	101.5	115.5	93.6	112.6	
12(S)-HETE-d8	0.2 ^a	0.1	0.0	0.0	0.0	104.0	118.4	99.3	117.3	
15(S)-HETE-d8	0.3 ^a	0.1	0.0	0.0	0.0	104.1	118.9	100.7	117.5	
LXA4-d4	0.0	0.0	0.0	0.0	0.0	107.1	112.1	103.5	112.1	

^a Due to measurement in different analytical runs, listed data represent the maximum values calculated.

and prostaglandin D2 with a value of -14.7% RE. All analytes in spiked human plasma showed to be stable for at least 60 days during storage at $-80\text{ }^{\circ}\text{C}$. A second pool of samples was analyzed after 90 days at freeze temperature but in this case only 26 compounds were stable for such an extended storage time. With accuracy values ranging between -9 and -15% RE for lipoxin A4, 10(S),17(R)-DiHADa and 17(R)-HDoHe, these analytes only allow a maximum storage time of 60 days. The analytes thromboxane B2, 6-keto-prostaglandin F1 α and 15(R)-HETE each showed accuracy values of -14% RE and lower implying that a storage of 90 days is on

the acceptance limits of molecular degradation. Data are shown in [Suppl. Table 6](#).

3.9. Biological application

The developed method was applied to the determination of the studied analytes in human plasma taken from healthy male subjects after verifying that the analytes could not be found in plasma samples proceeding from patients with hepatic failure even though showing clinical signs of inflammation (data not shown). Thus,

healthy volunteers were chosen because of the capability of controlling and standardization of sampling parameters such as collection or sample processing time, which are essential for the determination of the studied analytes (due to the described instability problems) and very difficult to control in real clinical situations. In the studied samples, various analytes could be detected in one or more cases with values varying between the different donors. The analytes found were prostaglandin E2 (0.6–1.4 ng mL⁻¹), thromboxane B2 (1.5 ng mL⁻¹), 5(S)- and 12(S)-HETE (0.3–0.6 ng mL⁻¹ and 0.1–1.2 ng mL⁻¹, respectively), leukotriene B4 (0.3–0.6 ng mL⁻¹), protectin DX (0.7 ng mL⁻¹), (±)18-HEPE (2.5–17.0 ng mL⁻¹) as well as 14(S)- and 17(S)-HDoHe (3.5 ng mL⁻¹ and 0.7–1.4 ng mL⁻¹). Further analytes were not detected. The chromatogram of a plasma sample is shown in Fig. 3.

4. Discussion

Chiral liquid chromatography coupled to tandem mass spectrometry provides the required selectivity and sensitivity for the determination of all the chosen 34 PUFA metabolites and intermediates involved in inflammatory processes, including 11 analytes belonging to the SPM family. Its suitability has been shown by the full validation.

When working with LC-MS/MS, most methods published use reversed phase chromatography to separate SPM and other lipid mediators [13, 15, 32, 33]. However, this may lead to the possibly insufficient detection of chiral isomers resulting in single peaks instead of cleanly separated enantiomers, which may possess different biological applications and effects [28]. For example, the enantiomers 17(S)-HDoHe and 17(R)-HDoHe, as DHA-derived intermediates, have varying intrinsic activities and give raise to different D-series resolvins and ATR, respectively [34, 35]. Additionally, separation of isobaric compounds such as lipoxins A4 and B4 as well as 6-*epi*-LXA4 and ATL with similar to identical

fragmentation patterns and transitions (Fig. 4) is usually insufficient or not embedded in the methods at all. Thus, overlays may result in values above the expected concentrations and calibration range, and molecular interferences are prone to give false either positive or negative results. A usually highly selective technique, in the current case mass spectrometry alone is not sufficient for the qualification of analytes and lipid mediators due to the great number of isomeric molecules studied. Thus, improvement of the LC conditions is mandatory. The use of chiral chromatographic columns appears to be the method of choice for the determination of the analytes. Due to their different chirality and unequal position of their functional groups, the chiral stationary phase interfered differently with each isomer, showing a nice difference between the resulting retention times and overall leads to an increase of both selectivity and resolution. Furthermore, our developed method includes a great number of chosen pathway markers with involvement in inflammation. While several existing methods focus on LOX- and/or COX-catalyzed PUFA-derived metabolites secreted in the resolution phase of an inflammation, pro-inflammatory intermediates are seldom included. Besides their own intrinsic effects and cell signaling abilities, these intermediates serve as evidence of the activation of PUFA metabolism cascades that lead to pro-resolving mediators. Thus, by neglecting to analyze these precursors possible inflammatory reactions may go unnoticed when sufficient concentrations of SPM could not be raised.

The method has demonstrated its suitability for the determination and quantification of all studied analytes in human plasma over the course of only 25 min. All analytes were first characterized and analyzed separately in order to determine their specific MS-parameters. Through optimization of the analytical gradient and the choice of solvents the corresponding elution time and peak width per analyte could be evaluated to achieve a sharper and more distinct separation. Using these data, a scheduled MRM-method could be established providing a higher number of points per

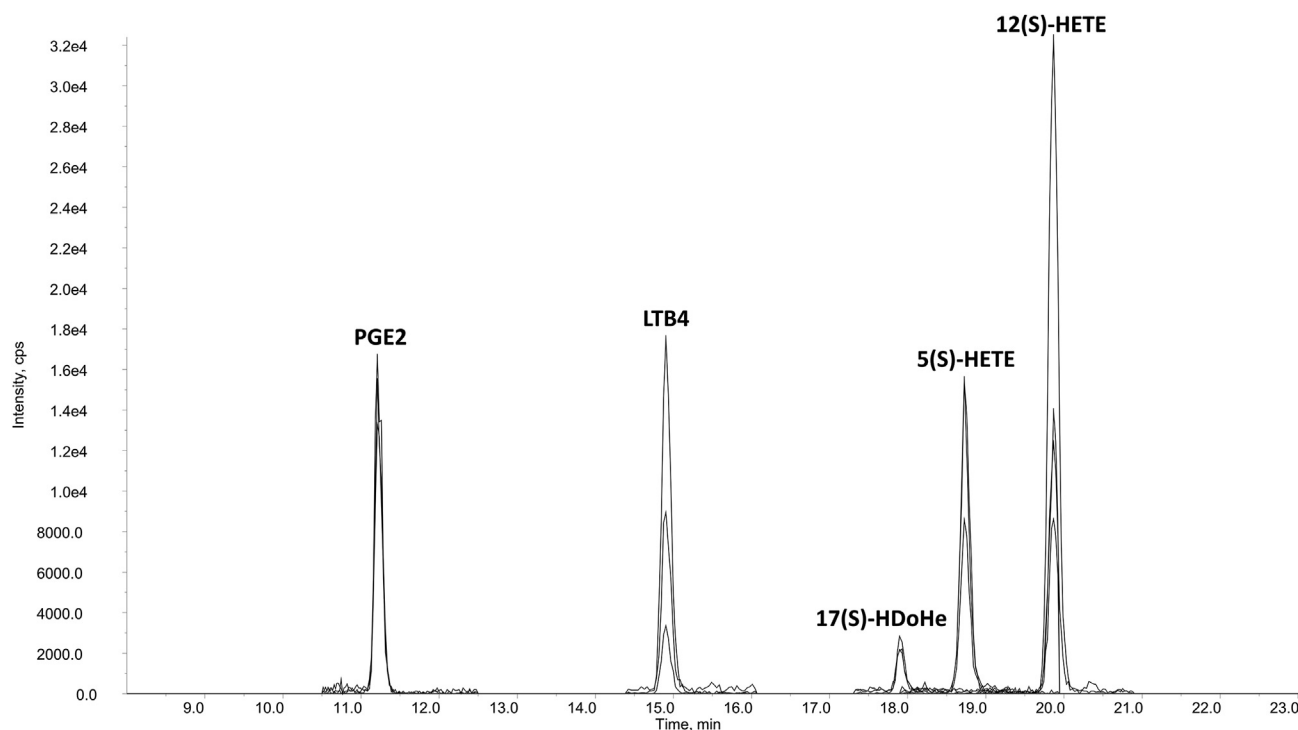


Fig. 3. Chromatogram corresponding to an extracted plasma sample. The analytes detected are prostaglandin E2 (0.6 ng mL⁻¹), leukotriene B4 (0.3 ng mL⁻¹), 17(S)-HDoHe (0.7 ng mL⁻¹), 5(S)-HETE (0.3 ng mL⁻¹) and 12(S)-HETE 0.7 ng mL⁻¹. For each analyte, three transitions are shown.

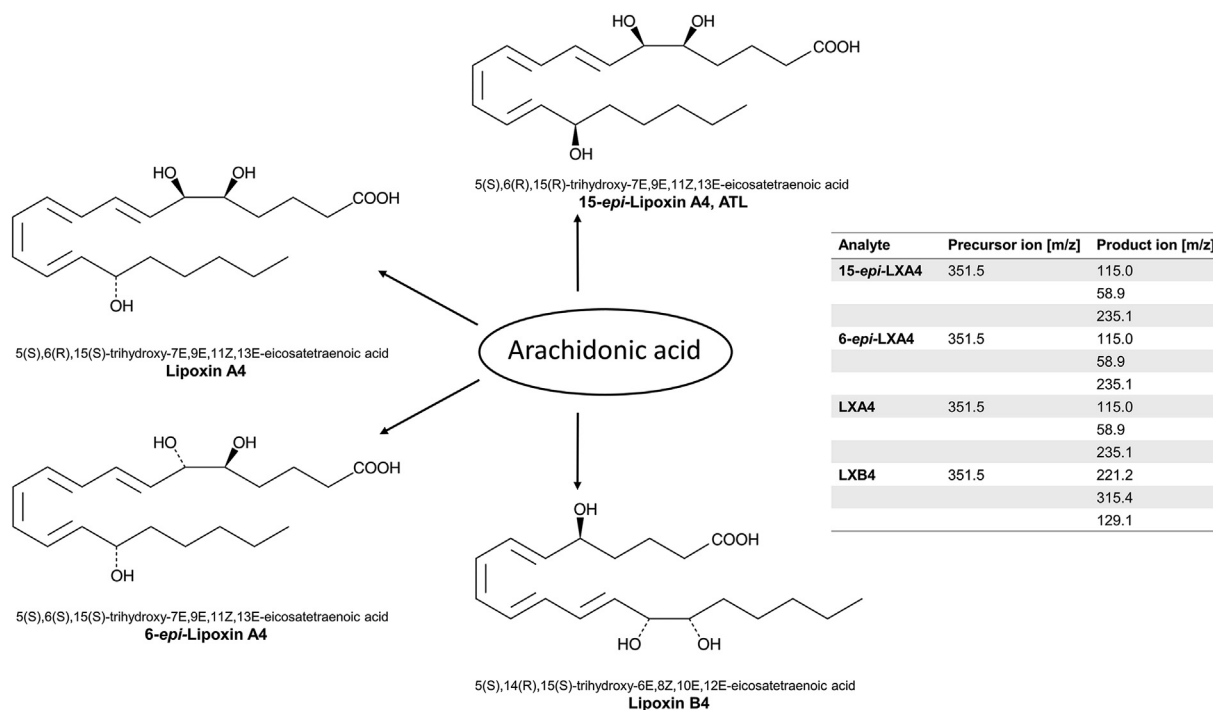


Fig. 4. Arachidonic acid-derived lipoxins sharing the same molecular formula C₂₀H₃₂O₅ and weight 352.5 g mol⁻¹.

peak for multi-analyte methods. Whereas a normal MRM-method investigates all chromatographic transitions throughout the whole run, a scheduled method only investigates the analytes and their transitions at specified elution times and thus increases the resolution of every peak. The use of solid-phase extraction for sample preparation measurements is an extensive pre-treatment procedure; however, it provides extracts with exceptional lower quantities of matrix when compared to a liquid-liquid extraction. Through a reduction of potential interferences from other endogenous components present in biological matrices, the selectivity of the analytes in given samples is increased and false positive results can be avoided. Extracted samples can be used directly, dried, stored and reconstituted in a different solvent, respectively, without further derivatization.

To correct for the loss of analyte during sample preparation or by molecular degradation, stable isotopically labelled internal standards each similar to the analyte of interest were added to the samples. For several analytes, the corresponding isotopically labelled derivatives were not available. In these cases, the best match of internal standard for each analyte was chosen in terms of molecular likeness and chromatographic behavior, even though they differed in configurations or chemical structures. Of the tested pairing none resulted in insufficient intensities and values for the lower limit of quantification of the analytes. Matrix effects and recovery studies also showed adequacy of chosen internal standards. Subsequently, suitability of the method was shown by all mentioned criteria and the developed method has been fully validated. Even though in other publications LLOQ values of 10 pg and lower have been established for biological matrices such as human milk and plasma [12,15], the use of chiral stationary phases allows enhanced separation qualities for both isobaric and isomeric compounds providing reproducible results with comparable sensitivity. Other methods already published which are also exploiting the lipid mediator's chirality for separation purposes show distinctly better separation properties than reversed phase chromatography alone, however, they were all limited in the

number of analytes studied simultaneously [16,29].

Although the detection may suffer from lower sensitivity, the separation of isomers using a chiral column is dramatically improved, reducing the risk of wrong assignment of analytical identities. LLOQ values achieved in the presented method were as follows: 0.1 ng mL⁻¹ for ATL, 6-*epi*-LXA4, Rv D1, ATR, PDX and NPD1, LTB4, 12-*epi*-LTB4, 5(S)-HETE and 12(S)-HETE; 0.2 ng mL⁻¹ for LXA4, LXA5, LXB4, Rv D2, MaR1, dinor-NPD1, tetranor-NPD1, PGE2, PGD2, PGJ2, TXB2, 11-dh-TXB2, 6-keto-PGF1 α , PGF2 α , 10(S),17(S)-DiHDPAn-3, 10(S)17(R)-DiHDPAn-6, 10(S),17(R)-DiHAdA, 15(R)-HETE, 15(S)-HETE, 20-HETE, 17(R)-HDoHe, 17(S)-HDoHe, (\pm) 18-HEPE and 14(S)-HDoHe.

5. Conclusion

Overall, this chiral method allows for the simultaneous separation of 34 intermediary pathway markers deriving from five different PUFAs, all of which are involved in inflammatory processes with 11 belonging to the group of pro-resolving lipid mediators. Through both high selectivity and sensitivity as well as a short analysis time, this robust method utilizing the analytes chiral characteristics for separation purposes may easily be applied for the routine analysis of endogenous lipid mediators in biological samples. By separation of both regioisomers as well as epimers under developed LC conditions, false positive or negative results can be avoided and the reproducibility of the method was increased. It is important to remark that the samples pending for analysis should be stored protected from light and air and at a temperature of -80 °C for a maximum period of 60 days.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.aca.2018.05.020>.

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