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# Ferromagnetic particles as a rapid and robust sample preparation for the absolute quantification of seven eicosanoids in human plasma by UHPLC–MS/MS

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ABSTRACT

We used ferromagnetic particles as a novel technique to deproteinize plasma samples prior to quantitative UHPLC–MS/MS analysis of seven eicosanoids [thromboxane $B_2$ ($TXB_2$), prostaglandin $E_2$ ($PGE_2$), $PGD_2$, 5-hydroxyeicosatetraenoic acid (5-HETE), 11-HETE, 12-HETE, arachidonic acid (AA)]. A combination of *ferromagnetic particle enhanced deproteination* and subsequent *on-line solid phase extraction* (on-line SPE) realized quick and convenient semi-automated sample preparation—in contrast to widely used manual SPE techniques which are rather laborious and therefore impede the investigation of AA metabolism in larger patient cohorts.

Method evaluation was performed according to a protocol based on the EMA guideline for bioanalytical method validation, modified for endogenous compounds. Calibrators were prepared in ethanol. The calibration curves were found to be linear in a range of 0.1–80 ng $mL^{-1}$ ($TXB_2$, $PGE_2$, $PGD_2$), 0.05–40 ng $mL^{-1}$ (5-HETE, 11-HETE), 0.5–400 ng $mL^{-1}$ (12-HETE) and 25–9800 ng $mL^{-1}$ (AA). Regarding all analytes and all quality controls, the resulting precision data (inter-assay 2.6 %–15.5 %; intra-assay 2.5 %–15.1 %, expressed as variation coefficient) as well as the accuracy results (inter-assay 93.3 %–125 %; intra-assay 91.7 %–114 %) were adequate. Further experiments addressing matrix effect, recovery and robustness, yielded also very satisfying results.

As a proof of principle, the newly developed LC–MS/MS assay was employed to determine the capacity of AA metabolite release after whole blood stimulation in healthy blood donors. For this purpose, whole blood specimens of 5 healthy blood donors were analyzed at baseline and after a lipopolysaccharide (LPS) induced blood cell activation. In several baseline samples some eicosanoids levels were below the Lower Limit of Quantification. However, in the stimulated samples all chosen eicosanoids (except $PGD_2$) could be quantified.

These results, in context with those obtained in validation, demonstrate the applicability of ferromagnetic particles for the sample preparation for eicosanoids in human plasma. Thus, we conclude that *ferromagnetic particle enhanced deproteination* is a promising novel tool for sample preparation in LC–MS/MS, which is of particular interest for automation in clinical mass spectrometry, *e.g.* in order to further address eicosanoid analysis in larger patient cohorts.



## 1. Introduction

In the constantly growing field of metabolomics, the lipidomics section plays an important role, addressing a large group of lipid mediators. Among these mediators are the oxylipins, which include polyunsaturated fatty acids (PUFA) and their various metabolites, also referred to as eicosanoids. These lipid mediators are synthesized from arachidonic acid (AA) and other polyunsaturated fatty acids by cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome P450 monooxygenases (CYPs), but they might also be generated through the non-enzymatic oxidation of unsaturated fatty acids [1].

Several oxylipins affect processes in health and disease, such as inflammation, coagulation, immune response and smooth muscle cell tonus, with partial opposing effects [2]. Thus, a quantita-

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tive multiparametric analysis of these compounds might have implications for the understanding of disease pathologies in various disciplines, such as neurology, cardiology, oncology, or pulmonology [3], as well as to determine the potential implementation of these compounds as biomarkers. However, the disease-associated changes and the diagnostic potential of these parameters have been poorly investigated so far, due to analytical difficulties and chemical and biological complexities. Astarita et al. recently reported in their review that the analysis of eicosanoids bears three major difficulties: low concentrations in human fluids, limited stability, and a large number of isomeric species [4]. Further preanalytical variables, *e.g.* possible *ex vivo* formation of eicosanoids, have to be taken into account [5].

Various assays based on different techniques have been developed. In recent years, liquid chromatography tandem mass spectrometry (LC–MS/MS) has become the most widely used technique for the quantification of eicosanoids, as the selectivity of this method and the less labour-intensive sample preparation are advantageous compared with previously used immunoassays or gas chromatography mass spectrometry (GC–MS) [6]. Some comprehensive profiling methods for the metabolomics of oxylipins have been previously described [6–11]. Solid phase extraction (SPE) has been used for sample preparation in the majority of LC–MS/MS methods, as this technique facilitates analyte enrichment and provides relatively clean extracts. Nevertheless, SPE is relatively expensive and requires multiple time-consuming steps, which makes this approach unsuitable for larger study cohorts.

Deproteination by means of ferromagnetic particles could be a promising alternative for this purpose, since it is a rapid procedure suitable for a large number of samples [12]. Additionally there is no need for centrifugation, vacuum, or pressure - steps usually impeding automation - and it can be combined with on-line SPE. A major advantage of *ferromagnetic particle enhanced deproteination* is the generic character of this technique. In contrast to liquid-liquid extraction or solid-phase extraction, the chemistry of the analyte is of minor importance since the particles primarily bind to the denatured proteins. Thus, this technique is a promising approach for multi-analyte panels involving molecules with a broad variability of physico-chemical properties, such as eicosanoids. Due to all these facts and our previous experience with this technique [12] we were encouraged to employ *ferromagnetic particle enhanced deproteination* to more challenging multiparametric LC–MS/MS assays for endogenous compounds.

The central aim of this study was the evaluation of *ferromagnetic particle enhanced deproteination* as a suitable tool in the analysis of eicosanoids. Thromboxane $B_2$ ($TXB_2$), prostaglandin $E_2$ ($PGE_2$), $PGD_2$, 11-hydroxyeicosatetraenoic acid (11-HETE), 12-HETE, 5-HETE, and AA were selected as parameters of interest in the present study as they are central representatives of COX or LOX pathway (Fig. 1. Additionally, these analytes were identified as potential biomarkers in sepsis, as an extreme type of inflammatory disease, making them particularly suitable for further investigation in large scale clinical studies [13–15].

Due to low abundance, pulsatile secretion and short half-life, a reliable quantification of eicosanoids in whole blood is very demanding. Therefore, it is often recommended to analyse eicosanoids in urine [5]. However, quantitation in urine is highly affected by urine concentration and in many patient groups, *e.g.* sepsis patients who are of particular interest for eicosanoid analysis, urine production is reduced or completely missing. Hence, in order to deal with these issues, eicosanoids were analyzed after lipopolysaccharide (LPS) whole blood activation as a model of inflammation [16]. A comparable application of whole blood stimulation assays was used for the evaluation of cytokine release [17]

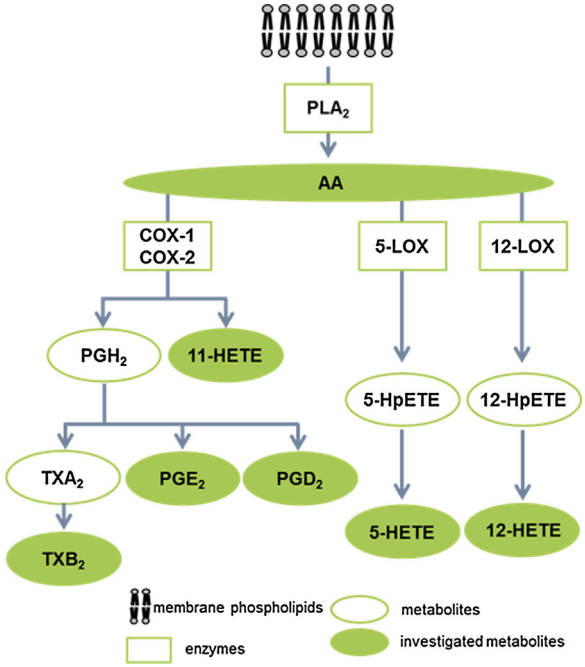



**Fig. 1.** Schematic representation of established metabolites and corresponding enzymes associated with arachidonic acid (AA) metabolism: released from membrane phospholipids, AA is the general precursor of eicosanoids and might also play an active role in inflammatory disease. 11-HETE directly reflects the activity of COX, thus representing a major eicosanoid pathway. Additionally this compound is differentially released in severe inflammatory diseases, such as sepsis. $TXB_2$, $PGD_2$ and $PGE_2$ represent important COX-associated downstream pathways. Together with 5-HETE and 12-HETE (representing 5-LOX and 12-LOX pathway), these compounds play a pathogenic role in milder inflammations, *e.g.*, occurring in cardiovascular disease.
(COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 2; 5-HETE, 5-hydroxyeicosatetraenoic acid; 11-HETE, 11-hydroxyeicosatetraenoic acid, 12-HETE, 12-hydroxyeicosatetraenoic acid; 5-HpETE, 5-hydroperoxyeicosatetraenoic acid; 12-HpETE, 12-hydroperoxyeicosatetraenoic acid; 5-LOX, 5-lipoxygenase; 12-LOX, 12-lipoxygenase; $PGD_2$, prostaglandin $D_2$; $PGE_2$, prostaglandin $E_2$; $PGH_2$, prostaglandin $H_2$; $PLA_2$, phospholipase $A_2$; $TXA_2$, thromboxane $A_2$; $TXB_2$, thromboxane $B_2$).

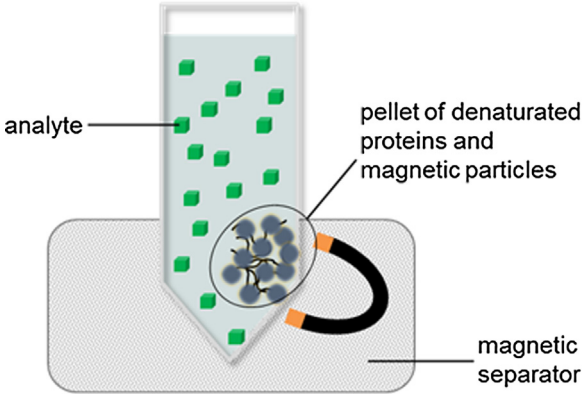



**Fig. 2.** Scheme of *ferromagnetic particle enhanced deproteination* using the magnetic separator.

and as well by our group for evaluation of eicosanoids in the field of sepsis [15].

As a proof of principle approach, to verify the applicability of the method to clinical samples, the novel LC–MS/MS assay including *ferromagnetic particle enhanced deproteination* was employed to investigate the capacity of AA metabolite release after whole blood stimulation in healthy blood donors.

## 2. Experimental

### 2.1. Materials

$TXB_2$, $PGD_2$, $PGE_2$, 5-HETE, 11-HETE, 12-HETE, AA, $TXB_2$-$d_4$, $PGD_2$-$d_4$, $PGE_2$-$d_4$, 5-HETE-$d_8$, 12-HETE-$d_8$, AA-$d_8$, and licofelone were obtained from Cayman Chemicals (Ann Arbor, Michigan, USA). Acetylsalicylic acid, ascorbic acid, and butylhydroxytoluol were purchased from Sigma-Aldrich (Steinheim, Germany). The ferromagnetic particles (MagSi-TOX$^{PREP}$ Type I) were obtained from Magna Medics (Geleen, The Netherlands). Acetonitrile (ACN), methanol (MeOH), water, and acetic acid were of ULC/MS grade and were obtained from Biosolve (Valkenswaard, The Netherlands). HPLC-grade ethanol (EtOH) was purchased from Merck (Darmstadt, Germany).

### 2.2. Preparation of primary standard and internal standard solutions

Stock solutions were prepared in EtOH at a nominal concentration of 50 mg $L^{-1}$ for 11-HETE, 5000 mg $L^{-1}$ for AA and 100 mg $L^{-1}$ for the remaining analytes. Aliquots of the stock solutions were sealed in glass vials under nitrogen and stored at −80°C.

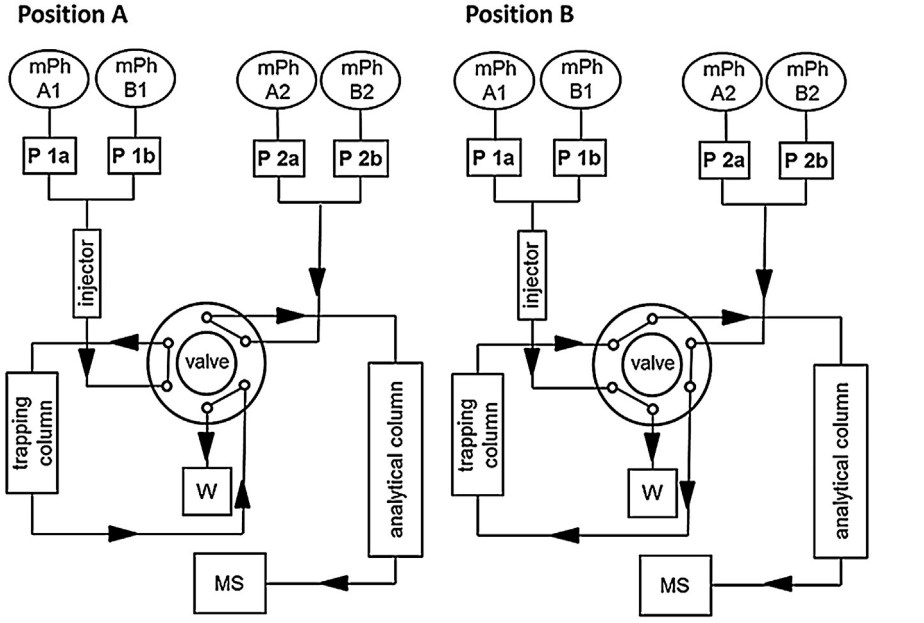
A master calibration solution ($TXB_2$ 990 ng $mL^{-1}$, $PGD_2$ 990 ng $mL^{-1}$, $PGE_2$ 979 ng $mL^{-1}$, 5-HETE 490 ng $mL^{-1}$, 11-HETE 490 ng $mL^{-1}$, 12-HETE 4,900 ng $mL^{-1}$, and AA 245,000 ng $mL^{-1}$) was used to prepare eight multi-calibrators (Cal) by serial dilution in EtOH (see Table 1 for exact concentrations).

Deuterated analogs of the analytes were combined to an ethanol based *internal standard working solution*, resulting in the following concentrations: 12.3 ng $mL^{-1}$ for $TXB_2$-$d_4$, $PGD_2$-$d_4$, and $PGE_2$-$d_4$; 24.5 ng $mL^{-1}$ for 5-HETE-$d_8$ and 12-HETE-$d_8$; and 1960 ng $mL^{-1}$ for AA-$d_8$. Because there was no commercially available stable isotope labeled analog for 11-HETE, 12-HETE-$d_8$ was used.

**Table 1**
Concentrations (ng $mL^{-1}$) of calibrators and controls.

| | $TXB_2$ | $PGE_2$ | $PGD_2$ | 5-HETE | 11-HETE | 12-HETE | AA |
|---|---|---|---|---|---|---|---|
| Cal 1 | 0.099 | 0.098 | 0.099 | 0.049 | 0.049 | 0.490 | 24.5 |
| Cal 2 | 0.198 | 0.196 | 0.198 | 0.098 | 0.098 | 0.980 | 49.0 |
| Cal 3 | 0.396 | 0.392 | 0.396 | 0.196 | 0.196 | 1.960 | 98.0 |
| Cal 4 | 0.990 | 0.979 | 0.990 | 0.490 | 0.490 | 4.90 | 245 |
| Cal 5 | 2.48 | 2.45 | 2.48 | 1.23 | 1.23 | 12.3 | 613 |
| Cal 6 | 9.90 | 9.79 | 9.90 | 4.90 | 4.90 | 49.0 | 2450 |
| Cal 7 | 39.6 | 39.2 | 39.6 | 19.6 | 19.6 | 196 | 9800 |
| Cal 8 | 79.2 | 78.3 | 79.2 | 39.2 | 39.2 | 392 | (19600) |
| QC L | 0.297 | 0.294 | 0.297 | 0.147 | 0.147 | 1.47 | 73.5 |
| QC M | 2.97 | 2.94 | 2.97 | 1.47 | 1.47 | 14.7 | 735 |
| QC H | 29.7 | 29.4 | 29.7 | 14.7 | 14.7 | 147 | 7350 |

Cal, calibrator; QC, quality control; L, low; M, medium; H, high; () excluded after validation.




**Solvents delivered by pumps 1a/b**

| time [min] | solvent flow [mL/min] | mobile phase A1 [%] | mobile phase B1 [%] |
|---|---|---|---|
| 0 | 2.0 | 100 | 0 |
| 0.6 | 2.0 | 100 | 0 |
| 0.7 | 0.1 | 100 | 0 |
| 4.8 | 0.1 | 100 | 0 |
| 4.9 | 0.1 | 0 | 100 |
| 5.1 | 2.0 | 0 | 100 |
| 6 | 2.0 | 0 | 100 |
| 6.2 | 2.0 | 100 | 0 |
| 7.5 | 2.0 | 100 | 0 |

**Solvents delivered by pumps 2a/b**

| time [min] | solvent flow [mL/min] | mobile phase A2 [%] | mobile phase B2 [%] |
|---|---|---|---|
| 0 | 0.5 | 60 | 40 |
| 2.5 | 0.5 | 60 | 40 |
| 2.7 | 0.5 | 30 | 70 |
| 3.7 | 0.5 | 30 | 70 |
| 3.8 | 0.5 | 10 | 90 |
| 5.8 | 0.5 | 10 | 90 |
| 6 | 0.5 | 60 | 40 |
| 7.5 | 0.5 | 60 | 40 |

**Fig. 3.** Schematic plan of the UHPLC–MS/MS system and corresponding solvent flows (mPh, mobile phase; MS, mass spectrometer; P, pumps; W, waste).

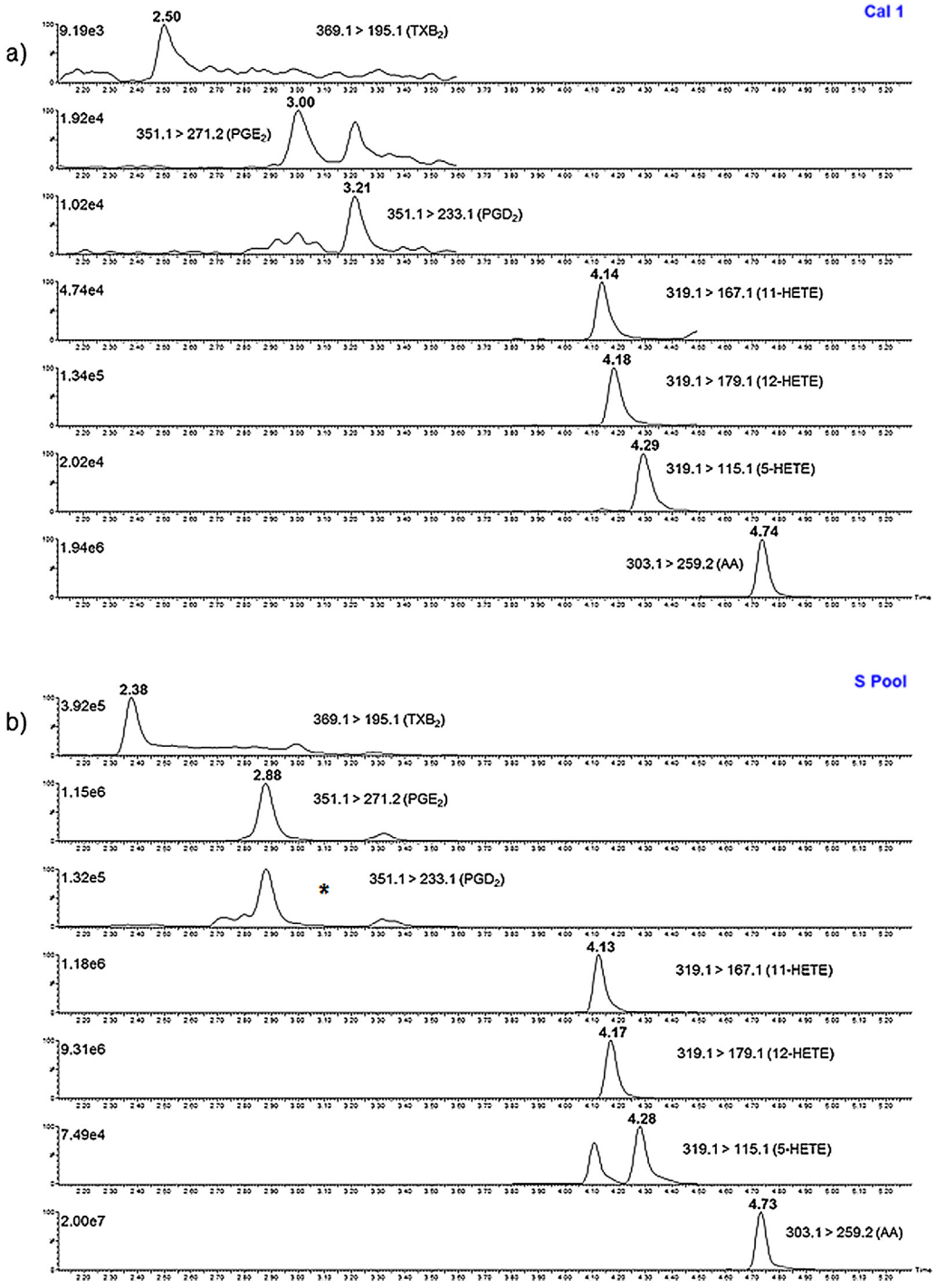



**Fig. 4.** Exemplary chromatograms of (a) Calibrator 1 (Cal 1), representing the LLOQ, and of (b) the stimulated pool employed as quality control (S Pool). (*, not detected).

### 2.3. In vitro *stimulation of human whole blood*

For the *in vitro* stimulation we used our previously described whole blood stimulation assay [15]: Briefly, 1 mL of heparinized whole blood was transferred into 6-well cell culture plates (tissue culture plate 6-well, Sarstedt, Nümbrecht, Germany), mixed with 0.5 mL RPMI 1640 medium (Biochrom, Berlin, Germany) containing 1% (m/v) penicillin/streptomycin (both Gibco life technologies, Darmstadt, Germany). A part of this mixture was spiked with LPS (from *Escherichia coli*; Sigma-Aldrich, St. Louis, USA). Whole blood medium mixture without LPS was immediately centrifuged at 4000 *g* for 10 min, serving as baseline. The aliquots containing LPS (final concentration of 100 ng $mL^{-1}$) were subsequently incubated at 37 °C and 5% $CO_2$. After 24 h of incubation, these aliquots were centrifuged as described above. All resulting supernatants were stored at −80 °C until quantitative analysis by UHPLC–MS/MS.

### 2.4. *Quality control samples*

Two quality control (QC) materials were used, ethanol and plasma. Three QC levels were spiked in ethanol at low (QC L), medium (QC M), and high (QC H) concentrations within the calibration range. The exact concentrations are summarized in Table 1.

The second QC type was based on the authentic matrix. Therefore a pool of heparinized whole blood from blood donors was used for *in vitro* stimulation as described in Section 2.3. The resulting baseline sample (Baseline Pool, "B Pool") and the resulting sample which was stimulated with LPS (Stimulated Pool, "S Pool") were each used as authentic matrix QCs.

### 2.5. Sample preparation

A 100 μL aliquot of the sample (calibrator, QC, Pool, or unknown) was transferred into a 1.5 mL Safe-Lock Tubes™ (Eppendorf, Hamburg, Germany) and mixed with 25 μL of *internal standard working solution* for 5 min on a horizontal shaker. The ferromagnetic bead suspension was re-suspended by vortexing vigorously. Subsequently, 40 μL of the ferromagnetic bead suspension was added to the sample, and the tube was vortexed again. To denature the proteins, 300 μL of ACN were added to the sample-bead mixture, and the tube was vortexed at high speed for at least 10 s to facilitate the binding of the denatured proteins to the surface of the particles. When the tube was placed on the magnetic separator (Magna Medics) the ferromagnetic particles together with the previously bound proteins were magnetically attracted to the permanent magnet integrated in this separator forming a pellet at the tube wall facing the magnet. This is illustrated by Fig. 2. After 1 min, 200 μL of the resulting supernatant were carefully transferred to a 2.0 mL Safe-Lock Tube™ (Eppendorf) without disturbing the pellet. Subsequently, the obtained supernatant was dried in a heat block (40°C) under a gentle stream of nitrogen. After resolving the residue in 100 μL of MeOH/$H_2O$ (50/50, v/v), 85 μL of this mixture were transferred to a brown glass vial containing a micro-insert (both Chromatographie Handel Müller, Fridolfing, Germany) and placed into the autosampler.

### 2.6. UHPLC–MS/MS

The UHPLC–MS/MS system comprised a Xevo TQ-S and an Acquity UPLC, including an autosampler, a switching valve, a column oven and two pairs of pumps (all Waters, Milford, Massachusetts, USA). Mass Lynx V4.1 (Waters) software was used to control the system.

Fig. 3 shows a scheme of the entire configuration with the trapping column (Oasis HLB Direct Connect 20 μm, 2.1 × 30 mm, Waters), the analytical column (Acquity UPLC BEH Shield RP18 1.7 μm, 2.1 × 100 mm, Waters) and the two potential switching positions.

The column oven for the analytical column was maintained at 55°C. The mobile phases for the trapping column were A1, $H_2O$/MeOH (90/10, v/v) and B1, ACN. For the analytical column, the mobile phases were A2, 0.025 % (v/v) acetic acid in $H_2O$ (pH∼3.5) and B2, ACN/MeOH (75/25, v/v). Details of the gradients are provided in Fig. 3. A total of 20 μL of the sample, prepared as described in Section 2.5, was injected into the UHPLC system in position A, where the analytes were retained on the trapping column (online SPE). The valve was switched to position B after 0.7 min, and by back-flushing the trapping column the analytes were eluted onto the analytical column for chromatographic separation. After 5.0 min, the valve was switched back to position A, and both columns were re-equilibrated. The entire run time, including re-equilibration, was 7.5 min.

Ionization was performed by electrospray in the negative mode with a capillary voltage of −2.0 kV. The cone voltage was 40 V, the source temperature was 150°C, the desolvation temperature was 600°C, the cone gas flow was 150 L/h, and the desolvation gas flow was 1000 L/h. The MS parameters, particularly the collision energy (see Table 2), were optimized for all analytes using post-column infusion of neat solutions. Multiple reaction monitoring (MRM) was employed: for each analyte two mass transitions were recorded, and for each internal standard one mass transition was recorded. Detailed information is provided in Table 2. To achieve an acceptable dwell time, the MRMs were scheduled.

The quantification method (Waters QuanLynx™ based) included a linear regression with a weighting function of $1/x^2$ and the exclusion of the origin. The analytes were quantified *via* the quotient of the area of the first transition (quantifier) and the area of the internal standard. The second transition (qualifier) was used to confirm the retention time of the analyte but did not contribute to the quantification results. The peaks were smoothed using the mean smoothing algorithm with two iterations and a smoothing width of three.

### 2.7. Evaluation of analytical performance

We based our evaluation protocol on the *Guidelines of bioanalytical method validation* of the European Medicines Agency (EMA) [18] and in part, also on the CLSI (Clinical and Laboratory Standards Institute) guideline (*Liquid Chromatography-Mass Spectrometry Methods; Approved Guideline, CLSI document C62-A*) [19].

The ethanol-based controls (QC L, QC M, and QC H) and the plasma pools (B Pool and S Pool) were employed as quality controls in several experiments, as detailed below.

#### 2.7.1. Linearity

Aliquots of the eight calibrators were processed as described in Section 2.5 and injected at the beginning of each batch. The data analysis was performed as previously described in Section 2.6, and the correlation coefficient and the slope of the calibration curves were monitored during the validation period.

#### 2.7.2. Accuracy and precision

The accuracy and the precision were assessed with one aliquot of each QC in five independent series (inter-batch). Correspondingly, for intra-batch accuracy and precision, five aliquots of each QC were individually processed and measured in one run. To determine the sole precision of the UHPLC–MS/MS method, multiple injections (n = 5) of one and the same processed aliquot of QC L and of QC H respectively were performed.

Since the nominal concentrations of the eicosanoids in the matrix-based controls B Pool and S Pool are not available due to the unknown endogenous amount of the respective target analytes, only the precision values were accessed for this type of QC.

#### 2.7.3. Stability

The stability of the eicosanoids in plasma as well as in ethanol was investigated for up to one month at different storing conditions in various container materials, *i.e.* polypropylene and glass. In detail, 1.5 mL Safe-Lock Tubes™ (Eppendorf), Matrix™ 0.5 mL ScrewTop Tubes for bio-banking (Thermo Scientific, Waltham, Massachusetts, USA), and 1.5 mL brown glass vials (Chromatographie Handel Müller) were employed to investigate analyte stability at −80°C. For storage at room temperature (+20°C) comprising light exposure as well as for storage in the refrigerator (+4°C) exclusively the 1.5 mL Safe-Lock Tubes™ (Eppendorf) were used.

Furthermore the stability of the processed samples was examined in the autosampler, *e.g.*, during a night batch at +8°C. The post-preparation stability at +20°C was also assessed.

Additionally, the stability of the intermediates of the sample preparation was investigated, using the deproteinated supernatant and the dried extracts, respectively.

#### 2.7.4. Robustness and ruggedness

The dilution integrity was assessed for values inside the calibration range (S Pool) and for those outside the calibration range, as

**Table 2**
MS/MS Parameters for the eicosanoids and corresponding internal standards.

| Analyte | Corresponding internal standard | Retention time [min] | Precursor ion [*m*/*z*] | Quantifier transition | | Qualifier transition | | Dwell time [s] |
|---|---|---|---|---|---|---|---|---|
| | | | | Product ion [*m*/*z*] | CE [eV] | Product ion [*m*/*z*] | CE [eV] | |
| $TXB_2$ | $TXB_2$-$d_4$ | 2.53 | 369.1 | 195.1 | 14 | 169.1 | 16 | 0.032 |
| $PGE_2$ | $PGE_2$-$d_4$ | 3.03 | 351.1 | 271.2 | 16 | 315.2 | 12 | 0.032 |
| $PGD_2$ | $PGD_2$-$d_4$ | 3.23 | 351.1 | 233.1 | 12 | 271.2 | 16 | 0.032 |
| 5-HETE | 5-HETE-$d_8$ | 4.32 | 319.1 | 115.1 | 14 | 203.2 | 16 | 0.028 |
| 11-HETE | 12-HETE-$d_8$ | 4.17 | 319.1 | 167.1 | 14 | 149.1 | 20 | 0.028 |
| 12-HETE | 12-HETE-$d_8$ | 4.20 | 319.1 | 179.1 | 14 | 208.2 | 14 | 0.028 |
| AA | AA-$d_8$ | 4.76 | 303.1 | 259.2 | 14 | 205.2 | 14 | 0.080 |
| $TXB_2$-$d_4$ | | 2.51 | 373.1 | 173.1 | 16 | | | 0.032 |
| $PGE_2$-$d_4$ | | 3.03 | 355.1 | 275.2 | 16 | | | 0.032 |
| $PGD_2$-$d_4$ | | 3.23 | 355.1 | 275.2 | 16 | | | 0.032 |
| 5-HETE-$d_8$ | | 4.29 | 327.1 | 116.1 | 16 | | | 0.028 |
| 12-HETE-$d_8$ | | 4.18 | 327.1 | 184.1 | 14 | | | 0.028 |
| AA-$d_8$ | | 4.75 | 311.1 | 267.2 | 14 | | | 0.080 |

CE, collision energy.

recommended in CLSI C62-A [19]. To simulate the latter case, an extra high ethanol-based control containing more than five times the amount of eicosanoids of Cal 8 was prepared. The *extra high QC* and the S Pool were processed and analyzed in triplicate without dilution. Subsequently, the samples were diluted with MeOH/$H_2O$ (50/50, v/v) before and after sample preparation; *i.e.*, three aliquots of both QCs were diluted prior to sample preparation; three other aliquots were processed normally as previously described, and the resolved residues were subsequently diluted.

The influence of different column lots was investigated by analyzing the same set of calibrators and controls twice on the same day. For each run a different lot of the analytical column was employed.

An injection of MeOH after the highest calibrator was used to assess the carry-over. By injecting the calibrators from highest to lowest the influence of potential carry-over on quantification accuracy was further investigated.

Additionally, the inter-operator variability was tested. For this purpose, the first operator, performing the evaluation experiments, and a second operator with no previous experience handling magnetic particles both prepared the same defined set of control samples (n = 10) and the results were compared regarding precision and accuracy.

#### 2.7.5. Matrix effect and recovery

The experiments described in the following were based on the recommendation of CLSI guideline C62-A [19], with slight modifications.

Experiments for the recovery and the matrix effect were performed according to Matuszewski et al. [20] using pre- and post-spiking in seven different lots of plasma. The matrix effect was calculated in consideration of the respective baseline value ($c_{baseline}$) of eicosanoids present in each plasma lot. The baseline values were determined by analyzing unspiked samples (n = 5 for each lot).

$$\text{Recovery} = 100\% \times c_{\text{plasma pre}}/c_{\text{plasma post}} \quad (1)$$

$$\text{Matrix effect} = 100\% \times (c_{\text{plasma post}} - c_{\text{baseline}})/c_{\text{solvent}} \quad (2)$$

According to the EMA *Guideline on bioanalytical method validation*, we also calculated the *IS normalised matrix factor* ($MF_{norm}$) using the Eqs. (3) and (4):

$$MF = \text{Area}_{\text{matrix}}/\text{Area}_{\text{solvent}} \quad (3)$$

$$MF_{norm} = MF_{\text{analyte}}/MF_{IS} \quad (4)$$

As recommended in the guideline, we subsequently calculated the variation coefficient of the $MF_{norm}$ for each analyte.

Furthermore, we performed post-column infusion according to Bonfiglio et al. [21]. Processed, unspiked plasma samples (containing only the baseline value of eicosanoids) were injected onto the column, while a solution of all analytes and all internal standards was infused directly into the mass spectrometer *via* T-tubing using a syringe pump. The different matrix lots used were either anticoagulated with heparin or EDTA, respectively.

A matrix mixing experiment referring to the CLSI guideline C62-A was the third part of the matrix effect determination. We mixed the S Pool with MeOH/$H_2O$ (50/50, v/v) in different proportions (25/75, 50/50, 75/25, v/v), *e.g.*, 25 μL of MeOH/$H_2O$ mixture and 75 μL of S Pool. Based on the previously determined concentrations of the analytes in undiluted S Pool samples, the theoretical concentrations resulting from dilution were calculated. After LC–MS/MS analysis of the dilutions, the determined concentrations of the analytes were plotted against their respective calculated theoretical concentrations. A linear regression of the plot was performed. Moreover the matrix mixing experiment was repeated with a second lot of stimulated plasma obtained from a different blood donor.

### 2.8. In vitro *stimulation of specimen from healthy blood donors*

As a proof of principle approach this assay was applied to investigate the release capacity of the targeted eicosanoids ($TXB_2$, $PGE_2$, $PGD_2$, 5-HETE, 11-HETE, 12-HETE, AA) in healthy blood donors (n = 5) using the aforementioned whole blood activation model (Section 2.3). Therefore, whole blood of healthy blood donors was stimulated according to Section 2.3 and subsequently analyzed according to Sections 2.5 and 2.6.

## 3. Results and discussion

### 3.1. Method development

#### 3.1.1. Calibration

Unfortunately, it was not possible to generate calibrators in the authentic matrix for two reasons. First, eicosanoid-free plasma was not commercially available. Also, several attempts to reduce the endogenous eicosanoid levels using physical approaches, *e.g.*, UV light, were not successful. A baseline value of relevant height was inevitable in authentic matrix, at least in the case of AA.

Secondly, we observed that after spiking with a mixture of the seven target eicosanoids, the resulting peak areas for $PGD_2$, 5-HETE, 11-HETE, and 12-HETE were markedly higher in plasma compared to corresponding peak areas in ethanol. The effect could not be easily explained, neither by the endogenous level nor by different solubilities, since ethanol is one of the optimal sol-

vents for eicosanoids [6]. The oxidation of AA to HETEs through oxygen from the surrounding atmosphere was also excluded, as this effect should also occur in ethanol or water. Consequently, this phenomenon was further investigated in spiking experiments at different concentration levels. Therefore each eicosanoid was individually spiked into plasma and ethanol, respectively. This experiment revealed that the sole addition of AA to plasma, led to increased concentrations of several other metabolites in a concentration-dependent manner (see Supplementary material, Fig. S1). All the other metabolites individually spiked did not affect the concentrations of other measured eicosanoids included in the assay. Tsikas et al. described in their review the formation of eicosanoids during and after blood sampling in whole blood samples [5]. However we did not expect this phenomenon to occur in plasma since it is free of platelets and other cells able to produce eicosanoids.

In the next step, we investigated the influence of antioxidants (butylhydroxytoluol, ascorbic acid) and inhibitors of COX and/or LOX (acetylsalicylic acid, licofelone) on this distorting formation of eicosanoids induced by the addition of AA to plasma. With the exception of ascorbic acid (at a final concentration of 1%, m/v) no reduction of the formation of $PGD_2$, 11-HETE, 12-HETE, and 5-HETE caused by AA spiking was observed (see Supplementary material, Fig. S2). Since even high concentrations of ascorbic acid could not suppress completely this adverse formation of other target eicosanoids, we decided to prepare the calibrators in a surrogate matrix, *i.e.* ethanol, to obtain reliable nominal concentrations.

### 3.1.2. Sample preparation

The main aim of high-throughput capability for the UHPLC–MS/MS method focused on a sample preparation involving a rapid, effective deproteination step in combination with an on-line SPE procedure involving a trapping column.

During our method development preliminary experiments with *ferromagnetic particle enhanced deproteination* demonstrated reproducible results for eicosanoids. Nevertheless, the high percentage of acetonitrile, necessary for protein precipitation, in the resulting supernatant caused a suboptimal peak shape. The dilution of the supernatant with water, a common approach to reduce the organic content prior to LC–MS/MS analysis, was in our case no option due to the low biological abundance of eicosanoids. Therefore, the supernatant was evaporated under a stream of nitrogen, and the residue was resolved in a different solvent. The temperature during evaporation and the resolving mixture were optimized, with respect to recovery and peak shape: good results were achieved for 40°C and a mixture of 50 % methanol in water.

Although application of on-line SPE has been described in the field of eicosanoid analysis for about thirty years [22], this technique has been rarely employed for these substances so far. Korecka et al. [23] and Willenberg et al. [24] both used on-line SPE for sample preparation, however, either investigated a rather small analyte panel of only two [23] respectively three [24] eicosanoids. Kita et al. combined on-line SPE with a manually, time and labour intensive off-line SPE step [25]. Kortz et al. employed a combination of manual protein precipitation and on-line SPE on a trapping column for sample preparation [11]. Unfortunately, this approach was accompanied with limited recovery rates for some analytes.

A combination of *ferromagnetic particle enhanced deproteination* and on-line SPE seemed a promising alternative to the sample preparation techniques described so far for eicosanoids. This approach combines a fast sample preparation with clean extracts suitable for LC–MS/MS analysis and at the same time, the generic character allows an application to a broad variety of analytes. This assumption was strengthened by our results for matrix effect and recovery for the chosen seven eicosanoids ($TXB_2$, $PGE_2$, $PGD_2$, 5-HETE, 11-HETE, 12-HETE, AA; for further information, see Section 3.2.5).

**Table 3**
Results for accuracy and precision.

| Sample | LLOQ | QC L | QC M | QC H | B Pool | S Pool |
|---|---|---|---|---|---|---|
| **$TXB_2$** | | | | | | |
| Target concentration [ng mL$^{-1}$] | 0.099 | 0.297 | 2.97 | 29.7 | (<0.099)* | (~4.90) |
| Accuracy inter-assay [%] | 100 | 104 | 104 | 109 | | |
| Accuracy intra-assay [%] | 99.4 | 103 | 102 | 105 | | |
| CV inter-assay [%] | 19.9 | 10.4 | 4.8 | 4.0 | | 5.2 |
| CV intra-assay [%] | 11.4 | 11.2 | 4.9 | 3.8 | | 2.5 |
| CV injection [%] | 9.3 | 4.2 | nd | 4.8 | nd | nd |
| **$PGE_2$** | | | | | | |
| Target concentration [ng mL$^{-1}$] | 0.098 | 0.294 | 2.94 | 29.4 | (<0.098)* | (~6.72) |
| Accuracy inter-assay [%] | 98.5 | 108 | 105 | 113 | | |
| Accuracy intra-assay [%] | 98.7 | 101 | 101 | 104 | | |
| CV inter-assay [%] | 8.3 | 8.5 | 6.7 | 6.8 | | 6.7 |
| CV intra-assay [%] | 8.9 | 5.2 | 4.6 | 5.3 | | 5.2 |
| CV injection [%] | 6.2 | 8.0 | nd | 1.8 | nd | nd |
| **$PGD_2$** | | | | | | |
| Target concentration [ng mL$^{-1}$] | 0.099 | 0.297 | 2.97 | 29.7 | (<0.099)* | (<0.099)* |
| Accuracy inter-assay [%] | 99.8 | 101 | 104 | 111 | | |
| Accuracy intra-assay [%] | 88.9 | 94.3 | 95.7 | 96.8 | | |
| CV inter-assay [%] | 8.7 | 6.1 | 2.9 | 5.3 | | |
| CV intra-assay [%] | 7.4 | 7.6 | 6.3 | 3.9 | | |
| CV injection [%] | 11.4 | 5.1 | nd | 1.6 | nd | nd |
| **5-HETE** | | | | | | |
| Target concentration [ng mL$^{-1}$] | 0.049 | 0.147 | 1.47 | 14.7 | (~0.097) | (~0.398) |
| Accuracy inter-assay [%] | 101 | 110 | 95.6 | 125 | | |
| Accuracy intra-assay [%] | 108 | 94.0 | 91.7 | 97.4 | | |
| CV inter-assay [%] | 9.2 | 12.7 | 15.2 | 15.5 | 18.7 | 14.6 |
| CV intra-assay [%] | 7.9 | 13.1 | 15.1 | 9.9 | 5.5 | 6.1 |
| CV injection [%] | 9.5 | 5.3 | nd | 1.3 | nd | nd |
| **11-HETE** | | | | | | |
| Target concentration [ng mL$^{-1}$] | 0.049 | 0.147 | 1.47 | 14.7 | (<0.049)* | (~1.61) |
| Accuracy inter-assay [%] | 95.9 | 104 | 95.0 | 117 | | |
| Accuracy intra-assay [%] | 96.3 | 93.3 | 92.8 | 99.1 | | |
| CV inter-assay [%] | 5.2 | 7.0 | 9.9 | 12.2 | | 14.6 |
| CV intra-assay [%] | 11.2 | 7.3 | 11.5 | 5.7 | | 4.6 |
| CV injection [%] | 3.8 | 2.6 | nd | 1.6 | nd | nd |
| **12-HETE** | | | | | | |
| Target concentration [ng mL$^{-1}$] | 0.490 | 1.47 | 14.7 | 147 | (~1.57) | (~50.9) |
| Accuracy inter-assay [%] | 99.5 | 107 | 107 | 114 | | |
| Accuracy intra-assay [%] | 100 | 103 | 102 | 106 | | |
| CV inter-assay [%] | 6.6 | 5.4 | 6.0 | 2.8 | 5.5 | 5.8 |
| CV intra-assay [%] | 3.0 | 3.0 | 3.7 | 2.5 | 3.9 | 2.6 |
| CV injection [%] | 3.3 | 0.9 | nd | 2.6 | nd | nd |
| **AA** | | | | | | |
| Target concentration [ng mL$^{-1}$] | 24.5 | 73.5 | 735 | 7350 | (~627) | (~933) |
| Accuracy inter-assay [%] | 96.3 | 119 | 116 | 93.3 | | |
| Accuracy intra-assay [%] | 98.9 | 114 | 111 | 92.6 | | |
| CV inter-assay [%] | 8.7 | 2.8 | 2.6 | 2.7 | 9.0 | 3.0 |
| CV intra-assay [%] | 0.7 | 4.0 | 3.4 | 6.4 | 3.1 | 3.0 |
| CV injection [%] | 0.5 | 0.6 | nd | 1.8 | nd | nd |

LLOQ, Lower Limit of Quantification; QC, quality control; L, low; M, medium; H, high; CV, coefficient of variation; (~x), approximate concentration determined over 5 series; nd, not determined; *, below LLOQ.

Consequently, we can state that *ferromagnetic particle enhanced deproteination* in combination with on-line SPE is a reliable semi-automated sample preparation, that is a time-saving alternative to off-line SPE in eicosanoid analysis. Additionally, this technique is also presumed to have less co-precipitation issues, compared to currently used protein depletion by centrifugation.

### 3.1.3. UHPLC–MS/MS method

We described an UHPLC–MS/MS method for the absolute quantification of seven eicosanoids of particular interest in human plasma. Other LC–MS/MS methods have included additional analytes, but these methods primarily focus on relative quantification, as typically employed in metabolomics. However, the aim of the

**Table 4**
Results of LC–MS/MS analysis of specimen from 5 healthy blood donors at baseline and after whole blood stimulation (all data are presented in ng mL$^{-1}$).

| | Baseline (no incubation, no LPS) | Stimulated (24 h incubation with LPS) |
|---|---|---|
| **$TXB_2$** | | |
| Donor 1 | <0.10* | 4.68 |
| Donor 2 | <0.10* | 10.5 |
| Donor 3 | <0.10* | 5.70 |
| Donor 4 | <0.10* | 5.00 |
| Donor 5 | <0.10* | 4.63 |
| **$PGE_2$** | | |
| Donor 1 | <0.10* | 0.62 |
| Donor 2 | <0.10* | 10.4 |
| Donor 3 | <0.10* | 1.40 |
| Donor 4 | <0.10* | 2.74 |
| Donor 5 | <0.10* | 5.41 |
| **$PGD_2$** | | |
| Donor 1 | <0.10* | <0.10* |
| Donor 2 | <0.10* | <0.10* |
| Donor 3 | <0.10* | <0.10* |
| Donor 4 | <0.10* | <0.10* |
| Donor 5 | <0.10* | <0.10* |
| **5-HETE** | | |
| Donor 1 | <0.05* | 0.19 |
| Donor 2 | 0.06 | 0.29 |
| Donor 3 | <0.05* | 0.29 |
| Donor 4 | 0.06 | 0.50 |
| Donor 5 | <0.05* | 0.32 |
| **11-HETE** | | |
| Donor 1 | <0.49* | 0.81 |
| Donor 2 | <0.49* | 2.94 |
| Donor 3 | <0.49* | 1.27 |
| Donor 4 | <0.49* | 1.31 |
| Donor 5 | <0.49* | 1.66 |
| **12-HETE** | | |
| Donor 1 | 0.66 | 19.8 |
| Donor 2 | <0.49* | 46.7 |
| Donor 3 | <0.49* | 19.3 |
| Donor 4 | <0.49* | 22.6 |
| Donor 5 | <0.49* | 18.7 |
| **AA** | | |
| Donor 1 | 151 | 417 |
| Donor 2 | 383 | 787 |
| Donor 3 | 263 | 568 |
| Donor 4 | 372 | 770 |
| Donor 5 | 228 | 839 |

LPS, lipopolysaccharide; *, below Lower Limit of Quantification.

present study was absolute quantification using a set of eight calibrators analyzed at the beginning of every batch and a deuterated internal standard for every analyte (if commercially available), as recommended in a recently published review [4]. Moreover, deuterated internal standards play a crucial role when the calibration matrix is different from the authentic matrix of the unknown samples, as in our method.

As many isomers are present among the eicosanoids, selectivity is an essential aspect for the development of UHPLC–MS/MS methods [26]. Unlike HETEs, which are isomeric but can be discriminated through mass spectrometry due to different fragmentation patterns [27], the prostaglandins $E_2$ and $D_2$ have to be chromatographically separated. We separated $PGE_2$ and $PGD_2$ to baseline, as shown for Cal 1 in Fig. 4a. An exemplary chromatogram for an authentic matrix control, the S Pool, is shown in Fig. 4b (unfortunately the $PGD_2$ level was still below the LLOQ; for further information see below).

Although only 100 μL of plasma were used and throughout the sample preparation the original eicosanoid level was diluted to a final concentration of approximately 40% from its origin, we realized an LLOQ of 0.1 ng mL$^{-1}$ (5.4 fmol on column) for $TXB_2$, of 0.1 ng mL$^{-1}$ (5.7 fmol on column) for $PGD_2$ and $PGE_2$, and of 0.05 ng mL$^{-1}$ (3.1 fmol on column) for 5-HETE and 11-HETE, respectively. Notably, the LLOQs for 12-HETE and AA were not based on the technical detection limits of this method but were selected as lower calibration limits based on the biological occurrence of these eicosanoids (12-HETE 0.5 ng mL$^{-1}$ and AA 24.5 ng mL$^{-1}$), as previously reported [7].

Regarding authentic matrix samples, $TXB_2$, $PGE_2$, all HETEs and AA could be quantified in our pool originating from stimulated whole blood (S Pool). Levels of 5-HETE, 12-HETE, and AA were actually high enough to be quantified in the baseline pool originating from whole blood without stimulation (B Pool). For detailed concentrations see Table 3.

### 3.2. Evaluation of analytical performance

There is a lack of official guidelines for the validation of LC–MS/MS methods addressing endogenous compounds, such as eicosanoids. Various approaches to cope with these problems were published in recent years [28–30].

The validation protocol for the assay described herein was designed based on the *Guidelines of bioanalytical method validation* by the EMA [18]. However, this guideline addresses xenobiotics, not endogenous compounds such as eicosanoids. Hence, to generate a validation protocol suitable for the intended endogenous analytes, we modified the protocol referring to the published recommendations mentioned above.

#### 3.2.1. Linearity

Our method was linear ($R^2 > 0.98$ for 5-HETE and AA; $R^2 > 0.99$ for all other analytes) for the whole calibration range (see Table 1).

In case of AA, the highest calibrator, Cal 8, was excluded because the detector response was no longer linear at this concentration. However, the resulting upper limit of quantification (ULOQ) of 9800 ng mL$^{-1}$ (Cal 7) should be sufficient to quantify the majority of biological samples according to previous studies [7,15].

#### 3.2.2. Accuracy and precision

For all five controls (QC L, QC M, QC H, B Pool, and S Pool), the resulting values for accuracy and precision (inter- and intra-assay) were within the limits (±15 %) of the EMA guideline. For 5-HETE, the results were slightly higher but remained under ±20 %. Regarding the lower limit of quantification (LLOQ), the results for all analytes were within the limits of the EMA guideline (±20 %). The detailed values for all analytes and QCs are provided in Table 3.

#### 3.2.3. Stability

The results of the stability experiments showed that the eicosanoids were stable at −80°C for at least one month in ethanol and plasma. Marked differences (> ±15 %) between Eppendorf Safe-Lock Tubes™, Thermo Scientific Matrix™ Tubes (both polypropylene), and glassware were not observed. The only exception is 5-HETE in stimulated plasma (S Pool): over four weeks a decrease of approximately 34 % was observed when stored in Matrix™ Tubes (n = 3) [compared with storage in Safe-Lock Tubes™ (n = 3)]. In further studies, the stability at −80°C should be investigated over a longer time period.

The data also showed that the analyte concentrations are neither impaired after three freeze and thaw cycles (−80°C), after storage for three days at +4°C nor after standing for one hour on the bench (room temperature, light) prior to sample preparation.

The readily processed samples are stable in the described glass vials for at least 72 h in the autosampler (at +8°C) and for 13 h at room temperature.

The data obtained from stability experiments using the sample preparation intermediates suggests that the best point to interrupt

– if necessary – would be after the deproteination step. Without bias, the obtained supernatant might be stored in the refrigerator for up to four hours prior to continuing the sample preparation.

#### 3.2.4. Robustness and ruggedness

The dilution experiment, employing the *extra high QC* (based on EtOH) and the S Pool, showed good accuracy values for pre-preparation and post-preparation dilution. Based on these findings, we selected post-preparation dilution as standard procedure in case an unknown sample having a concentration above the ULOQ might occur. This approach provides more reliability, as the internal standard is already present, and it is additionally more convenient.

Carry over (peak area of the analyte in a blank injection following the highest calibrator divided by the peak area of the analyte in the highest calibrator) was < 0.05 % for all analytes, except AA (1.2 %).

The EMA calculates a second value: the peak area of a blank injection following the highest calibrator is divided by the peak area of the lowest calibrator. Due to the wide calibration ranges of this method, this second value was approximately 20–30 % for the HETEs and 230 % for AA. If this value exceeds 20 %, the EMA recommends arranging the samples based on the concentration (when possible) or otherwise to inject a blank after high concentrations. Nevertheless, the reversed order of the calibrators starting with Cal 8 (highest calibrator) resulted in a calibration with similar parameters and equal quality as the usual curve starting with Cal 1 (lowest calibrator).

The *inter-operator variability* was within the usual variation determined through precision experiments. Also, the use of a different column lot did not affect the results.

#### 3.2.5. Matrix effect and recovery

Notably, the pre-/post-spiking experiments (see Section 2.7.5) according to Matuszewski [20] were performed for AA alone and in parallel for the remaining metabolites to prevent bias through the AA induced formation of $PGD_2$, 5-HETE, 11-HETE, and 12-HETE in plasma, as described in Section 3.1.1.

No relevant matrix effect was observed (98 % $TXB_2$, 101 % $PGE_2$, 98 % $PGD_2$, 107 % 5-HETE, 102 % 11-HETE, 94 % 12-HETE, and 109 % for AA). The variation coefficient of the *IS normalised matrix factor* ($MF_{norm}$) ranged from 1 to 7 %, which is clearly below the limit of the EMA (15 %).

Also recovery showed satisfying results: 90 % $TXB_2$, 95% $PGE_2$, 74% $PGD_2$, 93 % 5-HETE, 92 % 11-HETE, 91 % 12-HETE, and 93 % for AA.

Regarding the post-column infusion of blank plasma samples, no ion suppression or ion enhancement was observed, compared to a solvent injection. Because eicosanoids are endogenous compounds present in every plasma sample, a slight increase in the signal resulting from infusion was observed when endogenous AA (having relatively high natural abundance) from the blank plasma sample was eluted from the column.

In a third experiment, we employed matrix mixing to investigate whether a different amount of matrix influences the accuracy: plotting the determined and calculated theoretical concentrations of eicosanoids for the different mixing ratios resulted in a linear relationship with good correlation coefficients for both matrix lots ($R^2 > 0.99$).

The theoretical assumed compensation of matrix effects by the internal standard can be confirmed by these findings. This compensation is very important in LC–MS/MS methods, particularly when constructing the calibration curve in a matrix different from the authentic matrix of the samples.

### 3.3. In vitro *stimulation of specimen from healthy blood donors*

As a proof of principle approach our newly developed analytical LC–MS/MS assay employing *ferromagnetic particle enhanced deproteination* was used to investigate the release capacity of the targeted eicosanoids ($TXB_2$, $PGE_2$, $PGD_2$, 5-HETE, 11-HETE, 12-HETE, AA) in healthy blood donors (n = 5) using the whole blood activation model described in Section 2.3.

As shown in Table 4, target mediators were not detectable at baseline, with the exception of AA and for some donors also for 5-HETE and 12-HETE. It is noteworthy that the baseline pool (B Pool) used for validation was characterized by markedly higher endogenous levels for 5-HETE and 12-HETE (see also Table 3), compared to the found baseline concentrations of 5-HETE and 12-HETE in the unstimulated specimens of our five healthy blood donors, which were below or only slightly above the respective LLOQ. These obvious differences potentially indicate a broad variability of 5-HETE and 12-HETE at baseline among healthy people. However, further studies are necessary to underpin this assumption.

As expected, LPS whole blood activation resulted in a relevant increase of eicosanoids (compare Table 4). The intensity of the eicosanoid release markedly differed between the individual blood donors. As in the S Pool used during validation, $PGD_2$ was unfortunately not detected in stimulated samples of the 5 blood donors.

These results show that the newly developed multi-analyte LC–MS/MS assay employing *ferromagnetic particle enhanced deproteination* described herein is suitable for analysis of samples generated with the described whole blood activation model.

## 4. Conclusion

To the best of our knowledge, the herein presented study is the first to describe *ferromagnetic particle enhanced deproteination* in combination with on-line SPE for sample clean-up of eicosanoids in human plasma samples. This novel approach to semi-automated sample preparation was found convenient and rugged enabling reliable analyses of demanding analytes in complex biological matrices.

Prospectively, the complete automation of the sample preparation might be possible. Actually, *ferromagnetic particle enhanced deproteination* could be performed using a liquid handling system and also the evaporation step could be integrated with the appropriate instrumentation. Based on our promising validation data for eicosanoids we are convinced that this innovative approach will facilitate automation in UHPLC–MS/MS.

Due to its rather generic character and based on our validation results for eicosanoids as a demanding model group of endogenous analytes, we believe *ferromagnetic particle enhanced deproteination* is a very promising tool for the analysis of a broad variety of target analytes, including endogenous analytes as well as xenobiotica.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2016.03.022.

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