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# Quantitative profiling of lipid mediators in sperm cells through on-line dilution on-line polymer matrix-based solid-phase extraction liquid chromatography with mass spectrometric detection

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An automated on-line dilution on-line solid-phase extraction liquid chromatography-mass spectrometry method was developed for the straightforward and versatile quantitative profiling of lipid mediators such as various eicosanoids, endocannabinoids, and arachidonic acid in human sperm. The LC-MS/MS system utilized a polymer matrix-based trap column (TurboFlow Cyclone™) for sample pre-treatment, followed by chromatographic separation using an ODS 3 μm, 100 × 2 mm column, and detection *via* a triple-quadrupole mass spectrometer equipped with an electrospray interface. The sample preparation process employed a simple "dilute and shoot" approach, with a centrifugation step to remove proteins. This method has significantly improved sensitivity and selectivity while minimising matrix effects, with limits of quantification in the low picogram range for most analytes. Linearity, accuracy, precision, and recovery all met the required criteria for bioanalytical method validation. This LC-MS/MS approach was successfully applied to determine basal levels of selected eicosanoids, endocannabinoids, and arachidonic acid in human spermatozoa.

## Introduction

Lipid mediators play important roles in numerous physiological and pathophysiological processes.[1] The polyunsaturated fatty acid arachidonic acid serves as an important intermediate in both the biosynthesis and the degradation of these compounds. After release from phospholipids of cell membranes by a phospholipase $A_2$,[2] it is converted by cyclooxygenases and lipoxygenases to oxidized compounds like prostaglandins and leukotrienes, grouped together under the name eicosanoids.[3] These exert their effects mainly through interaction with G protein-coupled receptors.[4] Endocannabinoids such as *N*-arachidonoylethanolamide (AA-EA) and 2-arachidonoylglycerol (2-AG), which are amides and esters of arachidonic acid, also act through these types of receptors.[5,6] Their hydrolytic degradation by fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL) leads to arachidonic acid, which can then be further metabolised *via* the arachidonic acid cascade described above.

Prostaglandins were first detected in seminal fluid.[7–9] They are also thought to be produced in spermatozoa themselves.[10–12] Furthermore, AA-EA and 2-AG appear to play a role in the physiological function of these cells.[13] Indeed, these substances and their catabolic and anabolic enzymes have been detected in them but their physiological role has not been fully elucidated.[14,15] To investigate the profile of lipid mediators in human sperm or to study their metabolism, we aimed to develop a sensitive and easy-to-use LC-MS/MS method.

Several methods for the determination of eicosanoids,[16–22] endocannabinoids[17,23–28] and fatty acids[18–20,29–31] are described in the literature. These include mainly techniques such as ELISA,[32–34] TLC-UV,[35,36] GC,[37] GC-MS,[38–40] LC-MS[41] and LC-MS/MS.[17–19,21,29–31,42–50]

The primary drawback of ELISA is its lack of specificity, coupled with relatively poor precision and accuracy. Additionally, its inability to simultaneously determine multiple analytes is a significant limitation.[51–53] GC-MS methods show good separation performance with high analytical precision, accuracy and sensitivity, but often require extensive derivatisation.

LC-MS/MS technology enables accurate determination of eicosanoids, endocannabinoids and fatty acids in biological matrices, but also often requires sample preparation. In these, the analytes are usually first isolated by liquid–liquid extraction (LLE),[27,31,54–61] by off-line solid-phase extraction (off-line SPE)[17,19,20,30,59] or by on-line solid-phase extraction (on-line – SPE).[18,48,62] Simple protein precipitation (PPT) is used less frequently.[63] We have recently published a LC-MS method for some specific eicosanoids with on-line dilution/on-line SPE for automated sample pre-treatment.[64]

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In the literature, individual determination methods are often reported for the respective lipid class of eicosanoids, endocannabinoids and fatty acids. In the present paper, we describe an improved on-line dilution on-line SPE LC-MS/MS method. Compared to our previously published method,[64] the fully validated method can now also be used to quantify endocannabinoids and fatty acids. In contrast to the methods described in the literature for determining these analytes in semen, sample preparation is not performed by LLE or off-line SPE, but by a significantly less time-consuming, fully automated on-line SPE, combined with on-line dilution of the injected sample.

## Experimental

### Chemicals and standards

Prostaglandin $E_1$ ($PGE_1$), prostaglandin $E_1$-$d_4$ ($PGE_1$-$d_4$), prostaglandin $E_2$ ($PGE_2$), prostaglandin $E_2$-$d_9$ ($PGE_2$-$d_9$), prostaglandin $D_2$ ($PGD_2$), prostaglandin $D_2$-$d_4$ ($PGD_2$-$d_4$), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin $F_{2\alpha}$-$d_4$ ($PGF_{2\alpha}$-$d_4$), thromboxane $B_2$ ($TXB_2$), thromboxane $B_2$-$d_4$ ($TXB_2$-$d_4$), 19(*R*)-hydroxy-prostaglandin $E_1$ (19(*R*)-HO-$PGE_1$), 19(*R*)-hydroxy-prostaglandin $E_2$ (19(*R*)-HO-$PGE_2$), 15-hydroxyeicosatetraenoic acid (15(*S*)-HETE), 15-hydroxyeicosatetraenoic acid-$d_8$ (15(*S*)-HETE-$d_8$), arachidonic acid (AA), arachidonic acid-$d_{11}$ (AA-$d_{11}$), arachidonic acid-$d_8$ (AA-$d_8$), *N*-arachidonoylethanolamide (AA-EA), *N*-arachidonoylethanolamide-$d_8$ (AA-EA-$d_8$), 2-arachidonoylglycerol (2-AG), 2-arachidonoylglycerol-$d_5$ (2-AG-$d_5$), 2-arachidonoylglycerol-$d_8$ (2-AG-$d_8$) were purchased from Cayman Chemical (Biomol, Hamburg, Germany). Acetonitrile (LC-MS grade), methanol (gradient grade) and isopropanol (gradient grade) were purchased from Fischer Chemicals (Pittsburgh, USA). Purified water was prepared by a Milli-Q Advantage A10 from Merck (Darmstadt, Germany). Formic acid (p.a.) was provided by VWR (Darmstadt, Germany). Sperm samples were obtained from volunteers at the department of Clinical Andrology, Centre of Reproductive Medicine and Andrology, University Hospital Münster (Germany).

### Sample preparation

**Human sperm preparation.** Sperm samples from normozoospermic (according to WHO) voluntary donors were kindly provided by the group of Prof. Timo Strünker from the Centre of Reproductive Medicine and Andrology, University Hospital Münster, University of Münster. Experiments on human sperm were conducted in agreement with the standards set by the Declaration of Helsinki. The donors provided ejaculates with prior written consent and under approval of the institutional ethics committees of the medical association Westfalen-Lippe and the Medical Faculty of the University of Münster (4INie and 2021-402-f-S). Motile sperm were isolated from the ejaculate using the swim-up technique as described before and adjusted to a concentration of about $1 \times 10^6$ or $1 \times 10^7$ sperm per mL in human tubal fluid (HTF) medium.[65]

**Standard solutions**

*Stock and work solution.* A mixed DMSO stock solution of all eicosanoids, endocannabinoids and arachidonic acid, each with a concentration of 20 μg mL$^{-1}$, was prepared from the supplied solutions and stored at −20 °C. Working standards in the concentration range of 10–10 000 ng mL$^{-1}$ in DMSO were prepared from this stock solution and stored at −20 °C. An internal standard solution (ISTD) of the respective deuterated analytes at a concentration of 2 μg or 4 μg in 500 mL acetonitrile/methanol (1 : 1) was prepared and stored at −20 °C. The ISTD solution was ready for use (see SI).

*Solvent standards.* For each standard, 1 μL of the corresponding concentrated work solution was placed in an Eppendorf vial and mixed with 99 μL of acetonitrile. Subsequently, 300 μL ISTD solution was added. The solution was transferred to an autosampler vial and injected directly.

*Matrix standards.* A pellet of $1 \times 10^7$ spermatozoa stored at −80 °C was resuspended in 1000 μL HTF buffer. For each standard, 99 μL of this sperm solution was placed in an Eppendorf vial and mixed with 1 μL of the corresponding concentrated work solution. Subsequently, 300 μL ISTD solution was added and the sample was centrifuged. The supernatant was transferred to an autosampler vial and injected directly.

*Matrix matched standards.* A pellet of $1 \times 10^7$ spermatozoa stored at −80 °C was resuspended in 1000 μL HTF buffer. For each standard, 99 μL of this sperm solution was added to an Eppendorf vial, 300 μL of ISTD solution was added and then mixed with 1 μL of the correspondingly concentrated work solution. The sample was centrifuged. The supernatant was transferred to an autosampler vial and injected directly.

**Sperm preparation for the determination of basal levels of eicosanoids, endocannabinoids and arachidonic acid.** $1 \times 10^7$ sperm cells from the described human sperm preparation were centrifuged (12 000 rcf, 20 min, 37 °C). The supernatant was discarded and the pellet was resuspended with 1 mL HTF buffer. Subsequently, centrifugation was performed (12 000 rfc, 20 min, 37 °C) and the yielded sperm pellet was stored at −80 °C. The pellet samples were resuspended with 100 μL HTF on the day of measurement, mixed with 300 μL ISTD solution, briefly centrifuged, and then 150 μL of the solution was injected.

### LC-MS/MS-analysis

**On-line dilution on-line SPE-LC-MS/MS system.** Investigations were done on a high performance liquid chromatography system (Shimadzu, Duisburg, Germany) coupled with a triple quadrupole mass spectrometer (Sciex QTRAP 6500+, Darmstadt, Germany).

In detail, the on-line dilution on-line SPE-LC-System (Shimadzu, Duisburg, Germany) consisted of four ADXR20 pumps (one with a quaternary low pressure gradient unit), a SIL-30AC autosampler with 0.5 mL injection loop, CTO-20AC column oven with one Rheodyne 2-position 6-port valve, a SPD-20A UV-detector and a CBM-20A controller (Fig. 1).

**LC-method for the analysis of selected eicosanoids, endocannabinoids and arachidonic acid.** The quaternary pump 1, which was operated at a flow rate of 0.5 mL min$^{-1}$ (0.1%

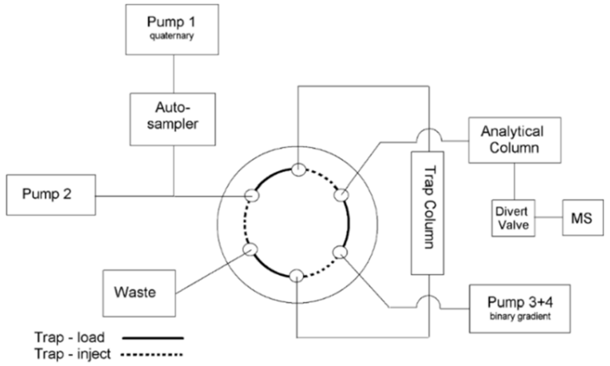

Fig. 1 Schematic diagram of the on-line dilution on-line SPE-LC-MS system. The injected 150 μL sample was transported to the trap column by the loading pump 1. Dilution pump 2 modified the composition of the mobile phase in such a way that the analytes were completely retained on the TurboFlow™ column. Once the analytes had been purified and concentrated, they were transported to the analytical column by switching the 6-port 2 position valve to the inject position using a back-flush process. After separation, detection was performed with a TQ-MS.

HCOOH/methanol (80/20, v/v)), transported 150 μL of injected sample volume towards the trap column. The sample was diluted by the 2 mL min$^{-1}$ flow (0.1% HCOOH) of pump 2 at a T-piece in front of the trap column. The on-line diluted sample was trapped on a TurboFlow Cyclone™ 1.0 × 50 mm column (Thermo Scientific, USA). After 2 min of sample concentration and sample purification on the trap column, the back-flush mode was activated by switching the valve in position trap-inject. Pumps 3 and 4 began analytical separation in binary gradient mode on a Prodigy ODS (3), 3 μm, 100 × 2 mm column (Phenomenex, Aschaffenburg, Germany) with SecurityGuard™ cartridge $C_{18}$ 4 × 3 mm (Phenomenex, Aschaffenburg, Germany). Mobile phase A consisted of acetonitrile/ultrapure water/formic acid (10/90/0.1, v/v/v) and acetonitrile/ultrapure water (95/5, v/v) for mobile phase B. The flow went through both the trap and analytical columns. The trap column was switched out of the analytical flow again by switching the valve to the trap-load position. Simultaneously with the further analytical separation on the analytical column, the trap column was regenerated. Finally, both columns were re-equilibrated to their initial conditions [method 1]. Since 15-HETE, endocannabinoids and arachidonic acid with their physicochemical properties are too different from the other analytes, a slightly different LC method had to be developed for their accurate quantification, which is described in detail in the SI [method 2].

**Configuration of the mass spectrometric detector.** QTRAP 6500+ system was operated in electrospray mode using a Turbo V™ Source Interface. The ESI conditions applied were curtain gas of 40 psi (nitrogen) and ionspray voltage of 5.5 kV and −4.5 kV for positive and negative modes, respectively. The entrance potential was set to ±10 V. The MS conditions were optimized to a source temperature of 500 °C and ion source gas pressure 1 and 2 with 50 and 70 psi, respectively. Multi reaction mode (MRM) transitions, collision energy (CE) and declustering potential (DP) were established for each analyte and listed in the SI. Scheduled MRM™ Algorithm in Analyst 1.7.2 was employed for data acquisition. MultiQuant™ 3.0 software was used for quantitative data processing.

**Investigations into the properties of the TurboFlow Cyclone™ SPE column.** In addition to injection linearity, the elution, retention and recovery behaviour of representative eicosanoids on the TurboFlow Cyclone™ trap column was investigated. The test conditions and results are detailed in the SI.

### Analytical validation

**Linearity and LLOQ.** Standard curves were prepared for all analytes with nine calibration points ranging from 0.01 to 5 ng. For $PGD_2$ the calibration range covered only up to 2 ng. A $1/x$ weighted linear regression analysis of the area ratios (peak area analyte/peak area of the internal deuterated standard) was performed. The calibration for 19(*R*)-HO-$PGE_1$ was conducted without considering of an ISTD. The lower limit of quantification (LLOQ) was set at the lowest concentration of the calibration standards.

**Recovery rate and matrix effect.** Matrix from six different donors was examined at low and high concentration (0.05 ng/5 ng) with respect to recovery and matrix effect. Recovery rates were calculated by comparing the peak areas of matrix standards with matrix-matched standards without considering the ISTD. The matrix effect was assessed by comparing the peak areas of matrix-matched standards with solvent standard solutions without considering the ISTD. For the 19(*R*)-HO-$PGE_1$ and 19(*R*)-HO-$PGE_2$, due to the high basal concentration in the matrix and the absence of deuterated derivate as a possible substitute analyte with identical properties, the recovery and matrix effect had to be determined by comparing the slopes of the corresponding calibration curves without considering ISTD.

**Accuracy and precision.** To determine accuracy and precision, quality control (QC) samples were prepared by spiking sperm matrix at four concentration levels ($n = 5$ each) in three different batches that covered the entire calibration range: 0.02 ng (2× LLOQ-QC), 0.05 ng (low QC), 2 ng (medium QC), 5 ng (high QC). The within-run accuracy was calculated as the percentage mean of the 5 QC samples of the respective concentration level. The between-run accuracy was calculated as the percentage mean of all 15 QC samples of the respective concentration level of three different batches. The within-run and between-run precision of the QC-samples is expressed as percent coefficient of variance (CV%).

**Stability.** Autosampler stability at 10 °C was determined *via* re-injection of previously measured samples ($n = 6$) after 24 h. The peak areas of the respective samples were put into a percentage ratio and from this the mean value and the relative standard deviation were calculated.

**Carry over.** Carry over was determined by injecting a solvent sample after a matrix, matrix matched and solvent calibration standard of high concentration and calculated as percent area.

## Results and discussion

The quantitative determination of eicosanoids, endocannabinoids and fatty acids in various biological material such as plasma and tissue is well-documented in literature. Profiling of the analytes with respect to physiological functions is often the key area of interest. Our project focused on the development of a LC-MS/MS method for the analysis of eicosanoids, endocannabinoids and fatty acids in sperm cells.

The aim was to quantify the physiological distribution of the lipid classes in spermatozoa and to create the possibility to study changes in the lipid profile due to physiological influences in the future. In order to develop a sensitive LC-MS/MS method, a targeted-MS approach was chosen. Lipids that have been described in the literature in sperm fluid and spermatozoa were selected as examples and analysed using mass spectrometric MRM experiments.[37,66]

For eicosanoids, endocannabinoids and fatty acids, besides liquid/liquid extractions, mainly off-line SPE methods for matrix removal are described. In both sample preparation techniques, dilution steps take place during execution, which can lead to sensitivity problems in the analyte determination. One way to avoid this is to concentrate the extraction solutions under a stream of nitrogen followed by re-constitution. This is a time-consuming and personnel-intensive procedure. In addition, the destruction of the analytes by thermal stress, insolubility in the reconstituting solvent and non-specific adsorption of the analytes on surfaces can occur as undesirable side effects. The use of on-line SPE as an automated, reliable and fast technique eliminates most of these drawbacks.[18,48,62,67]

In a previous work,[64] we used on-line dilution on-line SPE-LC-MS/MS analysis for the determination of a few selected eicosanoids in platelets, which allowed a "dilute and shoot" approach. This was made possible by the so-called on-line dilution technique. With this technique, sample solutions can be modified with an aqueous mobile phase using a dilution pump so that even analytes present in a strongly eluting solvent can be completely enriched on the trap column.[68]

We now utilized the described on-line SPE-LC-MS/MS system with on-line dilution to develop an analytical method for the quantification of eicosanoids, endocannabinoids and fatty acids in sperm cells. Unlike the previous method used for determining eicosanoids in platelets, this new approach has been fully validated and expanded to include a broader range of analytes. These encompass additional polar and lipophilic eicosanoids, key endocannabinoids, and arachidonic acid.

### Method development and validation

For method development and validation no "analysis-free" blank matrix of sperm was available. The analytes were always present at different levels in different matrix samples. Therefore, for some analytes with very high basal levels, a corresponding deuterated standard was used for validation. In order to be able to make reliable quantitative statements, the degree of deuteration was verified for these analytes by means of high resolution mass spectrometry.

Research into the properties of the TurboFlow Cyclone™ column formed the basis for method development. The elution, retention and recovery behaviour of representative eicosanoids on the trap column was investigated. The switching times of the 6-port, 2-position valve and the start conditions for gradient methods 1 and 2 were derived from these results. Furthermore, it was demonstrated that on-line dilution enables the injection of a large sample volume without significant loss of analytes on the trap column or reduction in the chromatographic separation efficiency on the analytical column (see SI). Simultaneously concentrating and purifying the sample on the trap column increased the sensitivity and specificity of the entire analytical method.

The method was validated with respect to recovery rate, matrix effect, linearity, with-in run and between-run accuracy and precision. Furthermore, studies on trap column capacity, stability and carry over behavior were performed.

As can be clearly seen in Fig. 2, all analytes can be chromatographed with method 1 with high separation efficiency. However, during validation it was found that the recovery rate and precision for 15-HETE, the endocannabinoids and arachidonic acid was slightly less sufficient. As the lipophilicity of these analytes differs significantly from that of the other oxylipid mediators, unspecific adsorption processes during chromatography were assumed to be the cause. This problem was solved by slightly adapting method 1 to more lipophilic gradient conditions. Method 2 was therefore used for the method validation of 15-HETE, the endocannabinoids and arachidonic acid.

**Recovery rate and matrix effect.** In order to determine a representative recovery rate, six different donor matrices in the low (next to LLOQ, 0.05 ng) and high concentration range (ULOQ, 5 ng) were spiked, measured and subsequently statistically evaluated. The mean values of the areas from the matrix samples ($n = 6$) were compared with the mean values of the matrix-matched samples ($n = 6$) without considering an ISTD.

For the determination of the matrix effect of each analyte, the quotient of the areas obtained from the matrix-matched samples ($n = 6$) and the solvent standards was calculated without considering an ISTD.

For 19($R$)-HO-PGE$_1$ and 19($R$)-HO-PGE$_2$, unfortunately, no deuterated analyte standard was available despite high basal values. Therefore, the slopes of the matrix regression curve, matrix-matched regression curve, and solvent regression curve from the linearity tests ($n = 3$, regression without consideration of an ISTD) were correlated here, respectively.

Absolute recoveries for all analytes ranged from 95.8 to 105.8% with a CV of <12.8%.

The matrix effect for most analytes ranged from 81.2 to 111.5% with a CV of <11.4%. Only the analytes 19($R$)-HO-PGE$_1$ and 19($R$)-HO-PGE$_2$ showed a high matrix effect of 153% and 167%, respectively (Table 1).

**Linearity and lower limit of quantification.** Quantification of eicosanoids, endocannabinoids and arachidonic acid was performed using the stable isotope dilution method. For each analyte to be quantified, an internal standard was selected and a $1/x$ weighted linear standard curve was constructed by plotting

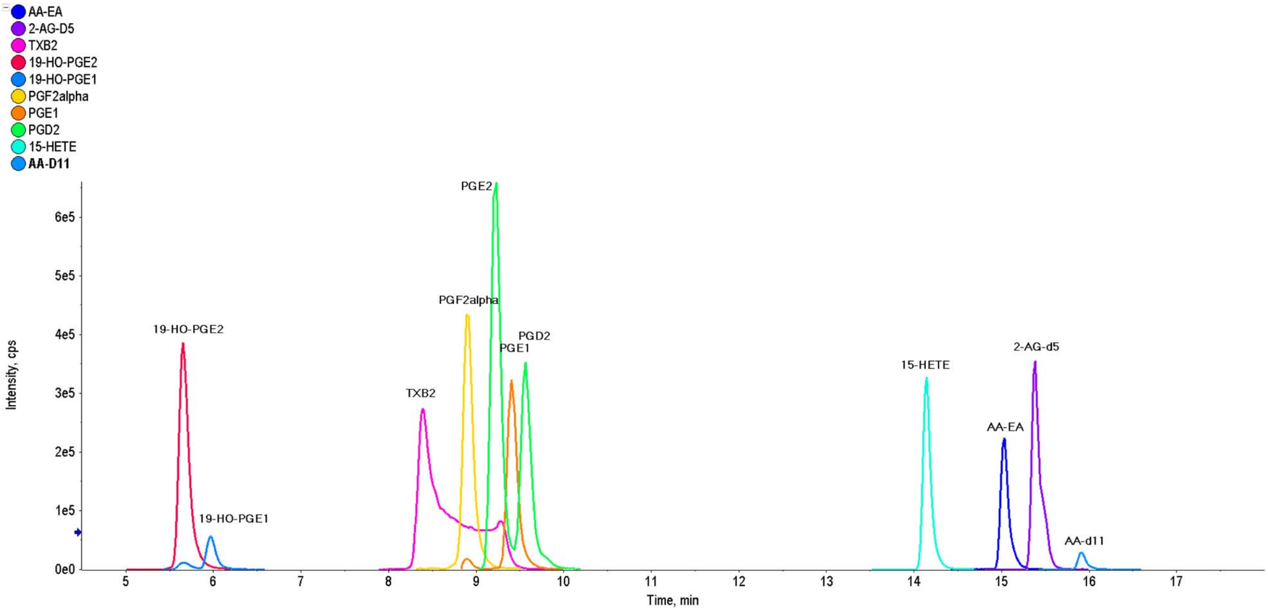

Fig. 2 Example ion chromatogram of the analytes without ISTD using method 1 (matrix standard, 500 pg/$10^6$ sperm).

Table 1 Matrix effect [ME] and recovery as percentage value at 0.05 ng and 5 ng/$10^6$ spermatozoa; for 19(*R*)-hydroxy-prostaglandin $E_1$ and 19(*R*)-hydroxy-prostaglandin $E_2$, the mean values of ME and recovery were determined by the slope of the respective regression lines ($n = 3$)

| | | Matrix effect | | Recovery | |
|---|---|---|---|---|---|
| Analyte | Conc. [ng] | Mean [%] | CV [%] | Mean [%] | CV [%] |
| $PGD_2$ | 0.05 | 107.6 | 5.7 | 105.8 | 11.4 |
| | 5 | 111.5 | 5.3 | 100.7 | 7.4 |
| $PGE_1$ | 0.05 | 111.1 | 3.8 | 101.5 | 4.1 |
| | 5 | 110.2 | 5.3 | 99.7 | 5.0 |
| $PGE_2$ | 0.05 | 106.6 | 7.3 | 104.5 | 7.1 |
| | 5 | 101.4 | 4.0 | 101.1 | 5.1 |
| $PGF_{2\alpha}$ | 0.05 | 102.5 | 7.4 | 103.5 | 8.1 |
| | 5 | 100.3 | 4.2 | 98.3 | 5.3 |
| $TXB_2$ | 0.05 | 103.1 | 4.1 | 103.9 | 4.8 |
| | 5 | 101.0 | 4.5 | 98.4 | 6.6 |
| 15(*S*)-HETE | 0.05 | 95.1 | 5.1 | 100.6 | 6.6 |
| | 5 | 94.5 | 6.0 | 99.2 | 5.5 |
| AA-$d_{11}$ | 0.05 | 92.8 | 11.03 | 95.8 | 6.4 |
| | 5 | 89.8 | 5.03 | 96.0 | 4.6 |
| 2-AG-$d_5$ | 0.05 | 92.9 | 7.1 | 96.8 | 5.2 |
| | 5 | 81.2 | 5.2 | 105.1 | 12.8 |
| AA-EA | 0.05 | 93.2 | 3.5 | 98.2 | 9.8 |
| | 5 | 93.3 | 4.9 | 97.6 | 5.6 |
| 19(*R*)-HO-$PGE_1$ | | 153.3 | 3.8 | 97.5 | 0.8 |
| 19(*R*)-HO-$PGE_2$ | | 166.5 | 2.9 | 98.5 | 0.9 |

the ratio of the peak area of the analyte standard to the peak area of the internal standard (ISTD) against the concentration of the analyte standard. Only for the determination of 19(*R*)-HO-$PGE_1$ no suitable ISTD was available.

Linearity was investigated for matrix standards, matrix-matched standards and solvent standards. All concentrations of the calibration standards back-calculated from the generated calibration curve were within ±15% of the nominal value. Only in the lower linearity range did individual values <20% occur for some analytes. In each case, the individual values and no mean values were used for evaluation. A 20% criterion for the ratio of quantifier and qualifier ions was met by all analytes.

The method provided a wide linearity range (0.01 ng to 5 ng) for all analytes. Only for $PGD_2$ the dynamic range was limited to 0.01 ng to 2 ng.

The LLOQ was set to the lowest value of linearity at 0.01 ng due to the generic method approach. The signal-to-noise (S/N) values at this concentration are >28 for all matrix standards. For many analytes, much lower sample concentrations are quantifiable (see SI).

**Accuracy and precision.** All analytes showed good results for both with-in run and between-run accuracy and precision. Even at LOQ, the nominal mean values of accuracy and the CV values of precision were below 15%. Table 2 shows the measured values for between-run accuracy and precision. The results from with-in run accuracy and precision are summarized in Table IV of the SI in detail.

Since the sperm cell matrix had a high basal level of 19(*R*)-HO-$PGE_1$ and at the same time no deuterated standard was available as a possible alternative analyte for validation, the precision and accuracy at the low concentrations of 0.02 and 0.05 ng were not valid.

**Stability.** To test the autosampler stability of matrix samples, they were stored in the autosampler for 24 h at 10 °C after an initial injection and then re-injected. The evaluation was performed by comparing the absolute respectively relative areas (without/with ISTD). Percent recoveries of 96–99% (without ISTD) and 100% (with ISTD) were found. Autosampler stability is therefore given at 10 °C for at least 24 h.

**Capacity studies of the trap column.** To optimize the recovery of analytes on the trap column, compositions of the

Table 2 Between-run accuracy and precision (batch 1–3, $n = 15$)

| | | Content [ng/$10^6$ spermatozoa] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0.020 | | 0.050 | | 2 | | 5 | |
| Analyte | ISTD | Mean [%] | CV [%] | Mean [%] | CV [%] | Mean [%] | CV [%] | Mean [%] | CV [%] |
| 19(*R*)-HO-PGE$_1$ | — | 100.3 | 58.2 | 100.3 | 31.6 | 100.9 | 3.8 | 98.4 | 4.4 |
| 19(*R*)-HO-PGE$_2$ | PGE$_2$-d$_9$ | 102.4 | 9.7 | 103.5 | 5.1 | 96.3 | 5.4 | 95.0 | 2.7 |
| PGD$_2$ | PGD$_2$-d$_4$ | 101.1 | 3.3 | 102.5 | 2.1 | 98.2 | 2.6 | 91.7 | 3.0 |
| PGE$_1$ | PGE$_1$-d$_4$ | 98.0 | 6.4 | 99.9 | 4.2 | 98.4 | 4.1 | 96.1 | 2.4 |
| PGE$_2$ | PGE$_2$-d$_9$ | 100.9 | 4.7 | 101.9 | 3.0 | 99.0 | 4.1 | 96.0 | 1.9 |
| PGF$_{2\alpha}$ | PGF$_{2\alpha}$-d$_4$ | 99.3 | 5.9 | 99.1 | 4.0 | 97.4 | 4.1 | 95.8 | 2.1 |
| TXB$_2$ | TXB$_2$-d$_4$ | 106.9 | 7.4 | 103.2 | 6.8 | 98.3 | 2.5 | 95.7 | 3.2 |
| 15-HETE | 15-HETE-d$_4$ | 101.6 | 6.7 | 99.1 | 5.7 | 98.8 | 4.3 | 102.1 | 1.3 |
| 2-AG-d$_5$ | 2-AG d$_8$ | 98.9 | 9.7 | 99.8 | 5.0 | 97.5 | 5.2 | 101.2 | 6.0 |
| AA-d$_{11}$ | AA-d$_8$ | 112.1 | 11.1 | 109.3 | 6.2 | 98.3 | 2.5 | 100.6 | 1.3 |
| AA-EA | AA-EA-d$_8$ | 99.8 | 4.9 | 98.8 | 4.8 | 97.8 | 3.6 | 99.2 | 2.5 |

Table 3 Concentrations of selected analytes found in sperm using the methods described above (*measured value must be corrected for the matrix effect, as the quantification had to be carried out without ISTD; #measured value is outside the validated calibration range and has been extrapolated)

| Analyte | Range [ng/$10^7$ spermatozoa] |
|---|---|
| 15(*S*)-HETE | 0.030–0.096 |
| 19(*R*)-HO-PGE$_1$ | 0.134–1.30* |
| 19(*R*)-HO-PGE$_2$ | 0.0067–0.064* |
| 2-AG | 0.030–0.47 |
| AA | 17.4–51.2# |
| AA-EA | 0.032–0.090 |

mobile phase of pump 1 were varied as part of the method development. It was found that both eicosanoids and endocannabinoids including arachidonic acid were well retained on the trap column quite independently of the lipophilicity of the mobile phase of pump 1 when sufficient on-line dilution with aqueous mobile phase delivered by pump 2 was performed.

**Carry over.** Carry over was determined by injecting a solvent sample after a matrix, matrix matched and solvent calibration standard of high concentration and calculated as area percent. Prostaglandins showed no carry over, endocannabinoids and arachidonic acid only a low carry over of max. 0.06%.

**Sensitivity.** Since the amount of biological material is often limited for many determinations, the sensitivity of a method is an important criteria. LC-MS/MS detection can be used to detect smallest amounts of analytes. Nevertheless, the use of an MS detector is often not sufficient to reach the desired concentration ranges. For this purpose, smart sample preparation for purification must be additionally combined with enrichment.

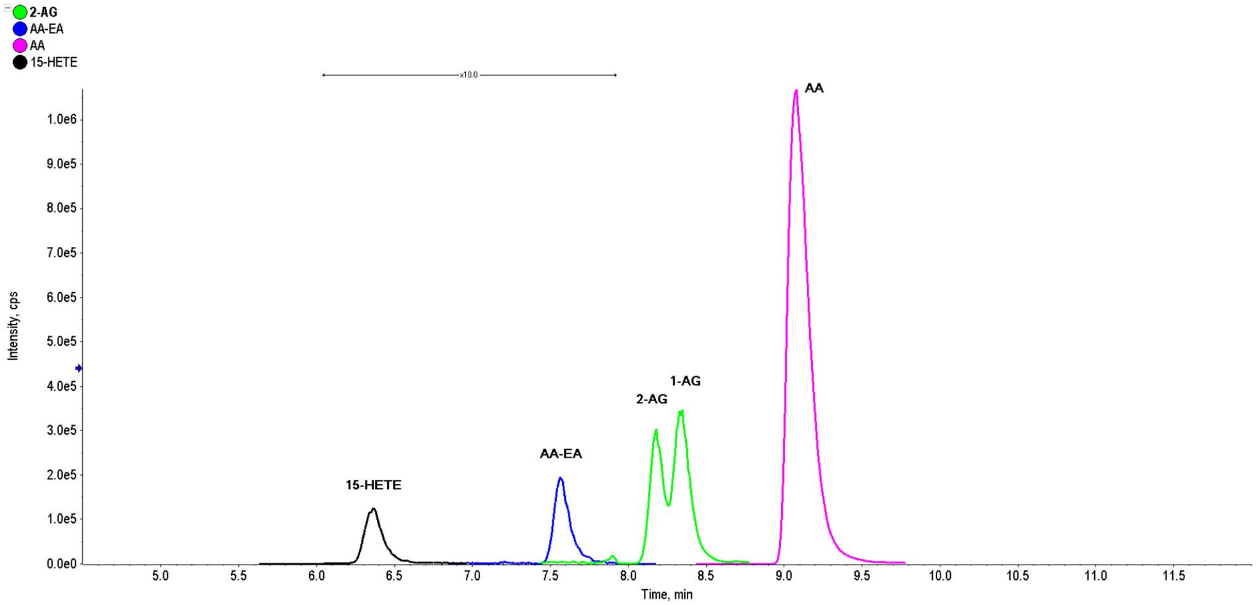

Fig. 3 Example ion chromatogram of processed sperm measured with method 2 (10× magnification of 15-HETE and AA-EA); spontaneous isomerisation of 2-AG to 1-AG is known from the literature,[69] both peaks are taken into account for quantification.

With the on-line dilution on-line SPE-LC-MS system presented here, injection volumes of 150 μL could be realized without loss of chromatographic separation efficiency. The high injection volume significantly increases the sensitivity of the method.

The S/N ratio of the matrix standard in the linearity tests indicates the low detection limit and high sensitivity of the method. At a concentration of 0.01 ng of analyte per $1 \times 10^6$ spermatozoa, the signal-to-noise values ranged from $S/N = 28$ for arachidonic acid to $S/N = 600$ for 19(*R*)-HO-PGE$_2$, depending on the analyte.

**Application of the method.** The on-line dilution on-line SPE-LC-MS/MS method described above was used in this research project to quantitatively determine the basal levels and profile of selected eicosanoids, endocannabinoids and arachidonic acid in sperm cells. For this purpose, carefully washed sperm pellets were analysed.

The analytes 2-AG, AA-EA, AA, 15(*S*)-HETE, 19(*R*)-HO-PGE$_1$ and 19(*R*)-HO-PGE$_2$ were found in the sperm cells. Other eicosanoids were only detected in very low concentrations or were completely absent (Table 3 and Fig. 3).

# Conclusions

So far, not much is known about the exact role of eicosanoids and endocannabinoids in sperm. For good profiling, a sensitive, selective and precise LC-MS/MS method is required due to the low concentrations of the analytes mentioned and the often limited availability of biological material.

Literature-known determination methods for eicosanoids, endocannabinoids and fatty acids in biological matrices often used LLE or off-line SPE with time-consuming and thermally stressful enrichment for sample preparation.

With the on-line dilution on-line SPE LC-MS/MS method presented here, a fast and valid analysis was possible despite a very simple "dilute and shoot" approach. Protein precipitation followed by centrifugation and direct injection of the sample onto a trap column were the few necessary sample preparation steps.

Despite this simple sample preparation, all validation parameters are very convincing in terms of linearity, precision, accuracy, recovery and matrix effect. On-line dilution with on-line SPE has proven to be an elegant analytical route with many advantages.

The eicosanoids, endocannabinoids and fatty acids quantified here are a selection based on previous studies in the literature. Despite the very different physicochemical properties of the analytes, all could be reliably quantified using an easily adaptable generic on-line dilution SPE-LC-MS/MS method. Therefore, no difficulties are expected in transferring the method to other lipid mediators. The methods were successfully used to detect or determine the basal levels of eicosanoids, endocannabinoids and arachidonic acid in human spermatozoa. Remarkably, some prostaglandins have not been found in significant concentrations in sperm, although their presence has been hypothesised in the literature.[10,11]

The lipid mediator profiling method presented here should help to identify potentially important lipid biomarkers in sperm and other biological matrices in the future and to better understand important metabolic pathways.

# Author contributions

Conceptualization: JF, ML. Investigation: JF. Methodology: JF. Project administration: JF, ML. Resources: ML. Supervision: ML. Validation: JF. Visualization: JF. Writing – original draft: JF, ML.

# Conflicts of interest

There are no conflicts to declare.

# Data availability

The data supporting this article have been included as part of the SI. See DOI: **https://doi.org/10.1039/d5ay00250h**.

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