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Assessment of Dried Blood Micro-Sampling Methods for Oxylin Analysis in Newborn Blood Using UPLC-MS/MS

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Abstract

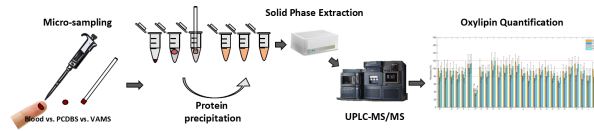
Background: Conventional sampling methods for clinical analysis make use of plasma or serum and require large blood volumes, cold storage, and specialized handling. This renders them impractical and invasive, especially when dealing with vulnerable groups of patients and the analysis of low-abundant and unstable compounds such as oxylin. Dried blood micro-sampling methods are designed for collecting minimally invasive small-volume blood samples (<100 µL), providing advantages in stability, handling, and storage. The aim of this study is to develop and compare micro-sampling approaches that can be readily implemented in clinical analysis circumventing the barriers of conventional sampling methods. (96)

Results: This study assessed the performance of Pre-Cut Dried Blood Spots (PCDBS) and Volumetric Absorptive Micro-Sampling (VAMS[®]) devices for determining oxylin, using small-volume liquid whole blood sampling as reference. A Liquid Chromatography - tandem Mass Spectrometry method was successfully developed and validated. Both PCDBS and VAMS[®] were prepared using 30 µL of spiked blood and dried for two hours before extraction and analysis. Optimal results were achieved by pre-treating devices with antioxidant before sample deposition and avoiding the internal standard to

undergo evaporation steps during preparation of micro-sampling devices. Long-term storage effects were assessed over a three-month period, and the micro-sampling devices were tested on umbilical cord blood samples from 35 newborns. For PCDBS and VAMS[®], eight and nine oxylipins, respectively, showed comparable results to those obtained from liquid whole blood samples with mean concentrations ranging between 0.7 and 6.3 nM. (140)

Significance: This study is the first to analyse oxylipins in umbilical cord blood using VAMS[®] and PCDBS micro-sampling devices, demonstrating their effectiveness for quantification at nM concentrations. It highlights the impact of sample collection, storage, and internal standard handling on lipid profiles. Stability is critical and cold storage (-20°C) is mandatory. Future research focusing on the standardization of protocols for comparability among laboratories and enhancing sensitivity with improved LC-MS/MS techniques to expand accessible oxylipins are needed. (75)

Keywords: *Micro-extraction, oxylipins, Dried blood spot, Volumetric Absorptive Micro-Sampling[®], Ultra-high performance liquid chromatography-electrospray ionization tandem mass spectrometry, inflammation biomarkers*



Journal Pre-proof

1. Introduction

The determination of oxylipins in blood is in the spotlight due to their key role in the onset and development of inflammatory processes. Oxylipins are oxygenated metabolites derived from the oxidation of polyunsaturated fatty acid precursors such as ω -3 fatty acids (FAs) (*i.e.*, docosahexaenoic acid, eicosapentaenoic acid, and α -linolenic acid) and ω -6 FAs (*i.e.*, arachidonic acid, adrenic acid, and linoleic acid). Prostaglandins, specialized pro-resolving mediators, such as maresins and protectins, and other mono-, di-, or tri-hydroxylated oxylipins are mainly formed via the cyclooxygenase, lipoxygenase (5-LOX and 12/15-LOX), and cytochrome P450 enzymatic pathways [1,2]. In addition, oxylipins derived from ω -6 FAs tend to increase inflammation, hypertension, and platelet aggregation, whereas those derived from ω -3 FAs exhibit anti-inflammatory, antiaggregatory, and vasodilatory effects [3]. In addition to their biological relevance, oxylipins exhibit notable instability, as they are highly susceptible to degradation under uncontrolled environmental conditions, particularly temperature fluctuations. This sensitivity poses a significant challenge for their analysis and preservation in clinical and biochemical studies, and it has recently been shown that repeated freeze-thaw cycles can alter their quantitative profile [4]. Accurate and precise quantification of oxylipins, along with proper identification and reporting, is essential for achieving clinical translation of biomarkers. Since certain oxylipins occur at very low endogenous levels, their measurement must strictly follow established best practices [5].

Conventional methods for quantifying endogenous oxylipins rely on plasma and serum samples. However, their applicability in clinical settings faces significant limitations, particularly due to the invasiveness of sample collection, which is especially challenging in vulnerable populations. Blood sampling from preterm infants requires the use of whole blood (WB) volumes below 500 μ L. The lack of advanced sample pre-treatment instrumentation, cold storage facilities, and adequately trained personnel in these settings further complicates the successful execution of both research studies and clinical analysis.

Dried blood micro-sampling methods enable the collection of small blood samples (<100 μ L), eliminating the need for ultra-cold storage (*e.g.*, -80°C) and improving sample stability suitable for both, targeted and untargeted metabolomics [6,7]. Additionally, this approach simplifies sample transportation and significantly reduces storage space requirements. Among the various dried blood micro-sampling techniques developed, Dried Blood Spots (DBS) remain the first and most widely used method. It involves the deposition of WB samples onto high-quality filter paper [6]. However, several factors, such as haematocrit (HCT), blood viscosity, analyte physicochemical properties, the

substrate of the DBS card, the paper chromatography (volcano) effect, spotting technique and conditions, and environmental conditions (e.g. temperature and humidity), contribute to variability in spot size and analyte distribution [8,9]. To overcome this issues, Pre-Cut Dried Blood Spot (PCDBS) techniques have been proposed as they allow the collection of accurate blood volumes, reducing variability associated with blood HCT [10,11]. In recent years, Volumetric Absorptive Micro-Sampling (VAMS[®]) has been introduced in Europe and the UK as a CE-IVD certified, commercially available product. The technique uses a sampler with a porous hydrophilic tip that enables the collection of small, accurate, and precise blood volumes of 10, 20, or 30 μL with less than 5% volumetric variation across a HCT range of 20 to 70% [12]. The use of VAMS[®] and PCDBS has been evaluated for venous micro-sampling in untargeted lipidomics studies, with findings indicating that VAMS[®] offers advantages in terms of HCT independence and overall reliability, however, both PCDBS and VAMS[®] have been shown to be viable and accessible options for lipid analysis [13].

The addition of stable isotope-labelled internal standards (ISs) is crucial in DBS-based bioanalysis, as it markedly enhances the accuracy and robustness of quantitative results in MS-based assays. These ISs effectively correct for variability during extraction and ionization processes and compensate for matrix effects and inconsistencies in sample volume or spot homogeneity. As highlighted by Van Baar et al., the co-spotting of isotope-labelled standards on the DBS card prior to extraction ensures that both the analyte and IS undergo the same conditions throughout the workflow, thereby improving method reliability [14]. When analysing liquid samples like serum or plasma, ISs are typically spiked into the sample before preparation and analysis. In contrast, for DBS methods, IS are added either to the extraction solvent or applied directly to the DBS sample before extraction. To enhance the accuracy of analytical methods, alternative procedures may be necessary, such as pre-treating micro-sampling devices with IS before blood addition, or the addition of IS to WB samples immediately after blood collection [15–17].

This study aimed to compare for the first time the performance of PCDBS and VAMS[®] sampling devices with conventional liquid small-volume WB samples for the determination of oxylipins by Liquid Chromatography - tandem Mass Spectrometry (LC-MS/MS). Additionally, the study assessed the effects of various factors on method performance, including different procedures for adding ISs, antioxidant stabilization with butylated hydroxytoluene (BHT), and solvent removal. Long-term storage stability was evaluated over a three-month period, and the method was applied to 35 umbilical cord blood (UCB) samples obtained in the delivery room to evaluate its performance in the context of a clinical study.

2. Materials and methods

2.1. Standards and reagents

All solvents used were HPLC grade including methanol and ethyl acetate from Labkem-Labbox (Barcelona, Spain), acetonitrile from Scharlab (Barcelona, Spain) and ethanol from Merck (Darmstadt, Germany). Formic acid (98% purity) was purchased from Panreac (Barcelona, Spain). Water was ultrapure grade water generated on a Milli-Q® integral system from Merck (Darmstadt, Germany). Dibutylhydroxytoluene (BHT) was obtained from Merck (Darmstadt, Germany).

Oxylipin standards included two derived from linoleic acid oxidation (12,13-DiHOME and 9,10-DiHOME), three from eicosapentanoic acid oxidation (18-HEPE, 14,15-DiHETE, 17,18-DiHETE), thirteen derivatives from arachidonic acid (8,15-DiHETE, LTB₄, PGF_{2α}, PGE₂, TXB₂, 14,15-EET, 11,12-EET, 8,9-EET, 5,6-EET, 8,9-DiHET, 14,15-DiHET, 5,6-DiHET, and 11,12-DiHET) and eight from docosahexaenoic acid (14-HDHA | 14-HDoHE, 17-HDHA | 17-HDoHE, Maresin-1, Maresin-2, Resolvin-D5, Neuroprotectin D1 (NPD1/PD1), 19,20-DiHDPA, and PDX), purchased from Cayman Chemicals (Ann Arbor, MI). In addition, two oxylipins derived from α-linolenic acid (9(R),16(S)-Linotrin and 9(R),16(R)-Linotrin), one from DHA (dinor-NPD1), two from docosapentaenoic acid (10(S),17(R)-diH n-6 DPA_{EEZ} and 10(S),17(R)-diH n-3 DPA_{EEZ}) and one from adrenic acid oxidation (10(S),17(R)-diH AdA_{EEZ}) were synthesized by *Synthèse de lipides bioactifs* group at *Institut des Biomolécules Max Mousseron* (Montpellier, France) [18–21].

The following deuterated internal standards were obtained from Cayman Chemicals: 12,13-DiHOME-d₄, 9,10-DiHOME-d₄, 12(S)-HETE-d₈, LTB₄-d₄, PGF_{2α}-d₄, 14,15-DiHET-d₁₁, Maresin 2-d₅, 11,12-DiHET-d₁₁, 8,9-DiHET-d₁₁, 11,12-EET-d₁₁, 8,9-EET-d₁₁, and 5,6-EET-d₁₁.

2.2. Sample collection and processing

This study was conducted in compliance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee for Biomedical Research of the Health Research Institute La Fe (Valencia, Spain) with approval number 2024-0754-1. Written informed consent was obtained from all study participants.

UCB was collected immediately after delivery from term infants at the University and Polytechnic Hospital La Fe. A sterile needle was inserted into the umbilical vein, allowing the blood to flow into an EDTA-coated tube to prevent coagulation. The tube was then gently inverted to ensure proper mixing of the anticoagulant and either processed immediately or stored at 4°C for up to one hour before oxylipin extraction and analysis. Micro-sampling devices employed for blood collection were VAMS®, 30 µL Quad-

Sampler devices (Neoteryx Mitra Clamshell, Torrance, CA, USA) and PCDBS with a diameter of 6 mm prepared by cutting filter paper cards (Revvity, Inc., Waltham, MA, USA) and dry micro-sampling tubes.

For method development, a 2 mL UCB aliquot was spiked with 200 μ L of a 250 nM oxylipin working solution containing all non-labelled oxylipins in $\text{CH}_3\text{OH}:\text{CH}_3\text{CN}$ (50/50, v/v). Spiked samples were homogenised (Vortex, 30 s), and VAMS[®] devices were impregnated with 30 μ L of blood, while another 30 μ L of blood were pipetted onto PCDBS cards or into micro-sampling tubes for further analysis. All sampling procedures were performed by triplicate, and samples were allowed to air dry at room temperature for two hours before extraction. 5 μ L of IS solution containing 12,13-DiHOME-d₄, 9,10-DiHOME-d₄, 12(S)-HETE-d₈, LTB₄-d₄ (0.5 μ M), PGF_{2 α} -d₄, 14,15-DiHET-d₁₁, Maresin 2-d₅, 11,12-DiHET-d₁₁, 8,9-DiHET-d₁₁, 11,12-EET-d₁₁, 8,9-EET-d₁₁, and 5,6-EET-d₁₁ (1.25 μ M) in $\text{CH}_3\text{OH}:\text{CH}_3\text{CN}$ (50/50, v/v) and/or 5 μ L of BHT (10 mg mL⁻¹ in $\text{CH}_3\text{CH}_2\text{OH}$) were added to correct potential matrix effects and prevent oxylipin oxidation, respectively. Different approaches for the addition of ISs and BHT were investigated across all three devices (VAMS[®], PCDBS or dry micro-sampling tube) as shown in **Figure 1**: (1) addition of IS solution and BHT to the liquid WB sample before application to the devices: no evaporation of IS or BHT, and no drying or storage of samples; (2) addition of ISs and BHT to the WB sample on the devices after drying and 2h of storage at RT: to evaluate the effect of drying and storage, in comparison to Approach 1; (3) evaporation of the ISs on VAMS[®], PCDBS or a dry micro-sampling tube, followed by addition of the WB sample, drying and 2h of storage at RT, and addition of BHT onto the device: to assess the impact of evaporating IS (but not BHT) onto the devices prior to sample addition, rather than before extraction, compared to Approach 2; (4) evaporation of BHT onto the devices, followed by addition of the WB sample, drying and 2h of storage at RT, and the addition of IS onto the device: to assess the impact of evaporating BHT (but not IS) onto the devices prior to sample addition, instead of before extraction, compared to Approach 2; and (5) evaporation of the ISs and BHT onto the devices followed by addition of the WB sample and drying and 2h of storage at RT: to evaluate the combined effect of evaporating both BHT and IS onto the micro-sampling devices before sample addition, in contrast to evaporating them prior to extraction as in Approach 2. WB processing as described for micro-sampling tubes within Approach 1 was selected as the reference method for comparison against results obtained with all other approaches (i.e., PCDBS and VAMS[®] in Approach 1, and micro-sampling tube, PCDBS, and VAMS[®] in Approaches 2 to 5).

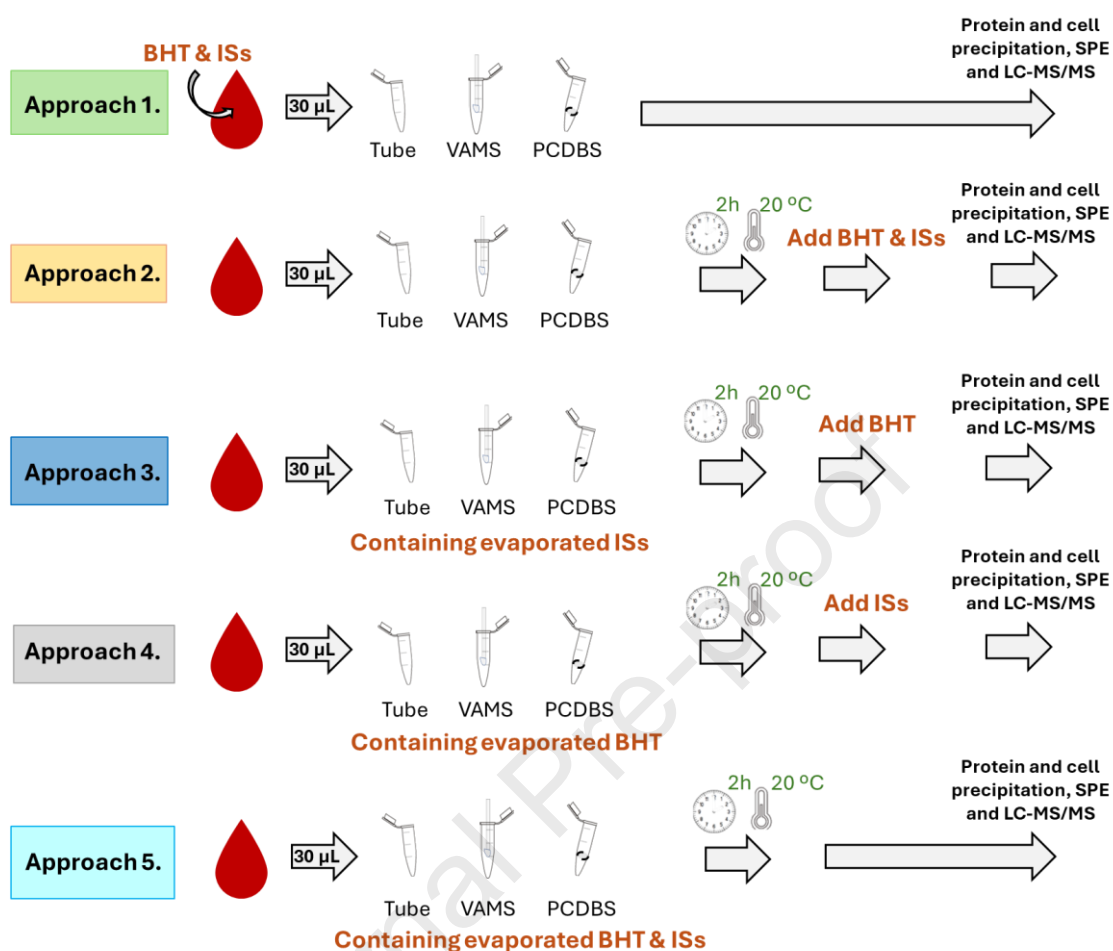


Figure 1. Schematic overview of the experimental setup used to compare micro-sampling devices and sample collection approaches. Note: Experimental details are described in the text.

The stability assay of oxylipins was conducted using a 3 mL UCB sample spiked with 0.5 mL of a mixture of 175 nM of each oxylipin in CH₃OH:CH₃CN (50/50, v/v). Samples were collected in PCDBS and VAMS[®], and were stored under three different conditions: at room temperature (RT), 4 °C, and -20 °C. At each specified time point (3, 7, and 14 days, and 3 months), the samples were extracted and subsequently stored at -80 °C until all samples could be analysed in the same batch. This approach was implemented to minimize batch effects and ensure consistent analytical results.

35 UCB samples were collected. For each UCB sample, WB was divided into three aliquots of 30 µL each. One aliquot was transferred into a tube, extracted, and stored at -80 °C until analysis. The second aliquot was applied onto a PCDBS, and the third aliquot onto a VAMS[®], both following approach 4 (see **Figure 1**). Both PCDBS and VAMS[®] samples were stored at -20 °C until extraction and further analysis.

2.3. Solid Phase Extraction (SPE) procedure

For protein and cell precipitation, 150 μL of cold solvent (CH_3OH with 0.1% v/v HCOOH) were added to each micro-sampling device. The tubes were sonicated for 10 min, followed by incubation on a thermal mixer at 4 $^\circ\text{C}$ and 800 rpm for 10 min. The samples were then centrifuged at 4000 $\times g$ for 10 min at 4 $^\circ\text{C}$. The resulting supernatants were diluted with 600 μL of H_2O prior to SPE. SPE employing Oasis HLB SPE 96-well plates (60 mg, Waters, Milford, MA, USA) was carried out as follows: SPE cartridges were conditioned with 1 mL ethyl acetate followed by 1 mL CH_3OH and 1 mL of H_2O (0.1% HCOOH , 5 % CH_3OH). Then, diluted supernatants were loaded into the SPE wells. The washing step consisted in rinsing each well with 1 mL H_2O (0.1% HCOOH , 5% CH_3OH). Cartridges were dried with room air and sample extracts were eluted with 250 μL of CH_3OH and 3 \times 250 μL of ethyl acetate which were subsequently evaporated using a miVac centrifugal vacuum concentrator (Genevac LTD, Ipswich, UK). The obtained residues were redissolved in 50 μL of $\text{CH}_3\text{OH}:\text{CH}_3\text{CN}$ (50/50, v/v) prior to UPLC-MS/MS analysis [19].

2.4. LC-MS/MS method for oxylipin analysis in WB samples

External calibration standards were prepared from serial dilution of the working solution in $\text{CH}_3\text{OH}:\text{CH}_3\text{CN}$ (50/50, v/v) (see concentrations in Table 1). Calibration standards and sample extracts were analysed using an Acquity-Xevo TQ-XS UPLC-MS instrument from Waters (Milford, MA, USA), operating in the negative electrospray (ESI-) mode. The source temperature was set to 150 $^\circ\text{C}$, while the desolvation temperature was set to 600 $^\circ\text{C}$. The nitrogen cone and desolvation gas flows were adjusted to 150 and 1000 L h^{-1} , respectively.

Separations were carried out using a Waters Acquity UPLC BEH C18 column (2.1 \times 100 mm; 1.7 μm particle size). A binary gradient system was employed, consisting of 0.1% v/v CH_3COOH as mobile phase component A, and a mixture of CH_3CN and isopropanol (in a ratio of 90:10 v/v) as mobile phase component B. The flow rate was set at 0.6 mL min^{-1} , while the column temperature and injection volume were maintained at 40 $^\circ\text{C}$ and 5 μL , respectively. The gradient elution method had a total runtime of 10.0 min. During the first minute, the composition of mobile phase B was set at 75% v/v. From 1.0 to 8.0 min, the percentage of mobile phase B gradually increased up to 95%. Between 8.0 and 8.5 min, the conditions were kept constant with 95% B, followed by a return to the initial conditions (*i.e.*, 75% B) between 8.51 and 10.0 min maintaining those conditions for 1.5 min for system re-equilibration.

2.5. Validation of UPLC-MS/MS oxylipin quantification

For method validation (reference method; approach 1 in tube), selectivity was assessed by injecting individual pure analytical standard solutions once, when available. The

lower limit of quantification (LLOQ) was defined as the lowest concentration at which the analyte's quantification showed a relative standard deviation (RSD) below 20% and a deviation from the expected value within $\pm 20\%$. The detection limit (LOD) was estimated as 3/10 of the LLOQ in standard solutions. Linear regression curves were constructed using the relative peak area of each compound to its corresponding IS, with a linearity acceptance criterion requiring a correlation coefficient >0.95 . Additionally, back-calculated standard concentrations were required to be within $\pm 15\%$ of the nominal value, with RSDs $<15\%$.

Intra-day and inter-day precision and accuracy were evaluated by analyzing individual oxylipins in standards and spiked WB samples (i.e. addition of standard solutions to UCB samples before extraction) in triplicate at three concentration levels (i.e., 25, 50, and 100 nM in extracts) across three different days. The acceptance criterion for precision, both within and between batches, was an RSD lower than $<20\%$. Recovery was expressed as a percentage, calculated using the formula: $\text{Recovery (\%)} = 100 \times (\text{Concentration in spiked sample} - \text{Concentration in unspiked sample}) / \text{Concentration of the addition}$. This approach allows for the evaluation of the method's ability to recover the analyte from the matrix after the addition of a known concentration. Acceptable recoveries ranged between 80 and 120% with RSDs $<20\%$. The matrix effect in WB across the three micro-sampling devices was assessed by spiking the extracts obtained after SPE at a single concentration level with a working solution in $\text{CH}_3\text{OH}:\text{CH}_3\text{CN}$ (50/50, v/v) to obtain concentration levels of 50 nM in extracts. It was calculated and expressed as percentage, using the formula: $100 \times (\text{Area spiked} - \text{Area non-spiked}) / \text{Area standard}$. Carry-over was evaluated by analyzing a solvent blank immediately after the analysis of a concentrated 225 nM oxylipin standard solution prepared in $\text{CH}_3\text{OH}:\text{CH}_3\text{CN}$ (50/50, v/v).

2.6. Data processing and statistics

For operation of the UPLC-MS/MS system and peak integration, MassLynx software version 4.2 from Waters was employed. Further data analysis was carried out using Matlab 2021a from Mathworks Inc. (Nattick, MA, USA), and the PLS Toolbox 9.1 from Eigenvector Research Inc. (Wenatchee, WA, USA) for Principal Component Analysis (PCA). Data were autoscaled and leave-one-out cross validation was used for the estimation of internal figures of merit.

3. Results and discussion

3.1. UPLC-MS/MS determination of oxylipins in WB

Table 1 summarizes the selected measurement conditions, ISs employed for linear regression curves, and retention times for the 31 oxylipins included in the quantitative

oxylipin method, selected based on previous results [22]. Oxylipins eluted with retention times varying between 2.90 and 6.43 min under the selected conditions. Retention times were highly reproducible for the whole set of oxylipins and ISs with standard deviations $\leq \pm 0.03$ min across analytical batches analysed on different days. Table 1 summarizes results obtained during the validation of oxylipin quantification. Linear regression curves spanning four orders of magnitude were obtained with R^2 values ≥ 0.990 . LODs and the LLOQs in standard solutions ranged between 0.13 and 0.3 nM and 0.4 and 0.9 nM, respectively, and LLOQs in WB samples ranged between 0.7 and 2 nM. No carry-over was observed for any analyte, as shown by peak areas in blanks analysed after a highly concentrated standard (*i.e.*, $< 5\%$ of the peak area value of a 225 nM standard solution). Table 1 shows figures of merit for the assessment of intra- and inter-day accuracy and precision. Accuracies within the established acceptance criteria as specified in '2.5 Validation of UPLC-MS/MS oxylipin quantification' were obtained in standard solutions for most oxylipins (*i.e.*, 77% at low, 100% at medium, and 100% at high concentration levels) with intra-day and inter-day accuracies ranging between 84 and 116% and 80 and 120%, respectively. Precision was evaluated for intra-day and inter-day ranging between 1 to 20% for 90% of assayed oxylipins across all concentration levels tested.

The matrix effect was evaluated in the three micro-sampling devices at one single concentration level of 50 nM in the extract obtained after SPE elution. No matrix effects (recoveries between 80 and 119%) were observed for most compounds, *i.e.*, 95, 86, and 90% of tested compounds in dry micro-sampling tube, VAMS, and PCDBS, respectively. For the remaining compounds recoveries ranged between 73 and 129%, indicating moderate matrix effects. Supplementary Table 1 details the results obtained from the evaluation of the matrix effect.

Intra-day and inter-day precision and accuracy were evaluated in spiked WB samples in triplicate at three concentration levels over three different days. Inter and intra-day accuracy ranged between 81 and 119% and 80 and 120%, respectively, falling within acceptance limits for 90%, 87%, and 90% of oxylipins at low, medium, and high concentration levels, respectively. Appropriate precision levels with $RSD < 20\%$ were obtained for 87% of compounds studied. However, low inter-day recoveries ($\leq 75\%$) were observed for dinor-NPD1, 10S,17R-diH AdA_{EEZ}, and 14,15-EET. As a result, these analytes were excluded from further analysis of blood samples to ensure accurate results. For the remaining 28 oxylipins originally included in the method, results demonstrate that the developed method is suitable for their quantification in UCB.

Table 1. Acquisition parameters and main figures of merit of the LC-MS/MS method. Intra- and inter-day accuracies and precisions of standard solutions and spiked UBC samples.

Compound Name	Internal Standard	RT (min)	Range (nM)	R ²	LOD (nM)	LLOQ (nM)	LLOQ* (nM)	Inter-day (Standard) Recovery ± RSD (%)			Intra-day (Standard) Recovery ± RSD (%)			Inter-day (Sample) Recovery ± RSD (%)			Intra-day (Sample) Recovery ± RSD (%)		
								Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
TXB ₂	14,15-DiHET-d ₁₁	2.90 ± 0.02	0.9-225	0.991	0.3	0.9	2.0	125 (18)	102 (13)	95 (6)	98 (7)	87 (24)	89 (11)	104 (13)	112 (6)	98 (14)	98 (5)	81 (7)	85 (1)
PGF _{2α}	PGF _{2α} -d ₄	3.14 ± 0.02	0.4-225	0.995	0.13	0.4	0.7	84 (16)	111 (7)	94 (11)	91 (7)	104 (14)	83 (11)	104 (8)	100 (14)	90 (4)	94 (12)	88 (19)	88 (9)
PGE ₂	PGF _{2α} -d ₄	3.21 ± 0.02	0.4-225	0.997	0.13	0.4	0.7	91 (1)	110 (5)	94 (13)	91 (7)	104 (15)	80 (18)	100 (11)	87 (8)	86 (4)	88 (10)	82 (15)	89 (10)
9(R)-16(RS)-Linotrin	12-HETE-d ₈	3.50 ± 0.02	0.4-225	0.995	0.13	0.4	0.7	106 (10)	114 (3)	98 (5)	98 (9)	109 (8)	101 (5)	84 (9)	94 (15)	91 (9)	99 (20)	79 (7)	85 (2)
dinor-NPD1	12-HETE-d ₈	3.803 ± 0.012	0.9-225	0.994	0.3	0.9	2.0	100 (12)	108 (2)	100 (6)	115 (15)	110 (12)	106 (14)	97 (6)	94 (8)	87 (13)	62 (6)	72 (14)	68 (8)
10(S),17(R)-diH n-3 DPA _{EEZ}	12-HETE-d ₈	4.273 ± 0.012	0.9-225	0.991	0.3	0.9	2.0	106 (16)	116 (1)	99 (6)	129 (2)	118 (3)	106 (12)	99 (15)	94 (13)	87 (9)	107 (5)	81 (9)	80 (3)
NPD-1	12-HETE-d ₈	4.33 ± 0.03	0.4-225	0.991	0.13	0.4	0.7	75 (15)	111 (3)	98 (4)	88 (9)	115 (5)	100 (6)	95 (13)	104 (10)	97 (8)	95 (11)	96 (13)	89 (2)
17,18-DiHETE	14,15-DiHET-d ₁₁	4.35 ± 0.02	0.4-225	0.992	0.13	0.4	0.7	99 (11)	110 (5)	94 (9)	106 (9)	107 (12)	84 (17)	109 (11)	110 (18)	110 (19)	102 (7)	87 (8)	87 (4)
Resolvin D5	8,9-DiHET-d ₁₁	4.38 ± 0.02	0.9-225	0.992	0.3	0.9	2.0	98 (7)	104 (6)	106 (1)	120 (10)	111 (10)	107 (10)	95 (16)	86 (7)	95 (13)	111 (10)	93 (3)	108 (8)
PDX	12-HETE-d ₈	4.38 ± 0.02	0.4-225	0.992	0.13	0.4	0.7	95 (18)	112 (3)	96 (4)	80 (9)	115 (6)	95 (1)	107 (4)	99 (11)	96 (12)	96 (11)	87 (10)	85 (2)
Maresin 1	12-HETE-d ₈	4.40 ± 0.02	0.4-225	0.991	0.13	0.4	0.7	114 (0)	113 (11)	101 (0)	114 (10)	113 (20)	101 (7)	107 (16)	95 (23)	99 (6)	95 (4)	80 (2)	94 (14)
8(S),15(S)-DiHETE	8,9-DiHET-d ₁₁	4.473 ± 0.012	0.4-225	0.992	0.13	0.4	0.7	113 (19)	115 (10)	100 (6)	87 (7)	105 (11)	95 (5)	103 (5)	101 (5)	97 (1)	109 (5)	97 (9)	98 (7)
14,15-DiHETE	14,15-DiHET-d ₁₁	4.473 ± 0.012	0.4-225	0.990	0.13	0.4	0.7	104 (13)	108 (4)	94 (11)	94 (9)	103 (10)	84 (15)	117 (12)	107 (9)	114 (16)	109 (6)	96 (7)	102 (5)
LTB ₄	LTB ₄ -d ₄	4.51 ± 0.02	0.4-225	0.994	0.13	0.4	0.7	105 (16)	112 (2)	96 (6)	86 (8)	115 (9)	90 (9)	104 (5)	104 (10)	109 (15)	111 (9)	99 (15)	95 (4)
12,13-DiHOME	12,13-DiHOME-d ₄	4.593 ± 0.012	0.4-225	0.992	0.13	0.4	0.7	114 (20)	108 (7)	95 (8)	91 (8)	109 (6)	87 (8)	117 (11)	95 (8)	93 (9)	105 (11)	90 (12)	88 (4)
10(S),17(R)-diH n-6 DPA _{EEZ}	12-HETE-d ₈	4.65 ± 0.02	0.9-225	0.990	0.3	0.9	2.0	102 (19)	113 (3)	97 (5)	122 (5)	110 (7)	94 (3)	102 (17)	94 (12)	95 (11)	102 (9)	81 (10)	85 (1)
Maresin 2	Maresin 2-d ₅	4.703 ± 0.012	0.9-225	0.990	0.3	0.9	2.0	104 (13)	114 (15)	97 (5)	117 (5)	103 (9)	91 (4)	97 (6)	105 (6)	94 (8)	93 (16)	86 (2)	89 (9)
9,10-DiHOME	9,10-DiHOME-d ₄	4.73 ± 0.02	0.4-225	0.993	0.13	0.4	0.7	95 (6)	109 (3)	97 (5)	98 (8)	108 (10)	92 (8)	102 (2)	97 (6)	109 (6)	103 (15)	93 (18)	88 (7)
10(S),17(R)-diH AdA _{EEZ}	8,9-DiHET-d ₁₁	4.793 ± 0.012	0.9-225	0.991	0.3	0.9	2.0	98 (18)	110 (1)	93 (9)	123 (23)	110 (14)	85 (8)	81 (10)	87 (23)	97 (24)	75 (9)	73 (4)	71 (6)
19,20-DiHDPA	14,15-DiHET-d ₁₁	4.810 ± 0.010	0.4-225	0.993	0.13	0.4	0.7	96 (17)	109 (4)	95 (8)	83 (9)	106 (11)	86 (14)	101 (2)	97 (11)	103 (20)	100 (8)	87 (8)	88 (6)
14,15-DiHET	14,15-DiHET-d ₁₁	4.82 ± 0.02	0.4-225	0.990	0.13	0.4	0.7	101 (11)	108 (3)	96 (9)	98 (8)	110 (11)	87 (10)	109 (4)	106 (13)	103 (9)	106 (3)	92 (7)	87 (3)
11,12-DiHET	14,15-DiHET-d ₁₁	5.00 ± 0.02	0.4-225	0.990	0.13	0.4	0.7	90 (3)	112 (3)	94 (5)	86 (9)	109 (14)	89 (8)	109 (8)	109 (15)	99 (11)	111 (16)	92 (15)	91 (6)
18-HEPE	12-HETE-d ₈	5.123 ± 0.012	0.4-225	0.995	0.13	0.4	0.7	106 (23)	113 (1)	96 (5)	91 (9)	114 (7)	94 (6)	105 (6)	115 (11)	105 (12)	110 (14)	101 (12)	96 (3)
8,9-DiHET	8,9-DiHET-d ₁₁	5.16 ± 0.02	0.4-225	0.992	0.13	0.4	0.7	84 (11)	110 (4)	98 (9)	77 (8)	113 (7)	92 (4)	98 (3)	99 (9)	89 (3)	101 (12)	90 (9)	86 (2)
5,6-DiHET	14,15-DiHET-d ₁₁	5.39 ± 0.02	0.9-225	0.990	0.3	0.9	2.0	110 (12)	111 (4)	92 (12)	130 (6)	110 (12)	81 (15)	105 (10)	104 (12)	94 (14)	105 (4)	90 (7)	87 (5)
17-HDHA 17-HDoHE	12-HETE-d ₈	5.65 ± 0.02	0.4-225	0.993	0.13	0.4	0.7	114 (20)	107 (4)	95 (6)	91 (8)	109 (8)	90 (8)	104 (9)	100 (13)	93 (14)	94 (9)	89 (19)	85 (3)
14-HDHA 14-HDoHE	Maresin 2-d ₅	5.743 ± 0.012	0.4-225	0.992	0.13	0.4	0.7	100 (13)	108 (3)	95 (9)	93 (9)	107 (9)	89 (7)	86 (13)	85 (4)	87 (23)	89 (25)	91 (22)	94 (15)
14,15-EET	11,12-EET-d ₁₁	6.12 ± 0.02	0.4-225	0.996	0.13	0.4	0.7	107 (11)	112 (8)	97 (7)	92 (7)	104 (8)	91 (9)	69 (9)	79 (12)	69 (14)	73 (8)	58 (9)	61 (5)
11,12-EET	11,12-EET-d ₁₁	6.30 ± 0.02	0.4-225	0.992	0.13	0.4	0.7	114 (19)	115 (6)	95 (9)	93 (7)	110 (12)	88 (12)	97 (5)	100 (9)	87 (1)	97 (15)	92 (9)	87 (2)
8,9-EET	8,9-EET-d ₁₁	6.35 ± 0.02	0.9-225	0.990	0.3	0.9	2.0	106 (16)	104 (7)	96 (8)	87 (8)	112 (12)	89 (14)	119 (5)	114 (10)	112 (10)	120 (17)	112 (15)	110 (4)
5,6-EET	5,6-EET-d ₁₁	6.43 ± 0.02	0.4-225	0.990	0.13	0.4	0.7	97 (15)	109 (5)	95 (8)	86 (7)	107 (12)	88 (12)	110 (7)	112 (23)	105 (14)	109 (2)	94 (13)	95 (5)

Notes: LOD: Limit of detection; LLOQ: Lower limit of quantification; LLOQ*: Lower limit of quantification applying dilution factor in sample; RT: Retention Time

3.2. Effects of IS and antioxidant in five different approaches

Figure 2 shows relative oxylipin concentrations, determined with respect to the reference (*i.e.*, dry micro-sampling tube, approach 1), in dry micro-sampling tubes (**Figure 2A**), VAMS[®] (**Figure 2B**), and PCDBS (**Figure 2C**) across sampling approaches 1 to 5. 14-HDHA was excluded from the analysis due to recoveries outside the acceptance limits (>200%), likely caused by the generation of this compound during the 2h drying process at RT. It is well known that 14-HDHA is an enzymatic degradation product from docosahexaenoic acid via 12-LOX and P450 as reported in literature[23]. However, the employed method did not include PUFA precursors, and hence it was not possible to confirm their oxidation in this study. For the remaining 27 oxylipins, recoveries obtained with approaches 3 and 5 for both dry micro-sampling tubes and PCDBS, were generally lower than those observed with approaches 1, 2, and 4. In approaches 3 and 5, the solvent of IS solution was evaporated before the sample was deposited onto the micro-sampling device. Conversely, for VAMS[®], lower recoveries were observed with approaches 2 and 4, where the ISs were added after 2 h of drying. This suggests that the timing of IS addition may impact the recovery of oxylipins from the VAMS[®] device. For example, the handling of the IS used for 5,6-EET (*i.e.*, 5,6-EET-d₁₁) is particularly critical due to its instability. Notably, consistently high recoveries (>140%) were obtained when 5,6-EET-d₁₁ was added either directly to the biological sample (approach 1) or evaporated onto the device prior to sample drying (approaches 3 and 5), suggesting a loss of this IS during the drying process leading to unexpectedly high recoveries.

Figure 3 presents PCA scores plots based on concentrations of 27 oxylipins obtained by the different approaches and three micro-sampling methods (**Figure 3A**) as well as for each device individually: dry micro-sampling tube (**Figure 3B**), VAMS[®] (**Figure 3C**), and PCDBS (**Figure 3D**). In agreement with the results shown in **Figure 2**, **Figure 3A** indicates that approaches 3 and 5, when using dry micro-sampling tubes and PCDBS devices, resulted in lower PC2 scores. Similarly, the PCA scores plots for the individual micro-sampling devices (**Figures 3B** and **D**) showed clear differences between results of approaches 3 and 5 compared to approaches 1, 2, and 4. Interestingly, for VAMS[®] (**Figure 3C**), approaches 1, 3, and 5 yielded similar results, whereas approaches 2 and 4 showed lower relative recoveries as shown by negative scores in the direction of the first PC.

Statistical differences between the reference method (approach 1, dry micro-sampling tube) and the different devices and approaches were assessed by employing the Dunnett's test (see **Supplementary Figure S1**). Among all tested combinations, only dry micro-sampling tubes with approaches 2 and 4 showed no significant differences from the reference procedure. On average, significant differences were observed for 14%,

11%, and 15% of the oxylipins when using dry micro-sampling tubes, VAMS[®], and PCDBS devices respectively, across all approaches. For all sampling devices, the percentages of oxylipins showing significant deviations were 6%, 7%, 26%, 3%, and 21% for approaches 1 to 5, respectively. These findings highlight that selection of the experimental procedure (*i.e.*, approaches 1 to 5) has a greater impact on the results than the selection of the sampling device (*i.e.*, dry micro-sampling tubes, VAMS[®], or PCDBS). Approaches 1, 2, and 4 consistently provided more accurate results than approaches 3 and 5 when evaluated against the reference procedure.

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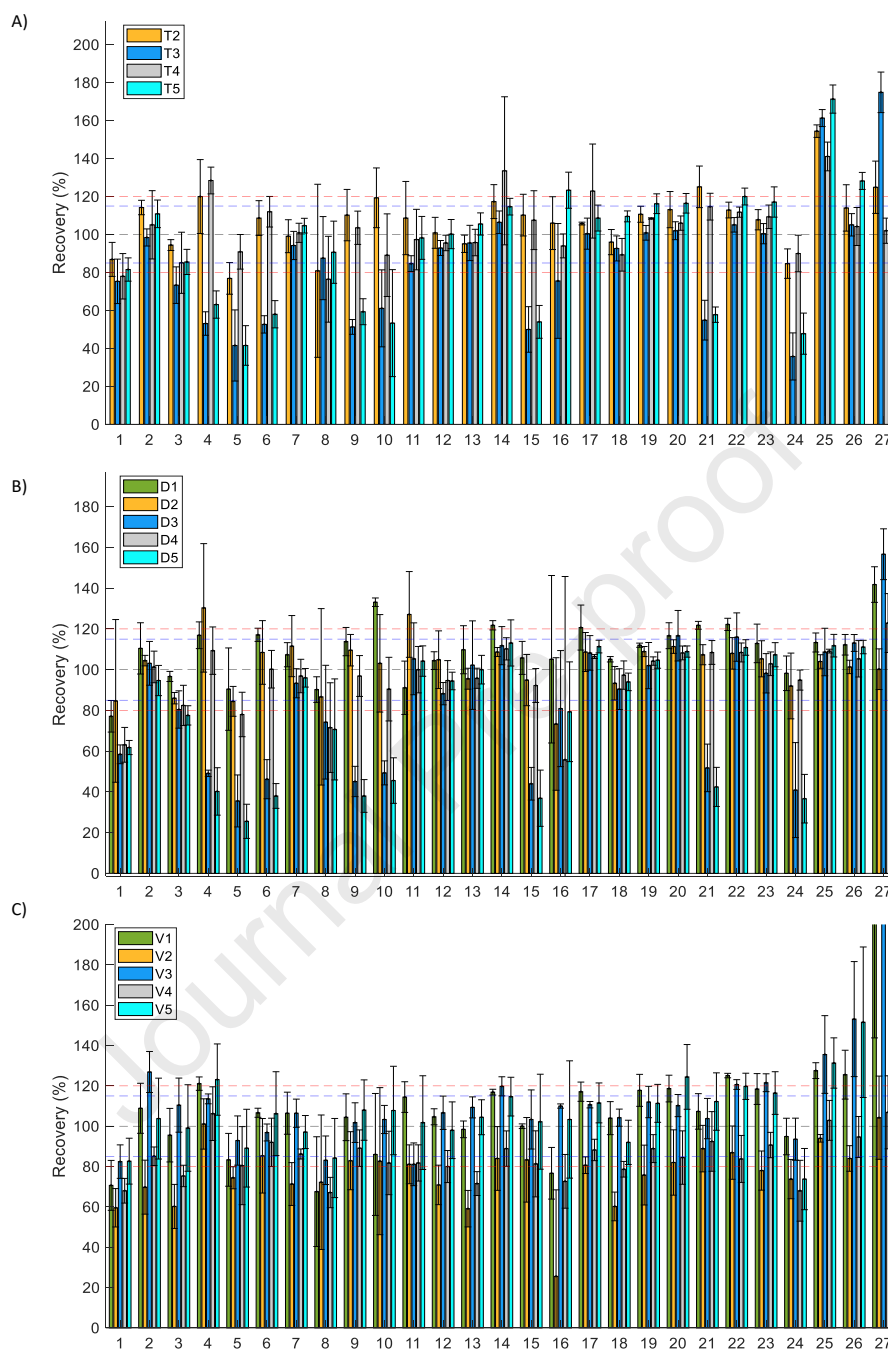


Figure 2. Relative oxylipin concentrations (mean \pm SD; $n=3$) calculated as percentage of the ratio between analyte concentration in each approach with respect to the reference method (*i.e.*, approach 1 using a dry micro-sampling tube) in micro-sampling tube (A), PCDBS (B), and VAMS[®] (C). Note. T: micro-sampling tube; V: VAMS[®]; D: PCDBS; numbers 1 to 5 indicate the approach used (see Figure 1); 1 – TXB₂; 2 – PGF_{2 α} ; 3 – PGE₂; 4 – 9(R)-16(RS)-Linotrin; 5 – 10(S),17(R)-diH n-3 DPA_{EEZ}; 6 – NPD-1; 7 – 17,18-DiHETE; 8 – Resolvin D5; 9 – PDX; 10 – Maresin 1; 11 – 8(S),15(S)-DiHETE; 12 – 14,15-DiHETE; 13 – LTB₄; 14 – 12,13-DiHOME; 15 – 10(S),17(R)-diH n-6 DPA_{EEZ}; 16 – Maresin 2; 17 – 9,10-DiHOME; 18 – 19,20-DiHDPA; 19 – 14,15-DiHET; 20 – 11,12-DiHET; 21 – 18-HEPE; 22 – 8,9-DiHET; 23 – 5,6-DiHET; 24 – 17- HDoHE; 25 – 11,12-EET; 26 – 8,9-EET; 27 – 5,6-EET.

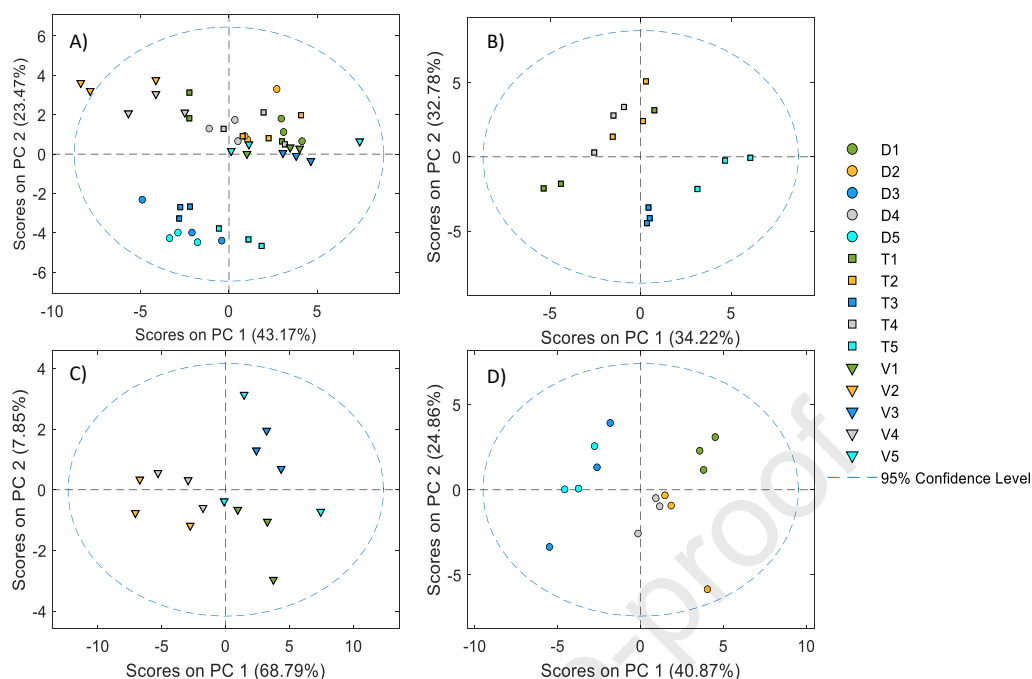


Figure 3. PCA scores plot from non-cyclic oxylipin concentrations determined employing approaches 1 to 5 across all micro-sampling methods (A), sampling by using dry micro-sampling tubes (B), sampling with VAMS® (C) and sampling with PCDBS (D). Note. T: micro-sampling tube; V: VAMS®; D: PCDBS; numbers 1 to 5 indicate the approach used (see Figure 1).

Based on the results obtained, approach 4 was selected for further experiments due to its overall superior performance in recovery experiments, demonstrating higher accuracy and precision. Furthermore, the addition of the antioxidant (BHT) in the sampling device prior to UCB collection might provide additional benefits for long-term storage, further supporting the selection of this approach.

3.3. Long-term storage assessment of oxylipins on micro-sampling devices

Figure 4 shows the recoveries of 27 oxylipins in spiked WB pool sample stored at different temperatures over a period of three months, expressed relative to the initial concentration measured on the day of sample collection. As shown in Figure 4, both storage temperature and duration have an impact on the recovery of oxylipins. Particularly, storage at RT and 4°C had a pronounced effect, leading to either the loss or over-estimation of several oxylipins, irrespective of the micro-sampling device employed. At RT, relative variations (%) ranged from 1 to 2620 and from 2 to 721 for PCDBS and VAMS® and coefficients of variation (CV%) were 232 and 98, respectively. At 4°C, relative variations (%) ranged from 2 to 754 and from 2 to 273 for PCDBS and VAMS® and CV% were 190 and 80, respectively.

Stability was better at -20°C, with 93% and 89% of oxylipins remaining within the established acceptability range of 80 to 120% for PCDBS and VAMS[®], respectively. However, when using PCDBS, 5,6-EET and 17-HDoHE showed consistent stability issues across all tested time points. Previous studies suggested that under physiological conditions, 5,6-EET is converted to a more stable lactone, namely 5,6- δ -DHTL [24], which could explain the low recoveries observed during storage due to compound degradation. For 17-HDoHE, previous research reported an increasing trend over time [25,26], likely due to enzymatic oxidation via 15-LOX from DHA present in the sample. Nevertheless, in this study, increasing concentrations of 17-HDoHE were only observed in PCDBS, but not in VAMS[®].

In VAMS[®] devices, 5,6-EET showed similar issues to those observed as in PCDBS, suggesting that this compound degrades during cold storage, even after three days. Furthermore, a consistent loss of TXB₂ and 8(S),15(S)-DiHETE was observed across tested time points. Previous studies on the stability of TXB₂ have reported inconclusive results: while TXB₂ in EDTA with BHT has been shown to remain stable for up to one year at -20 °C [26], a 10% reduction was observed within the first 2 h when comparing EDTA plasma to citrate plasma. For Maresin-1, a gradual decrease in recoveries was observed from three days to three months, indicating a slow degradation of the compound during storage on VAMS[®]. In summary, most oxylipins with exception of 5,6-EET and 17-HDoHE in PCDBS and 5,6-EET, TXB₂, 8(S),15(S)-DiHETE, and Maresin-1 in VAMS[®], showed acceptable levels of stability at -20 °C up to three months of storage. The recoveries obtained in the stability assay, shown in Figure 4, are available in Supplementary Tables 2 and 3 for PCDBS and VAMS[®], respectively.

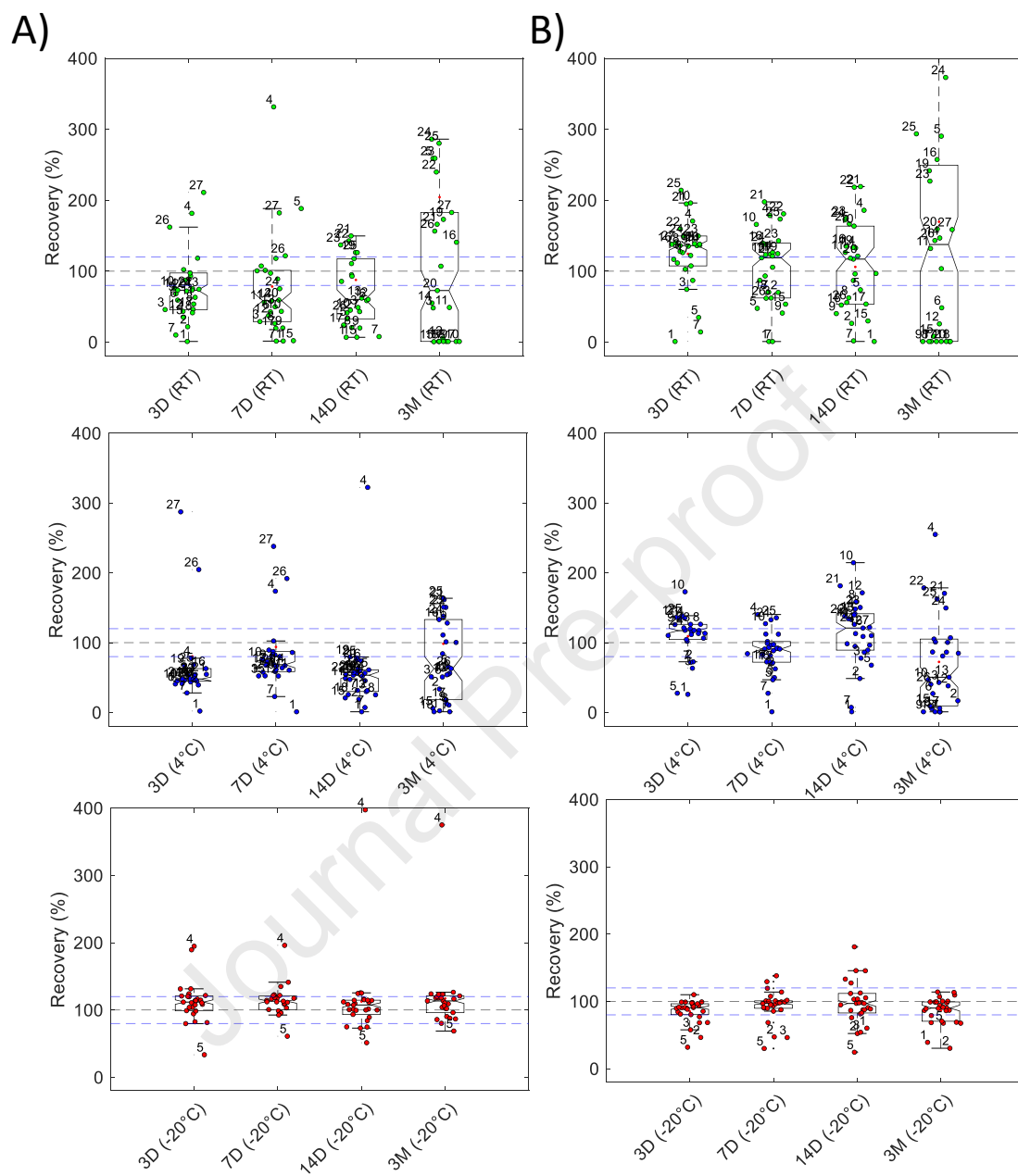


Figure 4. Stability assessment at three different temperatures (top: RT, middle: 4 °C, and bottom: -20 °C) in PCDBS (A) and VAMS® (B) over a period of three months in both micro-sampling devices. Note: 1 – TXB₂; 2 – Maresin 1; 3 – 8(S),15(S)-DiHETE; 4 - 17- HDoHE; 5 - 5,6-EET; 6 – PGF_{2α}; 7 – PGE₂; 8 - 9(R)-16(RS)-Linotrin; 9 - 10(S),17(R)-diH n-3 DPA_{EEZ}; 10 - NPd-1; 11 - 17,18-DiHETE; 12 - Resolvin D5; 13 – PDX; 14 – 14,15-DiHETE; 15 – LTB₄; 16 – 12,13-DiHOME; 17 - 10(S),17(R)-diH n-6 DPA_{EEZ}; 18 – Maresin 2; 19 – 9,10-DiHOME; 20 – 19,20-DiHDPA; 21 – 14,15-DiHET; 22 – 11,12-DiHET; 23 – 18-HEPE; 24 – 8,9-DiHET; 25 – 5,6-DiHET; 26 – 11,12-EET; 27 – 8,9-EET

3.4. Determination of oxylipin concentrations in UCB samples using micro-sampling devices

To evaluate the performance of micro-sampling devices, both PCDBS and VAMS[®] were used to analyse oxylipin profiles in 35 UCB samples from term infants, collected in the delivery room. The results were compared to those obtained using the validated method. Eleven compounds were present at detectable concentrations in the studied samples. Using Passing-Bablok regression (see **Supp. Figure. S2**)[27], a statistically significant linear relationship was observed between the results obtained from the micro sampling methods and the reference method. The regression coefficient, along with its 95% confidence interval (CI), included the value 1 in slope and 0 in the y-intercept, suggesting consistency between methods. Additionally, a Bland-Altman (see Supp. Figure. S2) difference plot with log-transformed data was used to evaluate the agreement between both micro sampling methods and the reference method. This analysis assessed the range within which 95% of the differences between the methods are expected to fall [28], providing insight into the limits of agreement. Several oxylipins (*i.e.*, PGE₂, 12,13-DiHOME, 9,10-DiHOME, 19,20-DiHDPA, 14,15-DiHET, 11,12-DiHET, and 8,9-DiHET) were found to be within acceptance limits when comparing concentrations found using PCDBS/VAMS[®] and the reference method. For others, method comparison was successful with one micro sampling device, but not the other. Specifically, TXB₂ could successfully be determined using PCDBS while 17-HDHA and 11,12-EET were validated in VAMS[®]. For the remaining two compounds, *i.e.*, 8,9-EET and 5,6-DiHET, the comparison failed and hence, those oxylipins cannot be considered quantifiable with the micro sampling devices under the assayed conditions.

The concentrations detected using PCDBS and VAMS[®] in the studied sample set are shown in Figure 5. Concentrations for all oxylipins were in the low nM range, as expected, with varying detection frequencies among tested compounds. Whereas some, such as 12,13-DiHOME and 9,10-DiHOME, were detected in all tested samples with both devices, others, such as PGE₂, showed a lower detection frequency ($\leq 20\%$). In summary, the oxylipin profiles obtained using PCDBS and VAMS[®] were comparable, with similar oxylipin profiles detected and overlapping detection frequencies for the majority of tested compounds indicating that both devices are equally suitable for the quantification of most oxylipins under the studied conditions.

This study has some limitations. While the method was successfully validated for a broad panel of oxylipins, its application to UCB samples resulted in the detection of only 12, 8, and 9 oxylipins in micro-sampling tubes, PCDBS, and VAMS[®], respectively. This is likely due to the small blood volumes used in combination with low endogenous levels of certain oxylipins in healthy term infants, often below the limits of detection of current

LC-MS/MS technology. Given the exploratory nature of this study, a lower detection frequency is expected, as commonly observed in small, early-phase studies.

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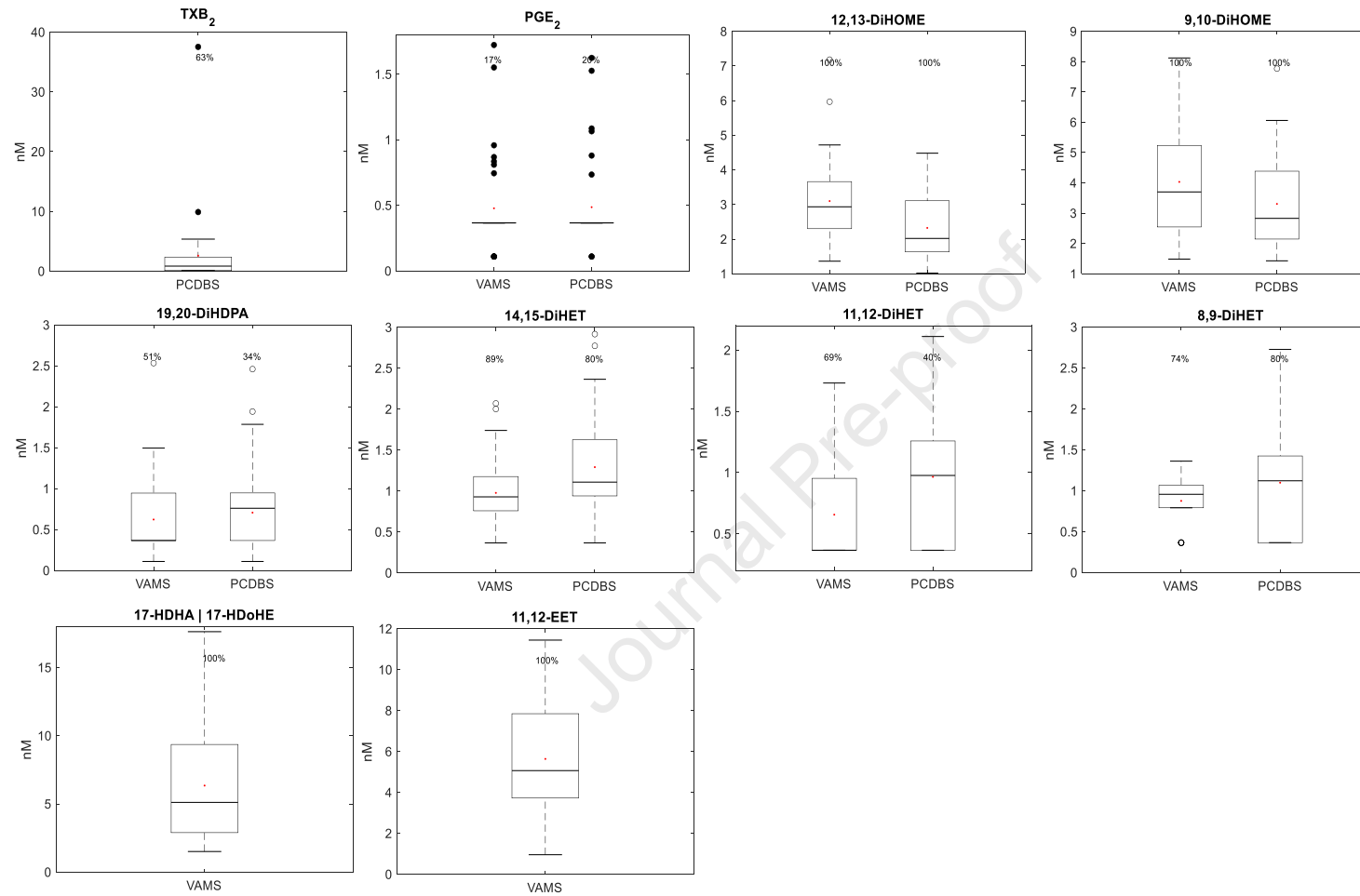


Figure 5. Concentrations of oxylipins found in 35 UCB samples from term infants using PCDBS and VAMS® devices.

4. Conclusions

To the best of our knowledge, this study represents the first report of oxylipin analysis in UCB samples using VAMS® and PCDBS devices in the research setting. The results demonstrate the applicability of these two blood micro-sampling devices for the quantification of oxylipins at nM concentrations in UCB samples. Our findings highlight the impact of sample collection and storage on the retrieved oxidized lipid profiles. In particular, the handling of IS was identified as a critical step, as solvent evaporation and variations in extraction efficiency relative to samples can impact the recovered concentrations. Cold storage (-20 °C) rendered necessary to ensure stability of most oxylipins for at least three months. Higher storage temperatures are not recommended, as even short storage durations of several days, increased the variability in the oxylipin concentrations.

The choice of micro-sampling device for clinical studies will depend on the trade-off between the practical advantages of these micro-sampling devices (*e.g.*, no need for the use of pipettes for VAMS®), the specific oxylipins of interest, and their cost. Finally, within the same study and across all participating entities, the chosen micro sampling device and standardized approaches for IS and antioxidant addition should be maintained to ensure comparability. Standardization of protocols will furthermore play an important role in enhancing comparability among laboratories and the successful completion of ring trials necessary to pave the way for the translation of oxylipins to routine biomarkers of inflammation assessment. Future studies should focus on extending the panel of studied oxylipins through the addition of metabolites as well as the use of more sensitive LC-MS/MS systems. Such innovations could enable the detection and quantification of oxylipins at concentrations below the LOQ of the current method, potentially enhancing the scope and utility of oxylipin analysis in neonatology and other areas of application.

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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Highlights

- Evaluated micro-sampling devices for oxylipin analysis in newborn blood.
- Targeted mass spectrometry method validated for oxylipins in micro-sampling.
- Antioxidant pre-treatment improved sample stability and accuracy.
- Long-term storage at -20°C preserved most oxylipins for three months.
- Micro-sampling devices provided comparable oxylipin profiles in newborns.

Journal Pre-proof