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Pre-analytical challenges for the quantification of endocannabinoids in human serum

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ABSTRACT

Endocannabinoids (ECs) are potent lipid mediators with high physiological relevance. They are involved in a wide variety of diseases like depression or multiple sclerosis and are closely connected to metabolic parameters in humans. Therefore, their suitability as a biomarker in different (patho-)physiological conditions is discussed intensively and predominantly investigated by analyzing systemic concentrations in easily accessible matrices like blood. Carefully designed pre-analytical sample handling is of major importance for high-quality data, but harmonization is not achieved yet. Whole blood is either processed to serum or plasma before the onset of analytical workflows and while knowledge about pre-analytical challenges in plasma handling is thorough they were not systematically investigated for serum.

Therefore, the ECs AEA and 2-AG, and closely related EC-like substances 1-AG, DHEA, and PEA were examined by LC-MS/MS in serum samples of nine healthy volunteers employing different pre-analytical sample handling protocols, including prolonged coagulation, and storage after centrifugation at room temperature (RT) or on ice. Furthermore, all analytes were also assessed in plasma samples obtained from the same individuals at the same time points to investigate the comparability between those two blood-based matrices regarding obtained concentrations and their 2-AG/1-AG ratio.

This study shows that ECs and EC-like substances in serum samples were significantly higher than in plasma and are especially prone to *ex vivo* changes during initial and prolonged storage for coagulation at RT. Storage on ice after centrifugation is less critical. However, storage at RT further increases 1-AG and 2-AG concentrations, while also lowering the already reduced 2-AG/1-AG ratio due to isomerization. Thus, avoidance of prolonged processing at RT can increase data quality if serum as the matrix of choice is unavoidable. However, serum preparation in itself is expected to initiate changes of physiological concentrations as standard precautionary measures like fast and cooled processing can only be utilized by using plasma, which should be the preferred matrix for analyses of ECs and EC-like substances.

1. Introduction

Endocannabinoids (ECs) are a very low concentrated but highly potent sub-group of lipid mediators. The two central ECs are arachidonoyl ethanolamide (AEA, anandamide) and 2-arachidonoyl glycerol (2-AG) which are endogenous ligands for the cannabinoid receptors 1

Abbreviations: ECs, endocannabinoids; AEA, arachidonoyl ethanolamide; 2-AG, 2-arachidonoyl glycerol; 1-AG, 1-arachidonoyl glycerol; CBR1/2, cannabinoid receptors 1/2; PEA, palmitoyl ethanolamide; DHEA, docosahexaenoyl ethanolamide; NAE, N-acetylethanolamide; MAG, monoacylglycerol; PAF, platelet-activating factor; STD, calibration standard; QC, quality control; IS, internal standard; LLOQ, lower limit of quantification; WB, whole blood; FT, storage on ice; RT, room temperature; IQR, interquartile range.

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and 2 (CBR1 and CBR2) [1,2]. Other structurally related compounds are for example palmitoyl ethanolamide (PEA), docosahexaenoyl ethanolamide (DHEA), and 1-arachidonoyl glycerol (1-AG). As these compounds don't or just slightly interact with the cannabinoid receptors they are named EC-like substances [3] and will be summed up together with AEA and 2-AG in the following within the term ECs. The ECs and the whole EC system have been studied intensively in the context of different diseases like major depression, multiple sclerosis, or inflammatory bowel disease [4–10] and physiological conditions like obesity or sepsis [11,12], indicating the high relevance of the EC system in mammalian organisms [3,11,13–17]. In consequence of this high relevance, using the EC system as a therapeutical target is investigated and discussed intensively [18,19].

However, it is still a demanding task to finally prove the relevance of these compounds in the pathogenesis of diseases and to evaluate their potential as a therapeutic target as reliable quantification of ECs is complex due to analytical and pre-analytical challenges. This is especially true for blood-based samples which are most frequently exploited to analyze ECs. Endogenous concentrations in blood-based samples are scarcely comparable between healthy controls of different studies and reference intervals seem only possible with highly characterized individuals [20–22]. In addition to dissimilar study populations, varying final sample types (plasma or serum) and pre-analytical sample processing methods are likely a part of the explanation. Determination of ECs is performed in human plasma (mainly K_3EDTA) [21,23–35] as well as human serum [8,17,36–39] as there is no consensus about the most suitable blood-based matrix. Serum is generated by activation of the coagulation cascade (e.g. through silica particles in blood tubes) which leads to the activation of platelets, the enzymatic formation of fibrin clots from fibrinogen, and, therefore, depletes the forming serum of clotting factors [40,41]. Complete clotting takes at least 20 min at room temperature due to its enzymatic nature and is required for the generation of serum samples before centrifugation. In contrast to serum samples, coagulation in plasma samples is inhibited by the use of anticoagulants such as K_3EDTA and centrifugation can be performed immediately after blood draw [41].

Overall, the wide variety of pre-analytical challenges for the determination of ECs in human (K_3EDTA) plasma are well characterized [20,42–45] as the impact of the anticoagulant [32,42,46,47], centrifugation parameters [43], and prolonged storage in anticoagulated whole blood [42,43] have been intensively examined. Nevertheless, data on influencing pre-analytical factors when generating serum samples are not available. The studies investigating plasma samples all highlight the need for rapid and continuously cooled pre-analytical sample processing to prevent *ex vivo* changes of analyte concentrations as well as isomerization from 2-AG to 1-AG, which results in changes of the 2-AG/1-AG ratio. Those routinely taken precautionary measures for generating plasma samples are impossible to implement for serum preparation due to mandatory and time-consuming clotting of blood samples at room temperature. Comprehensive analysis of the influence of pre-analytical sample handling on the metabolome in both matrices indicates that while several features show high comparability, few differ significantly, especially if they are involved during the coagulation cascade (e.g. lipid mediators thromboxane A_2 and lysophosphatidylinositol) [41,48,49]. As the monoacylglycerols 2-AG and 1-AG are released from human platelets upon activation of the platelet-activating factor (PAF) receptor, the connection between the coagulation cascade and changing analyte concentration has already been proven, suggesting inherently altered concentrations in serum for those analytes [50–52]. This is of great interest since 2-AG/1-AG ratios seem to be of physiological importance, and coagulation processes might bias observations [53]. Parallel quantification of *N*-acetylethanolamides (NAE, e.g. AEA) in serum and plasma either show similar concentrations [54] or suggests differences between both matrices [55] but were never systematically investigated within one study population.

As data about pre-analytical stability of EC concentrations while generating serum samples have not been reported yet, the main objective of this study was to investigate and identify critical pre-analytical factors during serum preparation to close this gap in knowledge about pre-analytical sample handling for the determination of ECs. Besides improving sample quality in studies employing serum samples, this knowledge can help translate reliable pre-analytical workflows into clinical practice. Furthermore, ECs in multiple serum and K_3EDTA plasma samples of the same healthy controls were analyzed to examine comparability between those two blood-based matrices regarding observed concentrations, the extent of 2-AG/1-AG isomerization, and overall suitability for EC analysis.

2. Materials and methods

2.1. Chemicals

Water (LC-MS-grade), acetonitrile (LC-MS-grade, purity $\geq$ 99.5%), hexane (UV/IR-grade, $\geq$ 99.5%) were purchased from Carl Roth (Karlsruhe, Germany), ethyl acetate (Reag. Ph. Eur. $\geq$ 99.9%) from Biosolve B. V. (Valkenswaard, Netherlands), and formic acid (98–100%) from AppliChem (Darmstadt, Germany). The ECs (AEA, $\geq$ 98%; PEA, $\geq$ 98%; 1-AG, $\geq$ 95%; 2-AG, $\geq$ 95%; DHEA, $\geq$ 98%) and their isotopically labeled internal standards (AEA-d_8, $\geq$ 99%; PEA-d_4, $\geq$ 99%; 1-AG-d_5, $\geq$ 99%; 2-AG-d_5, $\geq$ 95%; DHEA-d_4, $\geq$ 98%) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Deuterated sites are given in the supplementary material.

2.2. Instrumentation and data acquisition

The determination of EC concentrations was performed by LC-ESI-MS/MS. The triple quadrupole mass spectrometer QTRAP 6500+ was equipped with a Turbo Ion Spray source (both Sciex, Darmstadt, Germany) and operated in positive electrospray ionization mode. The MS was coupled to an Agilent 1290 Infinity II LC system with a binary HPLC pump, column oven, and autosampler (Agilent, Waldbronn, Germany). For chromatographic separation, an Acquity UPLC BEH C18 2.1 $\times$ 100 mm column from Waters (Eschborn, Germany) with a SecurityGuard ULTRA UPLC C18 pre-column 2.1 $\times$ 5 mm from Phenomenex (Aschaffenburg, Germany) were used. Water (solvent A) and acetonitrile (solvent B) including 0.0025% formic acid, respectively, were used for gradient elution as described previously [43]. The chromatographic gradient was shortened to 8 min (0.4 mL/min, solvent A-B in %; 0 min: 80–20, 0.8 min: 80–20, 1 min: 35–65, 5 min: 5–95, 6 min: 5–95, 6.01 min: 80–20, 8 min: 80–20) to increase sensitivity, especially for AEA. Isomeric pairs AEA/O-AEA and 2-AG/1-AG were baseline separated, while OEA/VEA was excluded from analysis due to coelution. DHEA was added to the method. Data acquisition and quantification were conducted with Analyst 1.7.1 and MultiQuant 3.0.3 (both Sciex, Darmstadt, Germany).

2.3. Standard preparation, sample extraction, and quality assurance measures

Standard preparation and sample extraction were performed as described previously with minor modifications [43]. The calibration standards (STDs) and quality control samples (QCs) were prepared in 200 µL phosphate-buffered saline (PBS) as a surrogate matrix. Pre-aliquoted plasma and serum samples (200 µL) were thawed in a fridge (4 °C) and extracted on ice alongside STDs and QCs. Blood samples were spiked with 20 µL IS working solution and 20 µL acetonitrile while STDs and QCs were spiked with 20 µL IS working solution and 20 µL of the respective standard working solution. All samples were vortexed for 1 min and centrifuged at 20.000 $\times$ g for 1 min at 4 °C before liquid–liquid extraction with 400 µL ethyl acetate:hexane (9:1, v/v) was performed. After vortexing for 1 min and subsequent centrifugation for 3 min at 20.000 $\times$ g and 4 °C, the upper layer was transferred into a new

polypropylene tube and evaporated at 45 °C under a constant stream of nitrogen. For reconstitution, 50 µL acetonitrile was added to the evaporated extract. After thorough mixing, the samples were transferred into vials with inserts, and 10 µL were injected into the LC-MS/MS system. Additional information regarding the concentration ranges of analytes and IS concentrations are given in the supplementary material.

Every analytical run consisted of a standard calibration curve and two sets of QC samples which enclosed the unknown samples. Furthermore, two pre-aliquoted control samples were employed to secure comparability between analytical runs. Blank signals had to be < 5% for the IS and < 20% for the LLOQ for analytes, which is why the LLOQ of PEA was increased to 375 pg/mL. The acceptance criteria for quantitative data and quality assurance measures are described in detail in a previous publication [43]. Data on the accuracy and precision of the employed LC-MS/MS method are given in the supplementary material.

2.4. Sample collection – Investigations on pre-analytical sample handling

Blood samples were collected from nine healthy volunteers after informed consent was given. The healthy volunteers comprised six female and three male participants with average weight and a mean age of 33 ± 8.6. Venous blood was taken using 2.7 mL S-Monovette K3EDTA for plasma samples and Z tubes for serum samples (Sarstedt, Nümbrecht, Germany). Blood draw took place between 12:30 and 14:30 o'clock on three consecutive days. Seven tubes for serum preparation and six tubes for plasma preparation were drawn consecutively from each participant. Whole blood (WB) for plasma preparation was directly stored on ice (FT, 0.68 ± 0.21 °C). WB for serum preparation was stored at room temperature (RT, 24.8 ± 0.19 °C) after collection. Different sample processing methods were applied to evaluate the effect of altered pre-analytical sample handling on EC concentrations besides a respective reference method following manufacturer's specifications (Fig. 1, A). The effects of prolonged storage of WB (Fig. 1, B) and storage after centrifugation without separating the respective layers (Fig. 1, C) were investigated. Samples for plasma preparation were permanently handled under chilled conditions (storage took place on ice, and the centrifuge was cooled to 4 °C). In contrast, storage of samples for serum

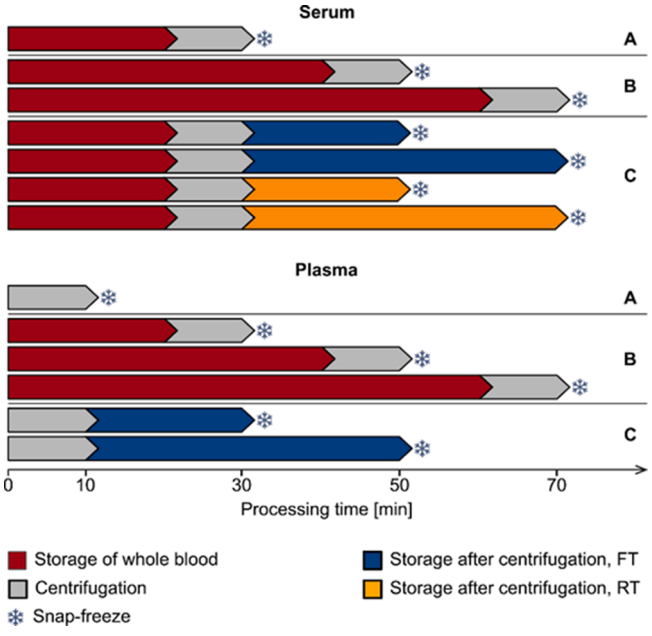

Fig. 1. Overview of serum and plasma processing methods investigated in this study. (A) depicts respective reference methods, (B) prolonged storage of whole blood before centrifugation at RT for serum and FT for plasma and (C) rapid processing in line with the reference method, but with additional storage after centrifugation (room temperature – "RT" or stored on ice – "FT").

preparation at RT is mandatory before centrifugation to allow for clotting. The centrifuge was tempered to 21 °C and solely the temperature for storage after centrifugation was evaluated at RT and FT. All samples were centrifuged at 2000 × g for 10 min. At the end of the respective sample processing methods, samples were snap-frozen on dry ice in aliquots of 200 µL and stored at −80 °C until analysis.

2.5. Statistical data evaluation

Data were analyzed using GraphPad Prism 8.3.1 (549) 64-bit (GraphPad Software, San Diego, CA, USA). All examined ECs were inspected visually for normality by employing quantile–quantile plots and log10-transformed, following general recommendations for data evaluation of generated blood-based samples [56]. One-way ANOVA was applied with uncorrected Fisher's LSD follow-up test for paired comparisons. Subsequently, *p*-values were adjusted for multiple comparisons employing the false-discovery rate (FDR) method of Benjamini and Hochberg [57]. Statistical significance is claimed and presented for calculated *q*-values as follows: ≤ 0.05 *, ≤ 0.01 ** and ≤ 0.001 ***. Due to chromatographic matrix interference, which could not be resolved employing the current method, the total number of samples for 1-AG and 2-AG in serum samples stored after centrifugation at RT for 40 min ($N = 7$) and 1-AG in samples stored under the same conditions for 20 min ($N = 8$) had to be reduced. Results given in the text are mean ± SD.

3. Results and discussion

3.1. Influence of pre-analytical sample handling of human serum on EC concentrations

The distinct influence of pre-analytical parameters on EC concentrations in blood-based samples is described in detail for plasma preparation [20,42–45]. By taking this knowledge into account, the same problems must be expected when generating serum samples. However, a detailed investigation on pre-analytics for ECs in serum has not been published yet. To identify critical pre-analytical factors during serum preparation, different sample handling protocols were investigated in this study (see 2.4). EC concentrations after prolonged coagulation of WB at RT and prolonged storage after centrifugation at RT or FT were determined in serum and subsequently compared with the results of a respective reference method. The results of this experiment indicate clearly that *ex vivo* formation of ECs is very prevalent for serum samples (Fig. 2). Prolonged coagulation at RT with an additional 20 or 40 min of WB storage before centrifugation (total processing times of 53 ± 1.8 and 74 ± 2.2 min) resulted in significantly increased concentrations of all NAEs (AEA, DHEA, PEA) and MAGs (2-AG, 1-AG). Nevertheless, no changes of NAE concentrations were observed for additional serum storage of 20 or 40 min at RT or FT after centrifugation (processing times of 50 ± 1.8 and 70 ± 1.9 min). In contrast, MAG concentrations increased significantly during storage for 20 or 40 min after centrifugation at RT. However, they were not altered after an additional 20 min of storage at FT. 2-AG was stable even after 40 min of additional storage of serum one ice.

The phenomenon of increasing NAE concentrations *ex vivo* was described multiple times in anticoagulated WB when generating plasma [20,42,43,46,58] and could be confirmed for plasma samples in our study (Fig. 3). Moreover, this time-dependent release of NAEs by blood components seems to be equally relevant for serum as for plasma. Only separating the cellular components into a separate phase by centrifugation stabilizes NAEs, likely by lessening the contact surface between blood cells and the forming serum or plasma. This procedure effectively stabilizes NAEs at RT and FT for 40 min of additional storage even if the serum is not removed from the lower cell layer. MAGs, on the other hand, are not stabilized solely by centrifugation if subsequent storage is necessary and takes place at RT. This indicates residual enzymatic

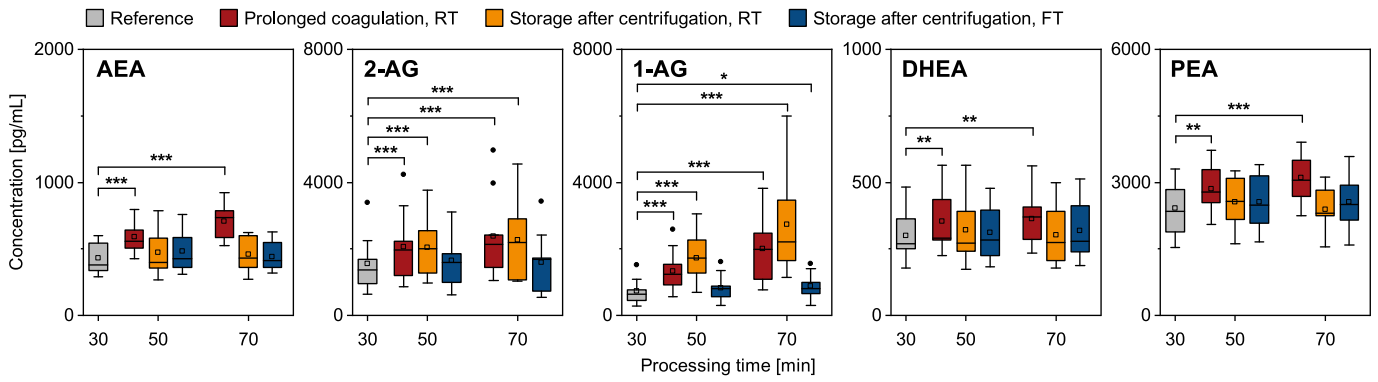

Fig. 2. Pre-analytical stability of AEA, 2-AG, 1-AG, DHEA, and PEA in serum samples under different processing conditions. Box and whiskers plot depicting Q1–Q3 with ± 1.5 IQR. Median (Q2) is plotted as a horizontal line and average as light square. Comparison of respective processing methods against a fast reference method. q-value ≤ 0.05 *, ≤ 0.01 ** and ≤ 0.001 *** (see 2.5).

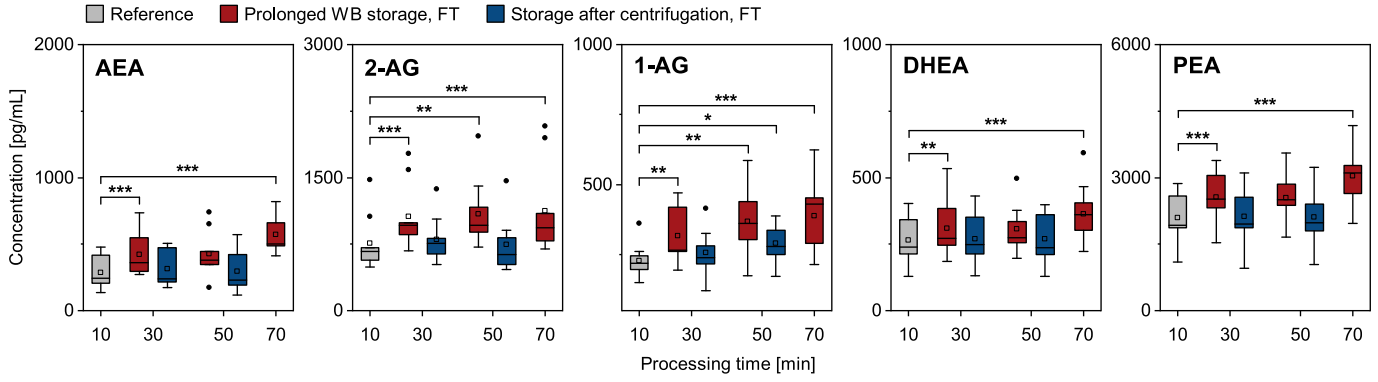

Fig. 3. Pre-analytical stability of AEA, 2-AG, 1-AG, DHEA, and PEA in plasma samples under different processing conditions. Box and whiskers plot depicting Q1–Q3 with ± 1.5 IQR. Median (Q2) is plotted as a horizontal line and average as light square. Comparison of respective processing methods against the reference. q-value ≤ 0.05 *, ≤ 0.01 ** and ≤ 0.001 *** (see 2.5).

activity that releases MAGs from soluble precursors like triglycerides by lipoprotein lipase [59]. Additionally, reduced contact surface to platelets after centrifugation can prevent internalization of 2-AG through a high-affinity transporter with subsequent hydrolysis, making this effect even more pronounced [52]. MAGs were stable in serum after centrifugation for an additional 20 min if storage takes place at FT and is thus presumably slowing down enzymatic conversion of soluble precursors. However, 1-AG concentrations were significantly increased after 40 min of storage at FT possibly due to isomerization of 2-AG to 1-AG and limits storage time after serum preparation at FT for MAG analysis to 20 min.

3.2. Comparability of endocannabinoid concentrations in serum and K3EDTA plasma

The comparison of EC concentrations determined in plasma and serum samples from healthy controls indicates differences between those blood-based sample types [51,55]. However, reported EC concentrations of healthy controls by independent research groups imply similar medians between serum and plasma samples for AEA, DHEA, and PEA while median 2-AG concentrations seem to differ (Table 1, Fig. 4, Table S2). Unfortunately, this literature-based approach is not able to exclude the influence of covariables such as sex, age, metabolic parameters, or different pre-analytical sample handling [60] and the impact of analytical pitfalls during analyses of ECs [61]. Furthermore, EC-like substances 1-AG, DHEA, and PEA are reported less frequently and, therefore, provide smaller sample sizes for comparison. This is further complicated by inconsistent reporting of 2-AG concentrations (either separate, as a sum of 2-AG + 1-AG, or without further specification) and the underlying issue of 2-AG isomerization or contamination

Table 1
Comparison of EC concentrations in serum and plasma samples from this study with literature data (Fig. 4, Table S2). Data reported as median (IQR) in ng/mL.

Analyte	Literature		This study	
	Serum	Plasma	Serum	Plasma
AEA	0.35 (0.24–0.61)	0.45 (0.24–0.88)	0.38 (0.34–0.54)	0.24 (0.21–0.42)
2-AG*	5.81 (1.71–11.52)	1.97 (0.74–4.14)	1.37 (0.96–1.69)	0.67 (0.57–0.71)
1-AG*			0.64 (0.46–0.76)	0.22 (0.20–0.25)
DHEA	0.28 (0.18–0.37)	0.39 (0.26–0.80)	0.27 (0.25–0.36)	0.24 (0.21–0.34)
PEA	3.78 (2.41–5.76)	2.63 (1.77–6.30)	2.34 (1.89–2.84)	1.92 (1.87–2.58)

* 2-AG/1-AG concentrations include 2-AG concentrations as reported by the respective research groups (separate, as sum or without further specification).

in case of PEA [37]. As a detailed investigation in samples from the same individuals is missing, plasma and serum EC concentrations were compared in samples from the same healthy controls (Fig. 5) using the fastest possible serum or plasma preparation protocol (Fig. 1, A). Total processing time after blood drawing until snap-freezing for plasma samples was 12 ± 1.9 min, and 31 ± 2.2 min for serum samples.

EC concentrations of AEA, 2-AG, 1-AG, DHEA, and PEA were significantly lower in plasma samples (Fig. 6) which is in good accordance with previous investigations reporting on both sample types for healthy controls [51,55] but in the case of NAEs contrary to the literature-based approach. Especially 2-AG/1-AG and PEA are lower

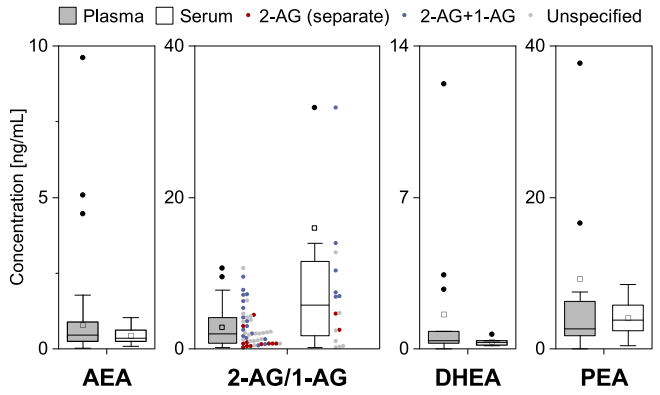

Fig. 4. Reported concentrations of AEA, 2-AG/1-AG, DHEA, and PEA (in ng/mL) in human blood samples from healthy volunteers or control cohorts by independent research groups. Box and whiskers plot depicting Q1–Q3 with ± 1.5 IQR. Median (Q2) is plotted as a horizontal line and average as light square. An outlier of 2-AG/1-AG in serum samples at 149.24 ng/mL is not shown. Color of data points indicates separate reporting of 2-AG (red), reporting of 2-AG + 1-AG as a sum (blue), or without further specification (light grey). Quantity of included values for plasma or serum samples: AEA (69, 21), 2-AG/1-AG (48, 16), DHEA (14, 5), and PEA (38, 11). Concentrations and respective references can be found in the supplementary material (Table S2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

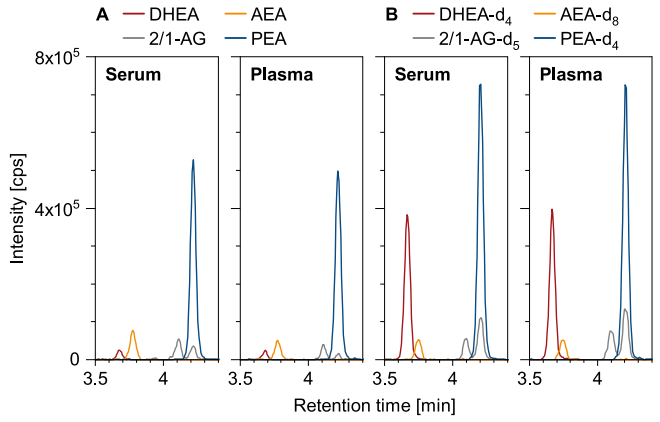

Fig. 5. LC-MS/MS chromatograms of a serum and plasma sample from the same healthy individual. Samples were generated by the respective reference method (Fig. 1A). (A) shows physiological EC concentrations (order of elution: DHEA (372.1/62.0), AEA (348.3/287.3), 2-AG/1-AG (379.2/287.3), PEA (300.2/62.2)) and (B) shows the respective internal standards (DHEA-d4 (376.1/66.0), AEA-d8 (356.2/295.2), 2-AG/1-AG-d5 (384.2/287.3), PEA-d4 (304.2/62.2)) used for stable-isotope dilution. Axes of serum and plasma samples are scaled equally.

within our study possibly due to fast sample processing and avoidance of PEA contaminants while AEA and DHEA are close to concentrations reported in the literature (Table 1).

By extending the storage time of WB samples for plasma preparation by 20 min (31 ± 1.6 min) to equate plasma and serum processing times, only 1-AG was significantly lower in plasma samples, indicating that serum EC concentration rises while storing WB for 20 min at RT. The underlying molecular mechanism of the time-dependent release of NAEs is still under debate and while proteins capable of transporting ECs were identified [62] passive diffusion across the plasma membrane following a concentration gradient is discussed [63]. 2-AG on the other hand is released during platelet activation and serum is therefore expected to yield higher 2-AG and by isomerization also 1-AG concentrations [52]. The approximation of 2-AG concentrations in serum (RT) and plasma

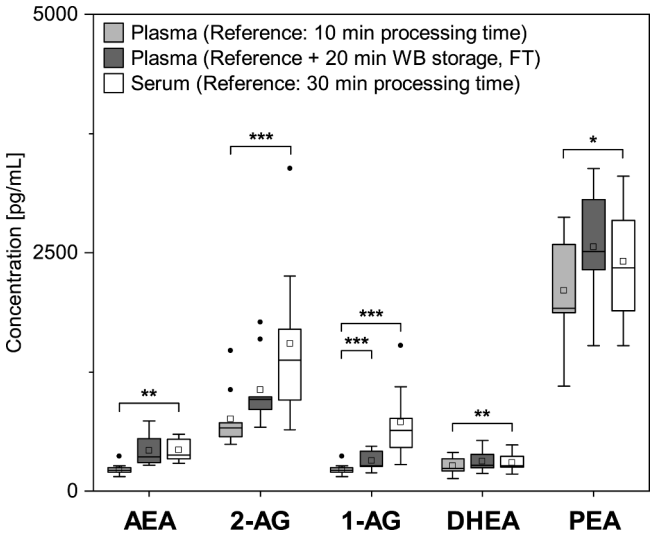

Fig. 6. Serum and plasma concentrations of AEA, 2-AG, 1-AG, DHEA, and PEA with respective reference methods and equalized processing time. Box and whiskers plot depicting Q1–Q3 with ± 1.5 IQR. Median (Q2) is plotted as a horizontal line and average as light square. Statistical evaluation against serum reference. q-value ≤ 0.05 *, ≤ 0.01 ** and ≤ 0.001 *** (see 2.5).

(FT) at equal processing times cannot be fully explained as 2-AG metabolism in platelets, release due to platelet activation and de-novo synthesis from soluble precursors presumably overlap (see 3.1). Also noticeable is the greater range of individual concentrations for 1-AG and 2-AG in serum, which could result from interindividual different capacities to release MAGs during coagulation from platelets (Table S1) [52].

3.3. *The ratio of 2-AG and 1-AG in blood-based matrices*

2-AG is one of the main objects of investigation when examining the relevance of the EC system in different pathological settings [4,5,12]. However, it is well known that 2-AG is very prone to isomerization into 1-AG [47,64], resulting in decreasing 2-AG and increasing 1-AG concentrations. Therefore, 2-AG and 1-AG are often evaluated as a summed parameter of both compounds as a surrogate, assuming that isomerization is the only process changing the concentration of 2-AG and 1-AG. Opposed to this, our analysis of the summed concentrations of 2-AG and 1-AG versus processing time indicates that the total concentration of 2-AG/1-AG is rising and can be therefore affected by pre-analytical sample handling (Fig. 7A). This is especially the case during serum preparation at RT but is also apparent to a lesser extent after prolonged WB storage at FT before plasma preparation. While the total concentration is rising with increasing processing time and storage at RT, the ratio of 2-AG/1-AG is decreasing (Fig. 7B), which is in good agreement with the previously described effect that the ratio of 2-AG/1-AG in processed blood samples is strongly temperature-dependent [47]. Cooled conditions stabilize the ratio over an extended period regardless of the chosen matrix. The initial 2-AG/1-AG ratio in plasma samples (3.3 ± 0.7) remained stable even if processing time was extended to 70 min and absolute concentrations of MAGs increased during WB storage. Overall ratios in serum were lower compared to plasma. While the storage of serum after centrifugation at FT stabilized the initially observed 2-AG/1-AG ratio (2.1 ± 0.3), it was further reduced by prolonged processing at RT. However, as initial storage at RT is unavoidable for coagulation, serum samples will presumably always lead to lower ratios compared to plasma samples that can be processed entirely at FT. By comparing absolute concentrations of 1-AG (Fig. 2, Fig. 3) and under the assumption that 1-AG originates solely from 2-AG, isomerization still takes place at FT and results in significant changes of 1-AG

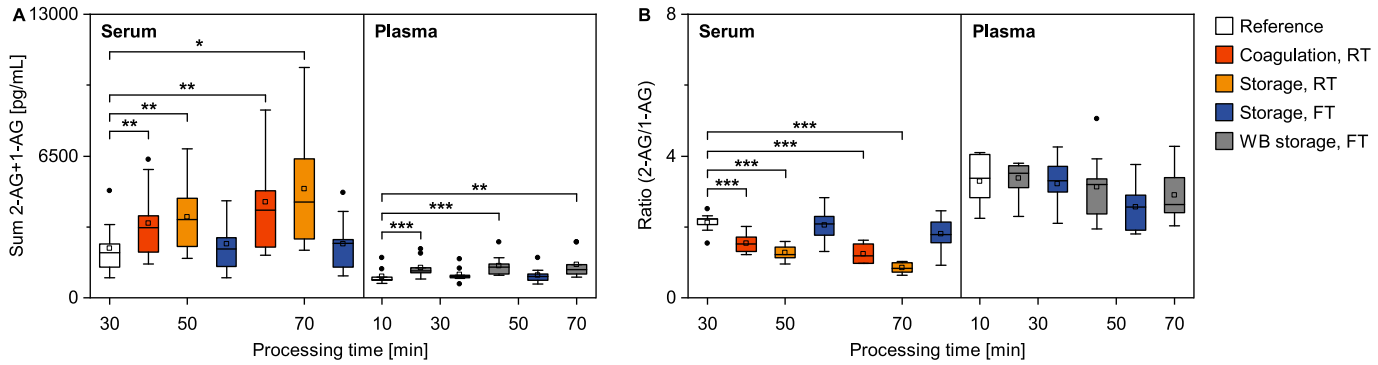

Fig. 7. Sum and ratios of 2-AG and 1-AG for different processing methods in serum and plasma. (A) shows absolute summed concentrations of 1-AG and 2-AG in pg/mL and (B) shows the respective underlying ratios of 2-AG/1-AG. Box and whiskers plot depicting Q1–Q3 with ± 1.5 IQR. Median (Q2) is plotted as a horizontal line and average as light square. Comparison against respective reference method, q-values of ≤ 0.05 *, ≤ 0.01 ** and ≤ 0.001 ***, respectively (see 2.5).

concentrations (processing time of 70 and 50 min, respectively). Therefore, short-term stable 2-AG/1-AG ratios could be just the result of an equilibrium between slow 2-AG release and 1-AG formation and highlights the need for fast sample processing.

The ratios for plasma observed here are higher than described in the literature and are presumably the result of swift and cooled sample processing [65]. Increased ratios seem to be closer to physiological conditions as there is no known mechanism to reverse 2-AG isomerization, and independent increases for 2-AG could not be observed. Additionally, previous studies of sodium fluoride/citrate plasma resulted in even higher average 2-AG/1-AG ratios [42] and demonstrate that there is still further potential for optimization regarding sample processing to capture accurate MAG ratios and concentrations. Recent research has highlighted the meaning of the 2-AG/1-AG ratio in regards to CBR1 activation [52]. While a distinct physiological function of 1-AG is still under debate, only accurate and separate determination of physiological 1-AG and 2-AG concentrations can further advance this field of research.

4. Conclusion

Potent lipid mediators such as ECs are highly regulated, prone to *ex vivo* changes of analyte concentration, and therefore need standardized and fully reported (pre-)analytical protocols. The presented data clearly indicate that already the selection of a blood-based matrix is incremental to resulting EC concentrations and should be made deliberately. Serum is not the matrix of choice when addressing the EC system as the initial storage of WB at RT for coagulation inevitably causes elevated EC concentrations. The initial storage time of WB before centrifugation is the most crucial step for high-quality data and should be standardized and performed under cooled conditions. Both plasma and serum can be stored short-term at FT after centrifugation even if the upper layer was not removed and allows for delays in (clinical) workflows without affecting initial and sample type-specific EC concentrations. By those means, even initial 2-AG/1-AG ratios can be maintained. While this ratio is not explanatory for the quality of pre-analytical sample handling itself, a decreased ratio can indicate prolonged storage at elevated temperatures, especially for serum samples. Fast and cooled pre-analytical sample processing can only be implemented when using plasma samples and should be employed if ECs are of interest. This avoids bringing more variability into the data than already present due to the high regulation of the EC system, and concentrations measured in this way are likely to reflect real physiological values closer. Knowledge about the limitations or ideally harmonization of pre-analytical processing has the potential to detect underlying trends in data from multiple sources and should therefore be of high priority for further studies.

CRediT authorship contribution statement

D. Kratz: Conceptualization, Investigation, Formal analysis, Visualization, Writing – original draft. **A. Sens:** Investigation, Formal analysis, Visualization, Writing – original draft. **S.M.G. Schäfer:** Investigation, Writing – review & editing. **L. Hahnefeld:** Writing – review & editing. **G. Geisslinger:** Supervision, Funding acquisition, Writing – review & editing. **D. Thomas:** Conceptualization, Project administration, Writing – review & editing. **R. Gurke:** Conceptualization, Writing – original draft, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2022.123102.

References

[1] C.J. Hillard, Circulating Endocannabinoids: From Whence Do They Come and Where are They Going? Neuropsychopharmacology 43 (1) (2018) 155–172, https://doi.org/10.1038/npp.2017.130.
[2] H.-C. Lu, K. Mackie, Review of the Endocannabinoid System, Biol. Psychiatry Cogn. Neurosci. Neuroimaging 6 (6) (2021) 607–615, https://doi.org/10.1016/j.bpsc.2020.07.016.
[3] K. Kleberg, H.A. Hassing, H.S. Hansen, Classical endocannabinoid-like compounds and their regulation by nutrients, BioFactors 40 (2014) 363–372, https://doi.org/10.1002/biof.1158.
[4] F.A. Iannotti, V. Di Marzo, S. Petrosino, Endocannabinoids and endocannabinoid-related mediators: Targets, metabolism and role in neurological disorders, Prog. Lipid Res. 62 (2016) 107–128, https://doi.org/10.1016/j.plipres.2016.02.002.
[5] L. Jean-Gilles, S. Feng, C.R. Tench, V. Chapman, D.A. Kendall, D.A. Barrett, C. S. Constantinescu, Plasma endocannabinoid levels in multiple sclerosis, J. Neurol. Sci. 287 (1-2) (2009) 212–215, https://doi.org/10.1016/j.jns.2009.07.021.
[6] A. Aran, M. Eylon, M. Harel, L. Polianski, A. Nemirovski, S. Tepper, A. Schnapp, H. Cassuto, N. Wattad, J. Tam, Lower circulating endocannabinoid levels in children with autism spectrum disorder, Mol. Autism 10 (2019) 2, https://doi.org/10.1186/s13229-019-0256-6.

[7] M. Grill, C. Högenauer, A. Blesl, J. Haybaeck, N. Golob-Schwarzl, N. Ferreirós, D. Thomas, R. Gurke, M. Trötzmüller, H.C. Köfeler, B. Gallé, R. Schicho, Members of the endocannabinoid system are distinctly regulated in inflammatory bowel disease and colorectal cancer, Sci. Rep. 9 (2019) 2358, https://doi.org/10.1038/s41598-019-38865-4.
[8] M.N. Hill, G.E. Miller, E.J. Carrier, B.B. Gorzalka, C.J. Hillard, Circulating endocannabinoids and N-acyl ethanolamines are differentially regulated in major depression and following exposure to social stress, Psychoneuroendocrinology 34 (8) (2009) 1257–1262, https://doi.org/10.1016/j.psyneuen.2009.03.013.
[9] I. Kaufmann, D. Hauer, V. Huge, M. Vogeser, P. Campolongo, A. Chouker, M. Thiel, G. Schelling, Enhanced anandamide plasma levels in patients with complex regional pain syndrome following traumatic injury: a preliminary report, Eur. Surg. Res. 43 (2009) 325–329, https://doi.org/10.1159/000235870.
[10] F.M. Leweke, A. Giuffrida, U. Wurster, H.M. Emrich, D. Piomelli, Elevated endogenous cannabinoids in schizophrenia, NeuroReport 10 (8) (1999) 1665–1669, https://doi.org/10.1097/00001756-199906030-00008.
[11] S. Engeli, J. Böhnke, M. Feldpausch, K. Gorzelniak, J. Janke, S. Bátkai, P. Pacher, J. Harvey-White, F.C. Luft, A.M. Sharma, J. Jordan, Activation of the peripheral endocannabinoid system in human obesity, Diabetes 54 (2005) 2838–2843, https://doi.org/10.2337/diabetes.54.10.2838.
[12] J. Lafreniere, C. Lehmann, Parameters of the Endocannabinoid System as Novel Biomarkers in Sepsis and Septic Shock, Metabolites 7 (4) (2017) 55, https://doi.org/10.3390/metabo7040055.
[13] L.K. Vaughn, G. Denning, K.L. Stuhr, H. de Wit, M.N. Hill, C.J. Hillard, Endocannabinoid signalling: has it got rhythm? Br. J. Pharmacol. 160 (2010) 530–543, https://doi.org/10.1111/j.1476-5381.2010.00790.x.
[14] E. Heyman, F.-X. Gamelin, M. Goekint, F. Piscitelli, B. Roelands, E. Leclair, V. Di Marzo, R. Meeusen, Intense exercise increases circulating endocannabinoid and BDNF levels in humans–possible implications for reward and depression, Psychoneuroendocrinology 37 (6) (2012) 844–851, https://doi.org/10.1016/j.psyneuen.2011.09.017.
[15] J.D. Meyer, K.M. Crombie, D.B. Cook, C.J. Hillard, K.F. Koltyn, Serum Endocannabinoid and Mood Changes after Exercise in Major Depressive Disorder, Med. Sci. Sports Exerc. 51 (9) (2019) 1909–1917, https://doi.org/10.1249/MSS.0000000000002006.
[16] E.C. Hanlon, E. Tasali, R. Leproult, K.L. Stuhr, E. Doncheck, H. de Wit, C.J. Hillard, E. van Cauter, Circadian rhythm of circulating levels of the endocannabinoid 2-arachidonoylglycerol, J. Clin. Endocrinol. Metab. 100 (2015) 220–226, https://doi.org/10.1210/jc.2014-3455.
[17] I. Matias, M.-P. Gonthier, S. Petrosino, L. Docimo, R. Capasso, L. Hoareau, P. Monteleone, R. Roche, A.A. Izzo, V. Di Marzo, Role and regulation of acylethanolamides in energy balance: focus on adipocytes and beta-cells, Br. J. Pharmacol. 152 (2007) 676–690, https://doi.org/10.1038/sj.bjp.0707424.
[18] O. Aizpurua-Olaizola, I. Elezgarai, I. Rico-Barrio, I. Zarandona, N. Etxebarria, A. Usobiaga, Targeting the endocannabinoid system: future therapeutic strategies, Drug Discov. Today 22 (1) (2017) 105–110, https://doi.org/10.1016/j.drudis.2016.08.005.
[19] D. Chanda, D. Neumann, J.F.C. Glatz, The endocannabinoid system: Overview of an emerging multi-faceted therapeutic target, Prostaglandins Leukot. Essent. Fatty Acids 140 (2019) 51–56, https://doi.org/10.1016/j.plefa.2018.11.016.
[20] F. Fanelli, V.D. Di Lallo, I. Belluomo, R. De Iasio, M. Baccini, E. Casadio, D. I. Gasparini, M. Colavita, A. Gambineri, G. Grossi, V. Vicennati, R. Pasquali, U. Pagotto, Estimation of reference intervals of five endocannabinoids and endocannabinoid related compounds in human plasma by two dimensional-LC/MS/MS, J. Lipid Res. 53 (3) (2012) 481–493, https://doi.org/10.1194/jlr.M021378.
[21] M. Blüher, S. Engeli, N. Klöting, J. Berndt, M. Fasshauer, S. Bátkai, P. Pacher, M. R. Schön, J. Jordan, M. Stumvoll, Dysregulation of the peripheral and adipose tissue endocannabinoid system in human abdominal obesity, Diabetes 55 (2006) 3053–3060, https://doi.org/10.2337/db06-0812.
[22] R. Jumpertz, T. Wiesner, M. Blüher, S. Engeli, S. Bátkai, H. Wirtz, A. Bosse-Henck, M. Stumvoll, Circulating endocannabinoids and N-acyl-ethanolamides in patients with sleep apnea–specific role of oleoylethanolamide, Exp. Clin. Endocrinol. Diabetes 118 (09) (2010) 591–595, https://doi.org/10.1055/s-0030-1253344.
[23] P. Monteleone, I. Matias, V. Martiadis, L. De Petrocellis, M. Maj, V. Di Marzo, Blood levels of the endocannabinoid anandamide are increased in anorexia nervosa and in binge-eating disorder, but not in bulimia nervosa, Neuropsychopharmacology 30 (6) (2005) 1216–1221, https://doi.org/10.1038/sj.npp.1300695.
[24] P. Caraceni, A. Viola, F. Piscitelli, F. Giannone, A. Berzigotti, M. Cescon, M. Domenicali, S. Petrosino, E. Giampalma, A. Riili, G. Grazi, R. Golfieri, M. Zoli, M. Bernardi, V. Di Marzo, Circulating and hepatic endocannabinoids and endocannabinoid-related molecules in patients with cirrhosis, Liver Int. 30 (2010) 816–825, https://doi.org/10.1111/j.1478-3231.2009.02137.x.
[25] V. Di Marzo, A. Verrijken, A. Hakkarainen, S. Petrosino, I. Mertens, N. Lundbom, F. Piscitelli, J. Westerbacka, A. Soro-Paavonen, I. Matias, L. van Gaal, M.-R. Taskinen, Role of insulin as a negative regulator of plasma endocannabinoid levels in obese and nonobese subjects, Eur. J. Endocrinol. 161 (2009) 715–722, https://doi.org/10.1530/EJE-09-0643.
[26] B. Gatta-Cherifi, I. Matias, M. Vallée, A. Tabarin, G. Marsicano, P.V. Piazza, D. Cota, Simultaneous postprandial deregulation of the orexigenic endocannabinoid anandamide and the anorexigenic peptide YY in obesity, Int. J. Obes. (Lond.) 36 (6) (2012) 880–885, https://doi.org/10.1038/ijo.2011.165.
[27] J.C. Sipe, T.M. Scott, S. Murray, O. Harismendy, G.M. Simon, B.F. Cravatt, J. Waalen, A. Vella, Biomarkers of endocannabinoid system activation in severe obesity, PLoS ONE 5 (1) (2010) e8792, https://doi.org/10.1371/journal.pone.0008792.
[28] C. Schroeder, S. Batkai, S. Engeli, J. Tank, A. Diedrich, F.C. Luft, J. Jordan, Circulating endocannabinoid concentrations during orthostatic stress, Clin. Auton. Res. 19 (6) (2009) 343–346, https://doi.org/10.1007/s10286-009-0026-1.
[29] M. Bilgin, L. Bindila, J. Graessler, A. Shevchenko, Quantitative profiling of endocannabinoids in lipoproteins by LC-MS/MS, Anal. Bioanal. Chem. 407 (17) (2015) 5125–5131, https://doi.org/10.1007/s00216-015-8559-8.
[30] M.S. Gachet, P. Rhyn, O.G. Bosch, B.B. Quednow, J. Gertsch, A quantitiative LC-MS/MS method for the measurement of arachidonic acid, prostanoids, endocannabinoids, N-acylethanolamines and steroids in human plasma, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 976–977 (2015) 6–18, https://doi.org/10.1016/j.jchromb.2014.11.001.
[31] M.G.J. Balvers, K.C.M. Verhoeckx, R.F. Witkamp, Development and validation of a quantitative method for the determination of 12 endocannabinoids and related compounds in human plasma using liquid chromatography-tandem mass spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877 (14-15) (2009) 1583–1590, https://doi.org/10.1016/j.jchromb.2009.04.010.
[32] A. Ozalp, B. Barroso, Simultaneous quantitative analysis of N-acylethanolamides in clinical samples, Anal. Biochem. 395 (1) (2009) 68–76, https://doi.org/10.1016/j.ab.2009.08.005.
[33] R. Ottria, A. Ravelli, F. Gigli, P. Ciuffreda, Simultaneous ultra-high performance liquid chromathograpy-electrospray ionization-quadrupole-time of flight mass spectrometry quantification of endogenous anandamide and related N-acylethanolamides in bio-matrices, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 958 (2014) 83–89, https://doi.org/10.1016/j.jchromb.2014.03.019.
[34] M.G.J. Balvers, K.C.M. Verhoeckx, J. Meijerink, H.M. Wortelboer, R.F. Witkamp, Measurement of palmitoylethanolamide and other N-acylethanolamines during physiological and pathological conditions, CNS Neurol. Disord. Drug Targets 12 (2013) 23–33, https://doi.org/10.2174/1871527311312010007.
[35] L. Lin, H. Yang, P.J.H. Jones, Quantitative analysis of multiple fatty acid ethanolamides using ultra-performance liquid chromatography-tandem mass spectrometry, Prostaglandins Leukot. Essent. Fatty Acids 87 (6) (2012) 189–195, https://doi.org/10.1016/j.plefa.2012.09.001.
[36] C. Schaefer, F. Enning, J.K. Mueller, J.M. Bumb, C. Rohleder, T.M. Odorfer, J. Klosterkötter, M. Hellmich, D. Koethe, C. Schmahl, M. Bohus, F.M. Leweke, Fatty acid ethanolamide levels are altered in borderline personality and complex posttraumatic stress disorders, Eur. Arch. Psychiatry Clin. Neurosci. 264 (5) (2014) 459–463, https://doi.org/10.1007/s00406-013-0470-8.
[37] R. Angelini, D.A. Argueta, D. Piomelli, N.V. DiPatrizio, Identification of a Widespread Palmitoylethanolamide Contamination in Standard Laboratory Glassware, Cannabis Cannabinoid Res. 2 (1) (2017) 123–132, https://doi.org/10.1089/can.2017.0019.
[38] M. Hill, G. Miller, W.-S. Ho, B. Gorzalka, C. Hillard, Serum endocannabinoid content is altered in females with depressive disorders: a preliminary report, Pharmacopsychiatry 41 (2) (2008) 48–53, https://doi.org/10.1055/s-2007-993211.
[39] D. Schreiber, S. Harlfinger, B.M. Nolden, C.W. Gerth, U. Jaehde, E. Schömig, J. Klosterkötter, A. Giuffrida, G. Astarita, D. Piomelli, F. Markus Leweke, Determination of anandamide and other fatty acyl ethanolamides in human serum by electrospray tandem mass spectrometry, Anal. Biochem. 361 (2) (2007) 162–168, https://doi.org/10.1016/j.ab.2006.11.027.
[40] J. Margolis, The effect of colloidal silica on blood coagulation, Aust. J. Exp. Biol. Med. Sci. 39 (3) (1961) 249–258, https://doi.org/10.1038/icb.1961.25.
[41] V.V. Hernandes, C. Barbas, D. Dudzik, A review of blood sample handling and pre-processing for metabolomics studies, Electrophoresis 38 (2017) 2232–2241, https://doi.org/10.1002/elps.201700086.
[42] L. Hahnefeld, R. Gurke, D. Thomas, Y. Schreiber, S.M.G. Schäfer, S. Trautmann, I. F. Snodgrass, D. Kratz, G. Geisslinger, N. Ferreirós, Implementation of lipidomics in clinical routine: Can fluoride/citrate blood sampling tubes improve preanalytical stability? Talanta 209 (2020) 120593, https://doi.org/10.1016/j.talanta.2019.120593.
[43] R. Gurke, D. Thomas, Y. Schreiber, S.M.G. Schäfer, S.C. Fleck, G. Geisslinger, N. Ferreirós, Determination of endocannabinoids and endocannabinoid-like substances in human K3EDTA plasma - LC-MS/MS method validation and pre-analytical characteristics, Talanta 204 (2019) 386–394, https://doi.org/10.1016/j.talanta.2019.06.004.
[44] W. Röhrig, S. Achenbach, B. Deutsch, M. Pischetsrieder, Quantification of 24 circulating endocannabinoids, endocannabinoid-related compounds and their phospholipid precursors in human plasma by UHPLC-MS/MS, J. Lipid Res. 60 (8) (2019) 1475–1488, https://doi.org/10.1194/jlr.D094680.
[45] J.T. Wood, J.S. Williams, L. Pandarinathan, A. Courville, M.R. Keplinger, D. R. Janero, P. Vouros, A. Makriyannis, C.J. Lammi-Keefe, Comprehensive profiling of the human circulating endocannabinoid metabolome: clinical sampling and sample storage parameters, Clin. Chem. Lab. Med. 46 (2008) 1289–1295, https://doi.org/10.1515/CCLM.2008.242.
[46] M. Vogeser, D. Hauer, S. Christina Azad, E. Huber, M. Storr, G. Schelling, Release of anandamide from blood cells, Clin. Chem. Lab. Med. 44 (2006) 488–491, https://doi.org/10.1515/CCLM.2006.065.
[47] A. Pastor, M. Farré, M. Fitó, F. Fernandez-Aranda, R. de la Torre, Analysis of ECs and related compounds in plasma: artifactual isomerization and ex vivo enzymatic generation of 2-MGs, J. Lipid Res. 55 (5) (2014) 966–977, https://doi.org/10.1194/jlr.D043794.
[48] J.R. Denery, A.A.K. Nunes, T.J. Dickerson, Characterization of differences between blood sample matrices in untargeted metabolomics, Anal. Chem. 83 (3) (2011) 1040–1047, https://doi.org/10.1021/ac102806p.
[49] Z. Yu, G. Kastenmüller, Y. He, P. Belcredi, G. Möller, C. Prehn, J. Mendes, S. Wahl, W. Roemisch-Margl, U. Ceglarek, A. Polonikov, N. Dahmen, H. Prokisch, L. Xie, Y.

Li, H.-E. Wichmann, A. Peters, F. Kronenberg, K. Suhre, J. Adamski, T. Illig, R. Wang-Sattler, Differences between human plasma and serum metabolite profiles, PLoS ONE 6 (2011) e21230. Doi: 10.1371/journal.pone.0021230.
[50] E.V. Berdyshev, P.C. Schmid, R.J. Krebsbach, H.H.O. Schmid, Activation of PAF receptors results in enhanced synthesis of 2-arachidonoylglycerol (2-AG) in immune cells, FASEB J. 15 (12) (2001) 2171–2178, https://doi.org/10.1096/fj.01-0181com.
[51] T.L. Pedersen, I.J. Gray, J.W. Newman, Plasma and serum oxylipin, endocannabinoid, bile acid, steroid, fatty acid and nonsteroidal anti-inflammatory drug quantification in a 96-well plate format, Anal. Chim. Acta 1143 (2021) 189–200, https://doi.org/10.1016/j.aca.2020.11.019.
[52] M. Maccarrone, M. Bari, A. Menichelli, E. Giuliani, D. Del Principe, A. Finazzi-Agrò, Human platelets bind and degrade 2-arachidonoylglycerol, which activates these cells through a cannabinoid receptor, Eur. J. Biochem. 268 (2001) 819–825, https://doi.org/10.1046/j.1432-1327.2001.01942.x.
[53] K. Dócs, Z. Mészár, S. Gonda, A. Kiss-Szikszai, K. Holló, M. Antal, Z. Hegyi, The Ratio of 2-AG to Its Isomer 1-AG as an Intrinsic Fine Tuning Mechanism of CB1 Receptor Activation, Front. Cell. Neurosci. 11 (2017) 39, https://doi.org/10.3389/fncel.2017.00039.
[54] T.H. Marczylo, P.M.W. Lam, V. Nallendran, A.H. Taylor, J.C. Konje, A solid-phase method for the extraction and measurement of anandamide from multiple human biomatrices, Anal. Biochem. 384 (1) (2009) 106–113, https://doi.org/10.1016/j.ab.2008.08.040.
[55] P.M.W. Lam, T.H. Marczylo, J.C. Konje, Simultaneous measurement of three N-acylethanolamides in human bio-matrices using ultra performance liquid chromatography-tandem mass spectrometry, Anal. Bioanal. Chem. 398 (5) (2010) 2089–2097, https://doi.org/10.1007/s00216-010-4103-z.
[56] L.F. Lacey, O.N. Keene, J.F. Pritchard, A. Bye, Common noncompartmental pharmacokinetic variables: are they normally or log-normally distributed? J. Biopharm. Stat. 7 (1) (1997) 171–178, https://doi.org/10.1080/10543409708835177.
[57] Y. Benjamini, Y. Hochberg, Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing, J. Roy. Stat. Soc.: Ser. B (Methodol.) 57 (1) (1995) 289–300.
[58] W. Jian, R. Edom, N. Weng, P. Zannikos, Z. Zhang, H. Wang, Validation and application of an LC-MS/MS method for quantitation of three fatty acid ethanolamides as biomarkers for fatty acid hydrolase inhibition in human plasma, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 878 (20) (2010) 1687–1699, https://doi.org/10.1016/j.jchromb.2010.04.024.
[59] S.G. Young, R. Zechner, Biochemistry and pathophysiology of intravascular and intracellular lipolysis, Genes Dev. 27 (5) (2013) 459–484, https://doi.org/10.1101/gad.209296.112.
[60] D. Kratz, D. Thomas, R. Gurke, Endocannabinoids as potential biomarkers: It's all about pre-analytics, J. Mass Spectrometry Adv. Clin. Lab 22 (2021) 56–63, https://doi.org/10.1016/j.jmsacl.2021.11.001.
[61] D. Tsikas, Quantitative analysis of eicosanoids in biological samples by LC-MS/MS: Mission accomplished? J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 1012-1013 (2016) 211–214, https://doi.org/10.1016/j.jchromb.2015.04.018.
[62] C.J. Hillard, H. Huang, C.D. Vogt, B.E. Rodrigues, T.S. Neumann, D.S. Sem, F. Schroeder, C.W. Cunningham, Endocannabinoid Transport Proteins: Discovery of Tools to Study Sterol Carrier Protein-2, Methods Enzymol. 593 (2017) 99–121, https://doi.org/10.1016/bs.mie.2017.06.017.
[63] C.J. Hillard, A. Jarrahian, Cellular accumulation of anandamide: consensus and controversy, Br. J. Pharmacol. 140 (2003) 802–808, https://doi.org/10.1038/sj.bjp.0705468.
[64] A.A. Zoerner, S. Batkai, M.-T. Suchy, F.-M. Gutzki, S. Engeli, J. Jordan, D. Tsikas, Simultaneous UPLC-MS/MS quantification of the endocannabinoids 2-arachidonoyl glycerol (2AG), 1-arachidonoyl glycerol (1AG), and anandamide in human plasma: minimization of matrix-effects, 2AG/1AG isomerization and degradation by toluene solvent extraction, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 883–884 (2012) 161–171, https://doi.org/10.1016/j.jchromb.2011.06.025.
[65] A.A. Zoerner, F.-M. Gutzki, S. Batkai, M. May, C. Rakers, S. Engeli, J. Jordan, D. Tsikas, Quantification of endocannabinoids in biological systems by chromatography and mass spectrometry: a comprehensive review from an analytical and biological perspective, Biochim. Biophys. Acta 1811 (11) (2011) 706–723, https://doi.org/10.1016/j.bbalip.2011.08.004.