Development of UHPLC–MS/MS method for determination and quantification of endocannabinoids in cerebrospinal fluid

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Abstract

Endocannabinoids (eCBs) are endogenous lipids that activate cannabinoid receptors. Their role in neuroinflammation is of a great interest due to the range of physiological effects they exhibit, potentially serving as bioindicators of pathologies. However, they are present at nanomolar concentrations in biological samples, posing challenges for detection and quantification. A method for quantitative analysis of eight exogenous eCB internal standards (anandamide-d4, docosahexaenoyl ethanolamide-d4, eicosapentaenoyl ethanolamide-d4, linoleoyl ethanolamide-d4, oleoyl ethanolamide-d4, palmitoyl ethanolamide-d5, stearoyl ethanolamide-d3 and palmitanilide) in cerebrospinal fluid is reported here. Six monophasic solvents (methanol, acetonitrile, chloroform/methanol/water, acetonitrile/acetone, isopropanol/acetonitrile/water, and ethanol) were compared in their extraction efficiency. Detection of analytes was performed using an ultra-high performance liquid chromatography method coupled to tandem mass spectrometry (UHPLC-MS/MS) in dynamic multiple reaction monitoring (dMRM) mode. The method was further optimised based on accuracy, precision, matrix effect, linearity, limits of detection and quantification. The method was applied to a cohort of healthy individuals (n=33) to identify and estimate the concentration of eCBs in CSF.

Keywords: Endocannabinoids, cerebrospinal fluid, UHPLC-MS/MS, method development, liquid-liquid extraction.

Abbreviations: eCB = endocannabinoid; CSF = cerebrospinal fluid; CNS = central nervous system; AD = Alzheimer's disease; UHPLC-MS/MS = ultra-high performance liquid chromatography coupled to tandem mass spectrometry; dMRM = dynamic multiple reaction monitoring.

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Introduction

The endocannabinoid system (ECS) is a widely distributed physiological signaling system throughout the body. It involves the participation of cannabinoid receptors CB_1 and CB_2 which are G-protein coupled receptors, along with their endogenous agonists called endocannabinoids (eCBs) [1].While CB_1 receptors are predominantly expressed on the neuronal and glial cells within the central nervous system (CNS), they also have a presence beyond the CNS [2]. CB_2 receptors are primarily located in circulating peripheral immune cells and macrophage derived cells outside the CNS [3]. The dysregulation of ECS has been associated with neurodegeneration [4], neuroinflammation, brain trauma, anxiety, depression [5] and pain perception [6].

Most of the existing clinical studies have primarily focused on quantifying eCBs in human plasma or human brain tissue [7,8] which presents limitations in our understanding of ECS. However, the cerebrospinal fluid (CSF) emerges as a better-suited biospecimen choice due to its close connection with the brain. CSF offers a deeper insight into the brain neurochemistry than blood samples because it is a better reflection of the metabolite levels in the brain [8]. Analysis of eCBs in CSF poses some challenges. Firstly, they are endogenous compounds, and there is an absence of a blank matrix for calibration and validation. Moreover, it is also important to note that eCBs exist as low-abundance metabolites, often found at exceedingly low concentrations in the CSF. Studies have reported presence of eCBs and their structural analogues in human CSF, with concentration ranging in the picomolar to even femtomolar concentration range [9]. Adding to the complexity is the limited volume of CSF that can be collected, as the average brain CSF volume is approximately 150 mL [10]. The combination of restricted sample volume and low abundance of these compounds poses significant challenges when it comes to identifying and quantifying the eCBs within the CSF, highlighting the need for a robust and sensitive analytical method to measure the metabolites with the highest level of reliability.

eCBs present in biofluids are typically analysed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) following solid-phase extraction or protein precipitation [11,12].While some study reports have used gas chromatography coupled to mass spectrometry (GC-MS) [13] to quantify the eCBs however, LC is preferred to GC due to the time-consuming derivatization step in GC, which can increase the variability [11]. Furthermore, LC-MS/MS employs a highly selective and sensitive analytical approach which can be further enhanced by employing the dynamic multiple reaction monitoring (dMRM) method developed to quantify the target metabolites with maximized coverage and sensitivity [14].

Hence, we aimed to develop a sensitive and robust UHPLC-MS/MS method to identify and quantify eCBs and their structural analogues in human CSF samples. To achieve this goal, the following steps were undertaken: (i) Compare the extraction efficiency of various monophasic solvents, methanol (MeOH), acetonitrile (ACN), chloroform/methanol/water ($CHCl_3$/MeOH/H_2O), acetonitrile/acetone (ACN/Ace), isopropanol/acetonitrile/water (IPA/ACE/H_2O) and ethanol (EtOH). (ii) Validate the analytical method for quantification of eCBs in human CSF, complying with regulatory standards and assessment of the results based on the parameters including, accuracy, precision, matrix effect, linearity, limit of detection (LOD) and limit of quantification (LOQ).

Experimental Section

Chemicals and reagents

LC-MS grade solvents and reagents were used. Acetonitrile (ACE), formic acid (FA), isopropanol (IPA), methanol (MeOH) and ultra-pure water were purchased from Fisher Scientific (Roskilde, Denmark). Ethanol (EtOH) and acetone (Ace) were acquired from Sigma-Aldrich (Copenhagen,

Denmark). All solvents and reagents were of ≥98% purity. FA was used as an ionising agent, ACN, IPA and ultra-pure water were utilised for the preparation of the chromatographic mobile phases.

The standard reagents of anandamide-d_4 (AEA-d_4), docosahexaenoyl ethanolamide-d_4 (DEA-d_4), eicosapentaenoyl ethanolamide-d_4 (EPEA-d_4), linoleoyl ethanolamide-d_4 (LEA-d_4), oleoyl ethanolamide-d_4 (OEA-d_4), palmitoyl ethanolamide-d_5 (PEA-d_5), and stearoyl ethanolamide-d_3 (SEA-d_3) were acquired from BioNordika (Herlev, Denmark). The standard reagents of palmitanilide (PAN) were obtained from TCI Europe N.V. (Zwijndrecht, Belgium).

Instruments and apparatus

A centrifuge 5427 R from Eppendorf (Hamburg, Germany) was utilised for centrifugation during sample preparation. A SPD130DLX SpeedVac cold trap concentrator from Thermo Scientific (Midland, MI, USA) was also used for sample preparation.

The analytes were separated by liquid chromatography, followed by electrospray ionization (ESI) in positive mode and dMRM MS/MS detection. The analytical system comprised of Agilent 1290 Infinity UHPLC system coupled with an Agilent 6460 triple quadrupole (QqQ) mass spectrometer from Agilent Technologies Inc. (Santa Clara, CA, USA). The data were processed using Agilent MassHunter quantitative software (version 10.2) for the analyte analysis.

Preparation of standards and internal standard solutions

A standard stock solution of various nonendogenous reference standards was prepared by dissolving each of the eight analytes in pure organic solvent (MeOH, EtOH or ACN) to reach an adequate concentration of ~1000 mg/L, and the samples were stored at –20°C. An internal standard (ISTD) mixture of the analytes at 10 mg/L was prepared in ethanol and stored at – 20°C. Seven calibration concentration levels were created from ISTD stock solutions at 0.12, 0.48, 1.9, 7.8, 31.25, 125 and 500 ng/mL. A multistandard working solution that contains all the standards was prepared at a concentration of 10 mg/L in methanol and stored at – 20°C in the dark for use within six months.

CSF samples

The CSF samples were collected and donated by the Danish Dementia Research Centre, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark. For method development a CSF pool was prepared by mixing leftover CSF samples from 10 anonymized individuals. For method application, CSF samples from a cohort of 33 individuals, females (n=19) and males (n=14) were collected for identification and quantification of endogenous endocannabinoids in human biofluid. The individuals were determined to be 'healthy' if they had no cognitive impairments. The samples were stored frozen at – 80 °C until processing.

Comparison of six monophasic extraction solvent systems

To evaluate extraction efficiency in pooled human CSF samples, six different monophasic extraction solvents were simultaneously compared. To cover a broad range of polarities, the following extraction solvents were examined: MeOH (extraction 1), ACN (extraction 2), ACN/Ace 1:1 (v/v) (extraction 3), $CHCl_3$/ MeOH/H_2O 2:5:2 (v/v/v) (extraction 4), IPA/ACE/ H_2O 3:3:2 (v/v/v) (extraction 5), and EtOH (extraction 6). The analytes were extracted using a monophasic extraction.

50 µL of CSF was aliquoted in an Eppendorf tube. ISTD mixture was diluted in EtOH to make up a concentration of 1 ng/µL. 10 µL of the nonendogenous ISTD mixture was added to each CSF sample prior to extraction. The ISTDs were extracted by adding a mixture of six different extraction solvent mixtures, individually to each CSF aliquot. The CSF samples were vortexed for 5 seconds, shaken at 1500 RPM for 5 minutes at 4 ºC and vortexed again for 5s before being centrifuged at 12,700 RPM for 5 min at 4ºC. The supernatants (450 µL) were evaporated to dryness with a

SpeedVac cold trap concentrator. All dried extracts were reconstituted in a 50 µL mixture of ethanol and toluene 9:1 (v/v). The reconstituted samples were analysed by UHPLC-MS/MS system and the extractions and analyses were carried out in six replicates.

UHPLC-MS/MS method

The separation of the target analytes was carried out using Waters HSS T3 (1.8 µm, 2.1 × 100 mm) column, protected by C18 HSS T3 VanGuard pre-column (100Å, 1.8 µm, 2.1 mm × 5 mm) from Waters (Taastrup, Denmark). The injection volume was 50 µL. The column temperature was set to 45°C. Mobile phase A was water with 0.1% formic acid, while mobile phase B was acetonitrile/isopropanol (v/v) with 0.1% formic acid. The flow rate was 0.4 mL min^{-1}.The following gradient profile was employed, in positive mode analysis: From 0 to 1 minutes 60% A and 40% B, the percentage of A was then reduced to 20% between 1 to 2 minutes, for the next 7 minutes the percentage of A was reduced to 0% and then ramped up quickly to the starting conditions at 60% between 9 to 12 minutes. The total analysis time was 12 minutes. Instrument-dependent parameters for MS/MS were set as follows: the nitrogen drying gas flow rate was 12 L/min, with a temperature of 325 °C; the capillary voltage was 3500; the nebulizer pressure was maintained at 45 psi; the nitrogen sheath gas flow rate was 11.0 L/min, with a temperature of 325 °C. The dMRM was used for data collection, it monitored and selected the precursor product ion transitions of each analyte (Table 1).

Table 1
MRM transitions and target specific MS parameters of target analytes (*n=8*) used for the UHPLC-MS/MS analysis method.

Analyte	Q_1 [m/z]	Q_3 [m/z]	Transition	Fragment (v)	CE (v)
AEA-d_4	352.3	66.2	Quantifier	92	13
		91.1	Qualifier	92	53
DEA-d_4	376.3	66.2	Quantifier	128	17
		91.1	Qualifier	128	57
EPEA-d_4	350.3	66.2	Quantifier	106	5
		91.1	Qualifier	106	57
LEA-d_4	324.2	62.2	Quantifier	106	13
		67.2	Qualifier	106	45
OEA-d_4	330.3	66.2	Quantifier	106	13
		55.2	Qualifier	106	49
PAN	332.3	94.1	Quantifier	196	45
		77.1	Qualifier	196	61
PEA-d_5	305.3	62	Quantifier	128	13
		57.2	Qualifier	128	37
SEA-d_3	331.3	62.2	Quantifier	128	13
		57.2	Qualifier	128	41

Q_1 – first quadrupole, Q_3 – third quadrupole, CE – collision energy.

Method validation

The method was optimised via the validation parameters outlined by the U.S. Food and Drug Administration (FDA) guidelines [15].

Recovery and precision were determined by spiking CSF and standards with ISTDs. Recovery % was evaluated as the ratio of the peak areas of CSF samples against peak areas of standards, multiplied by 100. The precision was determined by calculating % RSD. The inter-day recovery and precision were calculated based on the data collected from CSF and standards at three

concentration levels of QCs by analysing batches over a period of three validation days. QC samples at each concentration level were run in six replicates.

Matrix effect was determined based on CSF and standards spiked with ISTDs before extraction. The matrix interference was evaluated based on mean peak area ratios of CSF samples post extraction to standards in organic solvent.

Linearity was investigated by examining the calibration curve of ISTDs at seven concentration levels, fitted to a linear regression model. Visualizations were carried out in R version 4.3.0.

Ethical Approval

The current study received approval from the Ethics Committee of the Danish Capital Region (Journal No.: H-21051757) and The Danish Data Protection Agency (Journal No.: P-2022-97). Informed consent was obtained from all subjects selected for the study prior to their participation at the Copenhagen Memory Clinic, Copenhagen University Hospital, Rigshospitalet. A total of 33 subjects participated in the study as non-demented healthy controls (NDHC).

Results and Discussion

Solvent extraction efficiency comparison

The extraction efficiency of six different monophasic solvents was compared by assessing both extraction recovery and precision of the eight analytes.

The extraction recovery (%) was expressed as the mean peak area of the analytes extracted from CSF in relation to the mean peak area of analytes extracted from the internal standards prepared in organic solvent. The extraction recovery of the target analytes (*n=8*) (Figure 1) in MeOH was within the range of 95-132%, 87-115% in ACN, 65-95% in $CHCl_3$/MeOH/H_2O, 81-106% in ACN/Ace, 52-144% in IPA/ACN/H_2O, and 51-146% in EtOH. The acceptable recommended range for extraction efficiency is between 70-120% [16]. This parameter was satisfied by three out of six solvent mixtures, ACN, $CHCl_3$/MeOH/H_2O and ACN/Ace.

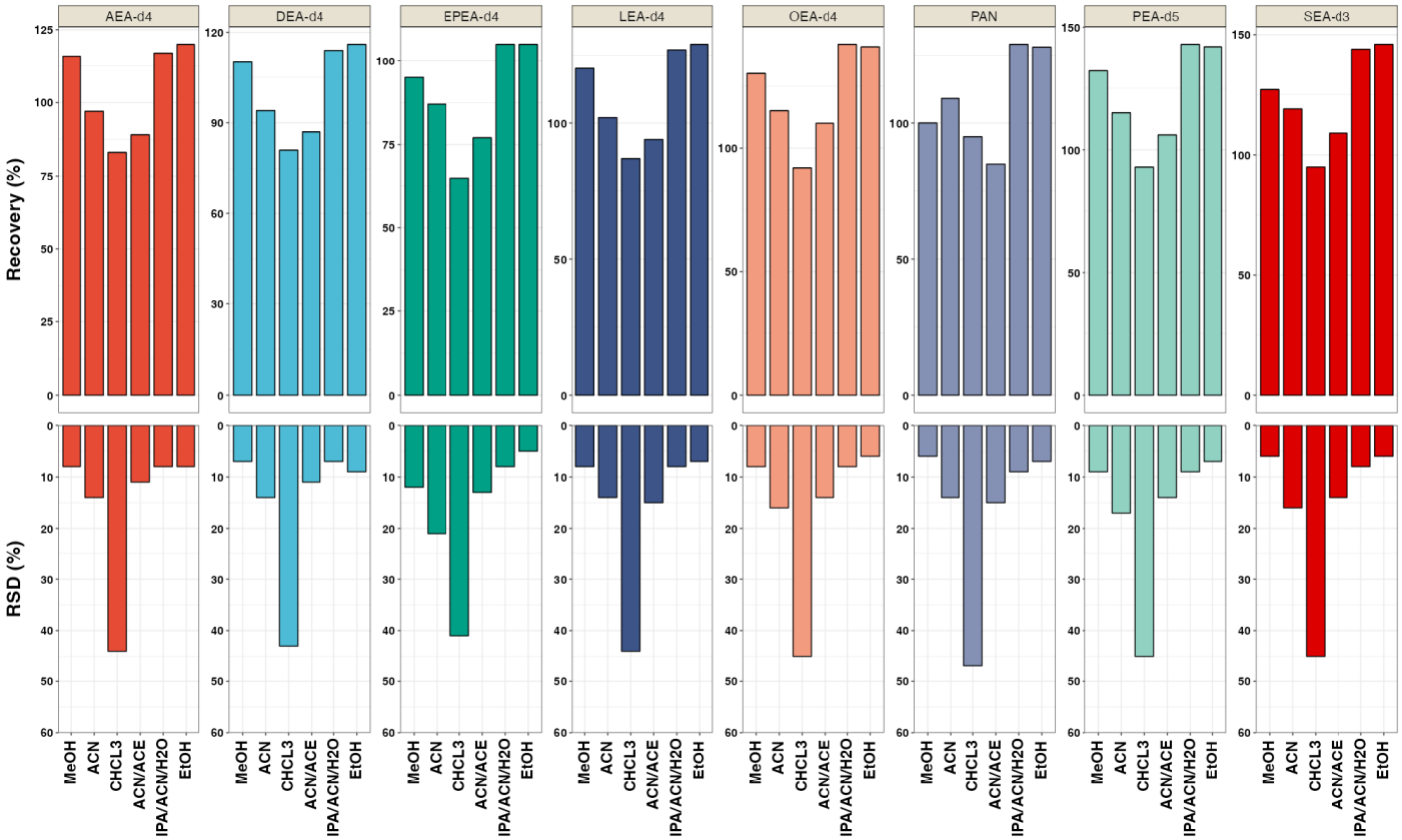
Evaluation of the precision of the solvents for each target analyte (*n=8*) was based on the calculation of the extraction recovery variability expressed as relative standard deviation (%RSD) [17]. The RSD (Figure 1) was in the range between 6-12% in MeOH, 14-21% in ACN, 41-47% in $CHCl_3$/MeOH/H_2O, 15-20% ACN/Ace, 7-9% in IPA/ACN/H_2O, and 5-9% in EtOH. Journal of Chromatography B guidelines require the precision of analytical methods to be within 20% RSD [18]. Four out of six solvents, MeOH, ACN/Ace, IPA/ACN/H_2O and EtOH, satisfied these requirements.

The recovery results were promising for $CHCl_3$/MeOH/H_2O and similar efficiency was observed by Reichl et al. [19] when utilizing similar method for the liquid-liquid extraction of endogenous lipids in CSF. However, the precision of the results was not satisfactory, with values exceeding 20% RSD. This suggests that there is high variability in the data therefore, this mixture was not selected as an efficient solvent for the extraction of eCBs from CSF. Similarly, ACN extraction recovery was within the accepted range however, the results had higher variability than the ACN/Ace mixture. One study reported that Ace tends to co-precipitate lipids with proteins, reporting 20-50% of the precipitate content being lipids [20]. Another report showed that Ace protein precipitation leaves low molecular weight proteins behind in the soluble fraction whereas, ACN tends to precipitate most of the proteins in the sample [21]. The combination of ACN/Ace solvent mixture showed acceptable recovery and precision results in comparison to the remaining five solvents (Figure 1). It seems that individually, Ace and ACN do not comply with the validation parameters. Although, this study does not explore the extraction efficiency of Ace by itself. Better performance of the ACN/Ace solvent mixture can be

due to the fact that ACN contributes to the efficient protein precipitation of the CSF matrix constituents while Ace is a good polar solvent for polar phospholipids like eCBs. However, the validity of this statement could be improved by comparing the extraction efficiency of Ace on its own against other monophasic solvents.

The conditions for determining the most efficient solvent were met by ACN/Ace solvent mixture. On the contrary, the five remaining solvents did not satisfy the requirements and, therefore, were not selected for further method validation.

Figure 1: Extraction recovery and precision (%RSD) of the target analytes (*n=8*) tested in six different monophasic solvents. MeOH = methanol. ACN = acetonitrile. CHCL3 = chloroform/methanol/water. ACN/Ace = acetonitrile/acetone. IPA/ACN/H2O = isopropanol/acetonitrile/water. EtOH = ethanol.



Inter-day recovery and precision of the method

The accuracy of the method was assessed by analysis of the mean results obtained in the non-spiked CSF aliquots of the samples in relation to known spiked (internal standards) concentrations. The target analytes (*n=8*) were analysed at low, mid and high concentration levels. Quality control samples (QCs) were run in six replicates on three consecutive days, as recommended by the FDA [15]. The analytes were extracted using ACN/Ace solvent mixture. The mean of the accuracy values reported as inter-day recoveries are listed in Table 3.

All target analytes reported interday recovery values ranging between 80-120% which is the recommended range [15]. Similarly, a study by Castillo-Petnado et al. [11] reported similar results.

The inter-day precision was reported as %RSD (Table 2). Inter-day precision varied between 3% to 12% which is within the recommended 20%. This suggests that the variability and repeatability of the results were within the tolerance limits. A study by Kantae et al. [22] reports similar results when developing quantification methods for eCBs using nano-flow LC-MS. Another study concluded equivalent results with micro-flow LC-MS method developed for quantification of eCBs [1].

Matrix effect

Matrix effect was evaluated at three QCs concentration levels (low, mid, high). The matrix interference was calculated based on the mean calibration of target analytes in biological matrix (CSF) against a calibration of the target analytes in the internal standards for parallelism, as recommended by the FDA [15]. The matrix effect results as listed in Table 3.
, range between 85-100%. The values of 100% were considered as no effect. If the response in the matrix was suppressed or enhanced by more than 20% it was concluded that the results were affected by the matrix effect [23]. No significant interference from the matrix was observed. The variability of the matrix interference results was within 20% RSD for all the target analytes (*n=8*). It is essential that there is no or minimum interference from the matrix. When handling CSF samples with an aim to extract a specific group of compounds, eCBs in this case, it is important to note that CSF is a complex biological fluid with various constituents.

A study by Aydin et al [24] reported slight ion enhancement in the matrix effect. They have used surrogate CSF which may have resulted in increased ionization efficiency of eCBs due to the presence of another compound. Since the surrogate CSF only mimics the concentration levels of its constituents and is only an estimate, it may not be an accurate representation of the human CSF composition. This study analysed real CSF samples which are more applicable in clinical settings.

Table 2
Mean inter-day recovery and precision (%RSD) of the target analytes (n=8) at three different concentration levels (low = 0.9, mid = 9, high = 90) ng mL^{-1} extracted with ACN/Ace over a period of three days.

Analyte	Mean Inter-day Recovery (%) ± RSD (%)								
	Day 1			Day 2			Day 3		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
EPEA-d_4	86 ± 7	100 ± 7	102 ± 8	90 ± 6	101 ± 7	93 ± 5	105 ± 11	104 ± 8	96 ± 4
DEA-d_4	90 ± 6	106 ± 8	107 ± 7	97 ± 10	104 ± 7	97 ± 4	101 ± 10	108 ± 7	98 ± 4
AEA-d_4	92 ± 9	102 ± 7	104 ± 7	97 ± 7	104 ± 6	97 ± 5	104 ± 15	103 ± 7	96 ± 4
LEA-d_4	97 ± 6	109 ± 7	107 ± 7	106 ± 6	111 ± 6	103 ± 4	118 ± 14	112 ± 8	101 ± 4
PEA-d_5	102 ± 8	113 ± 7	103 ± 10	102 ± 6	113 ± 7	103 ± 3	114 ± 12	118 ± 8	105 ± 4
OEA-d_4	98 ± 6	109 ± 8	104 ± 9	117 ± 4	120 ± 7	109 ± 5	112 ± 10	114 ± 6	101 ± 4
SEA-d_3	100 ± 5	114 ± 7	106 ± 10	108 ± 4	114 ± 5	105 ± 4	115 ± 9	117 ± 7	106 ± 5
PAN	93 ± 9	106 ± 7	92 ± 12	111 ± 5	119 ± 5	109 ± 11	101 ± 10	103 ± 7	91 ± 5

Limit of detection, limit of quantification and linearity

The method was further validated with evaluation of linearity, LOD and LOQ. The validation was carried out according to the FDA recommendations [15]. The LOD and LOQ were determined by spiking the CSF and internal standards with low concentrations of the standard compounds. The LOD and LOQ (Table 4) were calculated as three and ten times the concentration estimate standard deviation of the spiked samples, respectively [23].

The analytes were extracted using ACN/Ace and the data were collected over a period of three days. The LODs of the analytes extracted from CSF and standards ranged between 0.101-0.154 ng mL^{-1} and 0.023-0.071 ng mL^{-1}, respectively. The LOQs of the analytes extracted from CSF and standards ranged from 0.338 - 0.515 ng mL^{-1} and 0.077-0.237 ng mL^{-1}, respectively. The calibration curve was fitted by a linear regression. For all target analytes (*n=8*) the coefficient of determination (R^2) was 0.99 (Table 4). The linearity was determined using a calibration curve of spiked internal

standards with seven different analyte concentrations at 0.12, 0.48, 1.9, 7.8, 31.25, 125 and 500 ng mL^{-1}.

Specificity and sensitivity

The analytical method was optimized by ensuring that all target analytes (*n=8*) were identified. For assay specificity, dMRM transitions in positive mode of the eight endocannabinoids were acquired. The specificity was confirmed by injecting the individual calibrants and monitoring MRM transitions to identify ion fragmentations of each analyte. The CSF and internal standard samples were then compared against the individual ion fragments of each analyte for identification.

The LOQs of CSF and standards were similar. However, in the CSF the LOQs appeared to be at a slightly higher range (Table 4)

Table 3
Mean matrix effect of each target analyte (*n=8*) at three different concentration levels (low = 0.9, mid = 9, high = 90) ng mL^{-1} extracted with ACN/Ace.

Analyte	Mean Matrix Effect (%) ± RSD (%)		
	Low	Medium	High
EPEA-d_4	85 ± 2	89 ± 4	91 ± 3
DEA-d_4	91 ± 4	92 ± 4	94 ± 3
AEA-d_4	85 ± 4	88 ± 5	89 ± 2
LEA-d_4	87 ± 3	90 ± 4	94 ± 2
PEA-d_5	85 ± 4	88 ± 4	92 ± 3
OEA-d_4	85 ± 3	89 ± 3	92 ± 3
SEA-d_3	87 ± 3	90 ± 3	94 ± 3
PAN	90 ± 3	95 ± 4	100 ± 3

Performance comparison to other works

The LOQ of this method for OEA-d_4 (0.374 ng mL^{-1}) was lower in comparison to a previous approach using a similar method (3.62 ng mL^{-1}) [24]. The AEA LOQ of 1.07 ng mL^{-1} in human CSF was reported in another study [1] using a micro-flow LC-MS/MS approach. The LOQ of AEA presented by the method herein was significantly lower, 0.505 ng mL^{-1}.

The main proposed advantage of micro-flow system to UHPLC is obtaining similar or lower LODs. He et al. [1] obtained AEA LOD value of 0.312 ng mL^{-1}. This study reported lower LOD of AEA in CSF (0.152 ng mL^{-1}). Therefore, it can be suggested that UHPLC is an alternative approach when analysing low abundance compounds in biofluids with restricted volumes, such as CSF. Moreover, another study concluded that micro-flow LC-MS can take longer to run compared to UHPLC-MS/MS. In fact, the total analysis time was 12 min, slightly shorter in comparison to previous works that have utilised micro and nano flow LC-MS/MS, 16 min [1] and 20 min [22], respectively.

While it is clear that previous developed methods are robust and sensitive, it is worth noting that this method can have increased widespread use as micro-flow or nano-LC-MS/MS are not always an option in the analytical lab [26].

Table 4
Limit of detection (LOD), limit of quantification (LOQ) and coefficient of determination (R^2) values of target analytes (*n=8*) measured over a period of three days at 0.9 ng mL^{-1}.

Analyte	R^2	Standard		CSF	
		LOD (ng/ml)	LOQ (ng/ml)	LOD (ng/ml)	LOQ (ng/ml)
AEA-d_4	0.998	0.064	0.212	0.152	0.505
DEA-d_4	0.996	0.066	0.222	0.123	0.409
EPEA-d_4	0.999	0.064	0.214	0.115	0.384
LEA-d_4	0.995	0.040	0.132	0.154	0.515
OEA-d_4	0.999	0.035	0.116	0.112	0.374
PAN	0.997	0.071	0.237	0.120	0.402
PEA-d_5	0.999	0.062	0.205	0.144	0.481
SEA-d_3	0.996	0.023	0.077	0.101	0.338

Applications of the method

eCBs are lipid molecules within the eicosanoid family, resulting from the degradation of membrane phospholipids and triglycerides [27]. They act as neurotransmitters however, they are synthesized and released from postsynaptic neurons and travel back across the synaptic gap to interact with extracellular cannabinoid receptors on neighboring nerve terminals [28]. One of the characteristics that differentiates eCBs from conventional neurotransmitters is their lack of accumulation within the interior of the synaptic vesicles [27]. They act as messengers at the synapse by inhibiting neuronal transmission and undergo inactivation through reuptake or enzymatic degradation [29]. Arachidonoylethanolamine (AEA), also known as anandamide and 2–arachidonoylglycerol (2-AG) are the most extensively studied eCBs. Other structural analogues have also been identified , such as oleamide (OLE), *N*-linoleoyl ethanolamide (LEA), *N*-stearoyl ethanolamide (SEA) and many more. The primary site of eCB synthesis is in the brain [30] however, some studies have reported intracellular stores of anandamide outside of the synaptic vesicles [27]. Human adipocytes have been identified as a novel site of anandamide synthesis [30]. Anandamide binds intracellularly to peroxisome proliferator-activated receptors, PPARα and PPARγ, thereby promoting the ligand-dependent transcription of target genes that induce anti-inflammatory responses [31]. Furthermore, anandamide interacts with transient receptor potential vanniloid-1 (TRPV-1) channels which are responsible for detecting nociceptive stimuli in sensory nerve fibers [32]. Anandamide acts as a paracrine ligand of cannabinoid receptors, and intracellular ligand to PPARs and TRPV1 channel. The presence of extracellular anandamide transporters suggests that it is also an endocrine messenger [33]. On the other hand, the biosynthesis of oleamide is primarily localized within the brain microglia [34], and there is limited data available regarding its synthesis beyond the CNS. Oleamide is detectable in human plasma and notably individuals diagnosed with
Alzheimer's disease (AD) can exhibit elevated levels of oleamide within blood and blood exosomes [35,36]. Additionally *post-mortem* analysis of AD brains have revealed significant alterations in the composition and signaling of the ECS [27]. There is currently limited evidence on the eCBs role in neuroinflammation however, two hypotheses have been proposed on their neuroprotective role in AD. The first hypotheses suggests that when microglia, the immune cells of the brain, become activated by a toxin such as amyloid/tau in the case of AD, eCBs are subsequently released. These eCBs are then transported within the extracellular vesicles to neighboring neurons where they potentially modulate their cellular activity [35]. This modulation could be related to sedative effects or sleep-inducing effects, one of the known protective functions of eCBs, maybe preventing hyperactivity damage to neurons [37]. Another hypothesis, is that the stimulation of CB_2 receptors

can contribute to the removal of amyloid beta (Aβ) peptide by human macrophages [38], which is also the case with microglia [27].

The developed method was applied to CSF samples from 33 healthy individuals, females (n=19) and males (n=14). A profile of 17 endocannabinoids endogenous to the human CSF were identified and quantified. Their concentrations levels were within 0.1 – 1000 ng mL^{-1} (Figure 2). An interesting observation is the difference of endocannabinoid levels, oleamide, found in CSF and plasma samples. One study reports the concentration range of oleamide in plasma between 20 – 250 ng mL^{-1} [11]. The difference in concertation between the two biofluids could be because the major site of oleamide synthesis occurs in the brain microglia [34]. However, further research is required to confirm the sites of oleamide synthesis in humans.

This method showed acceptable sensitivity and reproducibility in quantification of endocannabinoids in human samples. It is a useful analytical method that has potential in clinical application for identification of these molecules in human CSF and improve our understanding of the endocannabinoid system and its role in neuroinflammation.

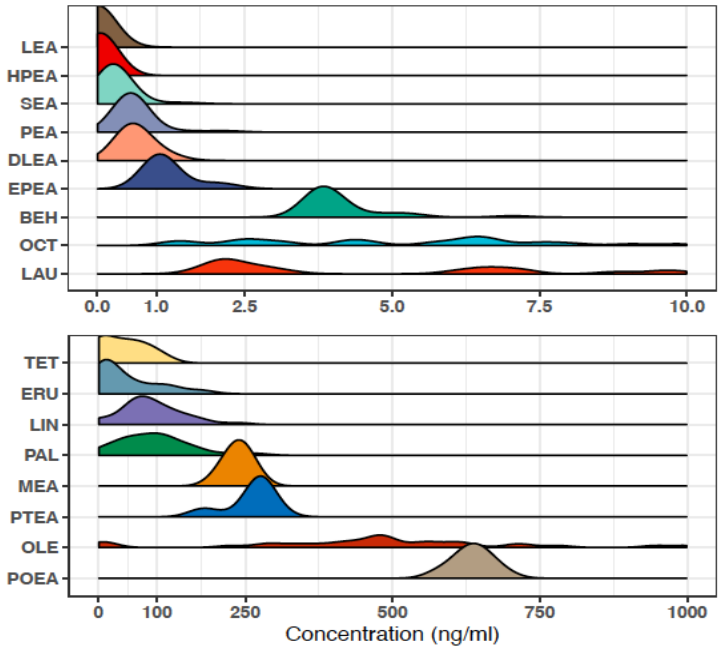


Figure 2: Concentration (ng mL^{-1}) of endogenous endocannabinoids in human CSF from 33 healthy individuals with no cognitive impairments.

Conclusion

In this study, a UHPLC-MS/MS method for quantification and identification of eCBs in human CSF was developed and optimised in compliance with the validation parameters proposed by the FDA. As part of the development process, six different monophasic solvents were compared for their extraction efficiency of endocannabinoids (n=8) from CSF. A solvent mixture of ACN/Ace yielded good extraction recovery results with good precision, in comparison to the remaining five solvents. The results were in alignment with the literature since ACN is an efficient solvent for protein precipitation which is important when analysing complex samples like CSF. Furthermore, Ace is known for its ability to efficiently dissolve polar phospholipids like eCBs.

The method presented in this study is simple and was further optimised, presenting high accuracy and precision based on acceptable %recovery and %RSD. Furthermore, this method acknowledges the complexity of human CSF as a biological fluid. Therefore, it was important to establish minimum interference from the matrix, which was the case in this study. The robustness and sensitivity of the method have been established by calculating the LOD and LOQ values, which were significantly lower in comparison to previous works.

Furthermore, the method is highly applicable to clinical trials. It has shown to be robust and sensitive method that can be applied to human CSF samples to better understand the biological purpose of the endocannabinoid system in neuroinflammation and cognitive function.

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