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Determination of endocannabinoids in nematodes and human brain tissue by liquid chromatography electrospray ionization tandem mass spectrometry

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ABSTRACT

A simple and highly sensitive liquid chromatography/tandem mass spectrometric (LC/MS/MS) method was developed to compare endogenous cannabinoid levels in nematodes and in brains of rats and humans, with and without prior exposure to ethanol. After liquid–liquid extraction of the lipid fraction from homogenized samples, a reversed-phase sub 2 μm column was used for separating analytes with an isocratic mobile phase. Deuterated internal standards were used in the analysis, and detection was made by triple quadrupole mass spectrometer with multiple reaction monitoring (MRM). Ionization was performed with positive electrospray ionization (ESI). The nematode *Caenorhabditis elegans fat-3* mutant, that lacks the necessary enzyme to produce arachidonic acid, the biologic precursor to 2-arachidonoyl glycerol and anandamide, was used as an analyte-free surrogate material for selectivity and calibration studies. The matrix effect was further investigated by in-source multiple reaction monitoring (IS-MRM) and standard addition studies. Selectivity studies demonstrated that the method was free from matrix effects. Good accuracy and precision were obtained for concentrations within the calibration range of 0.4–70 nM and 40–11,000 nM for monitored *N*-acylethanolamides (NAEs) and acyl glycerols, respectively.

1. Introduction

Endocannabinoids (ECs) are the molecular components of a lipid signalling system [1] that has been linked to a wide spectrum of physiological functions that include food intake, pain perception, cognition, emotion/motivation, and psychomotor control [2]. Recent studies have implicated the EC system in several neuropsychiatric disorders, and in particular the reinforcing effects of ethanol and some other drugs of use, misuse and abuse [3–5]. This signalling system comprises two G-protein-coupled cannabinoid receptors (CB1 and CB2), their endogenous ligands (the ECs), and enzymes that are involved in the biosynthesis and inactivation of the ECs [6]. CB1 receptors are the most abundant G-protein-coupled receptor species in the mammalian brain [7,8]. CB2 receptors are mainly located peripherally and are associated with the immune system [9]. Upon depolarization, ECs are released 'on demand' from postsynaptic neurons [10–12]. Anandamide (AEA), an *N*-acylethanolamide (NAE) derivative of arachidonic acid, was discovered to have high affinity for the CB1 receptor, and acts as endogenous lipid agonists [13]. The monoacyl glyceride (MAG) of arachidonic acid, 2-arachidonoyl glycerol (2-AG), was subsequently identified as an agonist of both CB1 and CB2 receptors [14,15]. Since then, new endocannabinoids and cannabimimetic compounds have been identified, such as 2-arachidonyl glycerol ether (noladin, 2-AGE), a selective CB1 agonist, and *N*-arachidonoyl dopamine (NADA), a selective CB1 agonist and a potent agonist of vanilloid receptors [16–19].

Several analytical methods have been published for the determination of ECs and their congeners in various biological samples [20]. ECs are present at pmol to nmol levels per gram of biological

Abbreviations: 2-AG, 2-arachidonoyl glycerol; 2-AG-d8, deuterated 2-arachidonoyl glycerol; AEA, arachidonoylethanolamide (anandamide); AEA-d8, deuterated arachidonoylethanolamide; *C. elegans* N2, *Caenorhabditis elegans* wild type; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; *fat-3*, Δ6 desaturase activity lacking mutant; FWHM, full width at half maximum; GPChos, glycerophosphocholines; LPA, lysophosphatidic acid; Lyso-2-GPCho, lysophosphatidylcholine; MAGs, monoacyl glycerides; NAEs, *N*-acylethanolamides; *N*-ArPE, *N*-arachidonoylphosphatidylethanolamide; PUFAs, polyunsaturated fatty acids; RE%, the relative error; ΣRE, the sum of the absolute values of the relative error; RSD, the relative standard deviation.

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material and therefore require highly sensitive analytical methods and instrumentation for their measurements. The formation of AEA from *N*-arachidonoylphosphatidylethanolamide (*N*-ArPE) in rat testis and 2-AG levels in rat tissue were initially studied using HPLC with fluorometric detection after converting *N*-acylethanolamides (NAEs) and monoacylglycerols (MAGs) to their respective anthroyl derivatives using treatment with 1-anthroyl cyanide and quinuclidine [21,22]. The majority of current analytical methods for ECs favor selective and sensitive mass spectrometric detection over other detection systems. NAEs, with and without MAGs, have been measured using gas chromatography with mass spectrometric detection (GC–MS) as silylated or acylated derivatives with splitless injection, non-polar stationary phase coated capillary columns, and electron impact (EI) ionization with quantification by selected ion monitoring (SIM) [23–28]. Maccarrone et al. [29] analyzed underivatized ECs by GC–MS with EI ionization. ECs and *N*-ArPE were measured by GC–MS as silylated and halogenated derivatives with positive [30] and negative [31–33] chemical ionizations, respectively. The simultaneous GC–MS measurement of ECs together with other eicosanoids, e.g., prostaglandins and thromboxanes, was accomplished after a multistep sample preparation and derivatization with diazomethane, *O*-hydroxymethylamine and dimethylisopropylsilyl imidazole [34]. ECs have been analyzed without derivatization by LC–MS by atmospheric pressure ionization techniques, i.e., electospray ionization (ESI) [35,36] and atmospheric pressure chemical ionization (APCI) [37,38]. These ionization techniques are soft, and both are suitable for the direct analysis of thermolabile analytes. These LC–MS methods used either the molecular ion $[M+H]^+$ or the sodium adduct $[M+Na]^+$ in the SIM mode. A more sensitive and selective method of quantification is the multiple reaction monitoring (MRM) mode, which uses a triple-quadrupole mass spectrometer (MS/MS) for detection [39–41]. In addition to LC methods, a GC/MS/MS method for AEA and other NAEs has been reported [33]. The use of silver cation adducts of 2-AG and AEA has been reported to provide highly sensitive detection with LC-ESI–MS/MS [42,43]. The LC methods mentioned above were based on reversed phase chromatography and gradient elution of ECs. Also, normal phase separation has been reported [37]. Subsequent LC–MS/MS methods by Bradshaw et al. [39], Richardson et al. [40], and Williams et al. [41] were able to detect multiple ECs and cannabimimetic compounds in a single run. To date, these are the most comprehensive methods reported for the quantitative targeted analysis of ECs and cannabimimetic compounds. In addition, untargeted lipidomics provide a powerful tool for the analysis of total lipid extracts and the discovery of new pathways involved in the biotransformation of lipid-derived signalling molecules [44,45].

ECs have been measured from a wide variety of biological materials, e.g., the cerebrospinal fluid (CSF) of acute paranoid-type schizophrenic patients [46], mammalian and rat plasma [26,34,35,43,47], mammalian and rat/mice tissues [23,25,27–30,32,38–41], rat brain microdialysate [48], and in cell cultures [24,29,42]. The lipid fraction of the sample, which includes ECs, is usually isolated from the biological material according to standard liquid extraction techniques [49,50]. Ethyl acetate and hexane have also been reported for separating lipid fractions from brain samples [40,42]. In many methods, the lipid fraction has been further purified before injection, with both normal phase [23–25,28,38,40] and reversed phase [30–32,39] chromatographic techniques. Hardison et al. [30] reported the superiority of reversed phase solid phase extraction (SPE) over normal phase in the purification of raw lipid extracts, due to better extraction recovery and lack of significant deuterium exchange of an isotopically labeled standard of AEA. Especially for single quadrupole MS instruments, in combination with SIM mode, biological samples require an additional purification of the lipid extract to remove interfering components in order to obtain cleaner sample extracts and a matrix effect free spray in the ion source [51,52].

A considerable disagreement in analytical results currently exists for ECs between different instruments (e.g. LC–MS vs. GC–MS), which could arise from a wide variety of reasons, e.g., variations in a samples physiology and pathology, strong post-mortem effects, sampling, calibration, sample preparation methodologies and instrumentation. The high lability, high lipophilicity and low concentrations of ECs conspire to make the development and validation of analytical methods for biological applications especially challenging. In a present study we developed and validated a LC-ESI–MS/MS targeted method for the accurate and precise analysis of ECs from human brains, rat brains and whole nematodes (*Caenorhabditis elegans*). After the single step liquid extraction of ECs from these tissues, a reversed-phase sub 2 μm column was used for separating analytes with an isocratic mobile phase. During method development, special attention was focused on the selection of a reversed phase column for the specific resolution of ECs from phospholipids that could possibly cause ion suppression. Retention of ECs and glycerophosphocholines (GPChos) in different columns were followed by in-source multiple reaction monitoring (IS-MRM) [53]. Selectivity and matrix effects (e.g., ion suppression) were also studied by standard addition [54], post-column infusion [55], and sample dilution [51] techniques. In addition, selectivity and calibration of the method were studied with the *C. elegans fat-3* mutant, which lacks Δ^6 desaturase activity and, therefore, is unable to produce arachidonic acid—the fatty acid precursor to both AEA and 2-AG [56]. This method was validated in terms of selectivity, linearity, precision, accuracy, recovery, and stability, and was proven to be appropriate for the determination of ECs in these biological samples. The method described in this report developed from earlier studies that investigated the influence of ethanol on the EC system in the brains of alcohol preferring AA rats [57] and on EC production in nematodes [56]. The method described in this report was also used for the determination of ECs in post-mortem brains of Cloninger type 1 and 2 alcoholics [58], and also in human adipocytes, human skeletal muscle cells, and cell culture media [59], and further in several targeted towards ongoing lipidomic studies of *C. elegans*.

2. Experimental

2.1. Chemicals and materials

Arachidonoylethanolamine (anandamide, AEA), arachidonoylethanolamide-d8 (AEA-d8), 2-arachidonoyl glycerol (2-AG), 2-arachidonoyl glycerol-d8 (2-AG-d8), *O*-arachidonoyl ethanolamine HCl (virodhamine), 2-arachidonyl glycerol ether (noladin, 2-AGE), *N*-arachidonoyl dopamine (NADA), dihomo-γ-linolenoyl ethanolamide (LEA), docosahexaenoyl ethanolamide (DHEA), palmitoyl ethanolamide (PEA), and oleoyl ethanolamide (OEA) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). 1-Oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (18:1 Lyso-2-GPCho) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Fatty acid free bovine serum albumin (BSA) was purchased from Sigma (Sigma–Aldrich Chemie GmbH, Steinheim, Germany). Acetonitrile (ACN) and methanol (MeOH) were from J.T. Baker (Deventer, The Netherlands). Chloroform and formic acid were purchased from Riedel-de Haën (Seelze, Germany). All solvents and chemicals were of HPLC grade or higher. Water was purified by a Milli-Q Gradient system (Millipore, Milford, MA, USA).

Worms (*C. elegans*) were grown as described [60] on nematode growth media (NGM) plates and maintained at 20 °C, with *Escherichia coli* (OP50) as a food source [61]. Worms were syn-

chronized by bleaching and embryos were allowed to grow until forming mixed populations (4–5 days). Nematodes were washed off the plates with M9 solution, washed 3–4 times with distilled H_2O, centrifuged (2000 rpm, 1 min), and the aqueous supernatant was removed. The sample was centrifuged at 6500 rpm (1 min) and then at 8000 rpm (2 min), removing the aqueous supernatant after each centrifugation. Samples were stored at −20 °C until analysis.

2.2. Preparation of calibration and quality control samples

Analytical standards were used as ready-made standard stock solutions or as solutions prepared from solid substances, which were stored at −80 °C. Stock solutions of AEA, AEA-d8, 2-AG, 2-AG-d8, and NADA were prepared and stored in ACN, and solutions of other NAEs, virodhamine, and noladin were prepared and stored in ethanol. Further dilutions of each analyte were made with ACN at eight different calibration levels and always used or discarded within ten weeks after storage at −20 °C. Calibration standards covered the concentration range between 0.2–70 and 40–11,000 nM for NAEs and acyl glycerols (2-AG and noladin), respectively. The internal standards AEA-d8 and 2-AG-d8 were used as received by diluting to concentrations of 50 and 460 nM with ACN, respectively. Calibration standards were prepared by taking 50 μl of the calibration standard working solution and adding 50 μl of both internal standard solutions. The resulting standard solution was then evaporated to dryness under nitrogen and dissolved in 50 μl of ACN, followed by the addition of 20 μl water and stored in HPLC sample vials. The calibration curves also included a blank sample and a zero sample (i.e., the blank standard contained both internal standards). Each day of analysis two sets of calibration standards were analyzed at the beginning and the end of the sequence, and the calibration curves for each analyte was made with a weighted ($1/x$) least square linear regression.

To determine the accuracy and precision of the method during the study, quality control (QC) samples at four concentration levels of the analytes were analyzed within each day of analysis. Quality control samples were prepared in the same way as the calibration standards, but were extracted as described in Section 2.3. Each day, at the beginning of the sequence, the compatibility of the method and instrumentation were determinated with the system suitability test (SST) by analyzing five replicate injections from the same vial containing QC standard level 3.

2.3. Sample extraction

A modified extraction method of Folch et al. [49] and Hardison et al. [30] was used for the isolation of analytes from the sample matrix. The samples were kept on ice while they were being processed. This step, and the study protocol, is described more carefully in Malinen et al. [57] and Lehtonen et al. [58] for rat brain and human brain samples, respectively. Briefly, the concentrations of AEA and 2-AG were measured from female and male alcohol-preferring AA (Alko, Alcohol) rats, which were trained to drink 10% (v/v) alcohol. Following the establishment of stable alcohol drinking, half of the rats were sacrificed immediately before the next daily ethanol access, while the other halves were sacrificed after the drinking session. A separate control group consisted of water drinking rats (n = 11–12 per group). Rat brains were quickly removed after decapitation and frozen in isopentane at −40 °C. Samples were then stored at −70 °C until processed. Frozen rat brains were sliced into 2 mm coronal sections, separate areas containing the medial prefrontal cortex (PFC), nucleus accumbens (NAcc), caudate putamen (CPu), amygdale (Amy), or hippocampus (HC) were dissected from these sections, and placed in pre-weighed Eppendorf test tubes. The vials were weighed again to determine the actual amount of tissue sample. The weight of the rat brain samples was between 10 and 30 mg.

The selection and collection of post-mortem human brains, psychological diagnostics and sample preservation methods have been described in detail [62,63]. Briefly, brains were removed, cleaned of the dura, and the left hemisphere was placed with the midsagittal plane on a glass plate before freezing at −75 °C and then cryosectioned into 100-μm horizontal sections. The sections were allowed to air dry before they were stored with dehydrating agents at −25 °C until analysis [63]. From these frozen post-mortem brain tissues, sizes of 1–3 mg samples were removed from the glass slide with a scalpel and transferred to a pre-weighed Eppendorf test tube, then weighed again to determine the precise amount of tissue sample added. ECs were measured in post-mortem brains of non-alcoholic controls (n = 10), Cloninger type 1 (n = 9) and 2 alcoholics (n = 8). ECs were measured from the PFC, NAcc, perigenual anterior cingulate cortex (pACC), Amy, HC, and white matter brain areas.

Worm samples (*C. elegans*) were accurately weighted into a pre-weighed Eppendorf test tube after the removal of excess water from the worm suspension with an automated pipette and centrifugation at 12,000 × g for 10 min at 10 °C. After the sample was transferred to the test tube, the tube was weighed again to determine the actual amount of sample added.

In all cases, half a millilitre of ice-cold methanol (500 μl) was added to samples, which were then homogenized with a Soniprep 150 homogenizer (MSE Ultrasonic Disintegrator; MSE Scientific Instruments, Manor Royal, Crawley, Sussex, England). Lipids were extracted by adding chloroform and water to yield a methanol/chloroform/water ratio of 1:2:1 (v/v/v), and centrifuged at 1500 × g for 10 min at 10 °C to achieve a sharp phase separation. The upper aqueous layer was discarded and the lower organic layer was transferred to a screw capped glass test tube. This liquid extraction was repeated once, and the organic layers were combined. The sample was then evaporated to dryness under nitrogen at room temperature and the residue was reconstituted in 50 μl of ice-cold ACN. The sample was allowed to dissolve for 5 min and then 20 μl of water was added. After another centrifugation at 12,000 × g for 10 min at 10 °C, the supernatant of the sample was then transferred to an HPLC sample vial.

2.4. LC/MS/MS instrumentation

The HPLC system consisted of an Agilent 1200 Series Rapid Resolution LC System (Agilent Technologies, Waldbronn, Germany) with a solvent micro vacuum degasser, a binary pump, a thermostatted column compartment, and an autosampler. The mass analysis was made with an Agilent 6410 Triple Quadrupole MS equipped with an electrospray ionization source (Agilent Technologies, Palo Alto, CA, USA). Data were acquired by Agilent MassHunter Workstation Acquisition software (Agilent Technologies, Data Acquisition for Triple Quad., version B.01.03).

Ten microliters of the sample solution was injected onto a reversed-phase HPLC column (Zorbax Eclipse XDB-C18 Rapid Resolution HT 2.1 mm × 50 mm, 1.8 μm) (Agilent Technologies, Palo Alto, CA, USA) using an isocratic mobile phase consisting of H_2O/ACN/formic acid (33:67:0.1, v/v/v), delivered at 150 μl/min. An in-line filter (RRLC In-line filter, 2 mm, max 600 bar, 0.2 μm, Agilent Technologies) was used for protecting the analytical column from possible contaminants. Column temperature was maintained at 40 °C and the autosampler tray temperature was set at 10 °C. The following ionization conditions were used: ESI positive ion mode, drying gas (nitrogen) temperature 300 °C, drying gas flow rate 10 l/min, nebulizer pressure 50 psi, and capillary voltage 4000 V. Analyte detection was performed by using multiple reaction monitoring (MRM) with a dwell time of 100 ms for each transition. The collision induced dissociation (CID) was made by nitrogen and

Table 1
MS detector parameters for MRM transitions (*m*/*z*), fragmentor voltage (V), collision energy (V), and mass resolution (FWHM) values for the studied ECs.

Compound	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Fragmentor voltage (V)	Collision energy (V)	Mass resolution (MS1/MS2, FWHM)
Time segment 1: 4–6.5 min					
AEA	348	62	120	10	2.4/1.2
AEA-d8 (IS)[a]	356	63	120	12	0.7/0.7
DHEA	372	62	130	10	0.7/0.7
Virodhamine	348	62	100	12	0.7/0.7
18:1 Lyso-2-GPCho[c]	184	184	325	0	0.7/0.7
18:1 Lyso-2-GPCho[c]	104	104	325	0	0.7/0.7
Time segment 2: 6.5–15 min					
2-AG	379	287	130	8	0.7/0.7
2-AG-d8 (IS)[b]	387	294	125	10	0.7/0.7
2-AGE	365	273	120	5	0.7/0.7
NADA	440	137	180	16	0.7/0.7
LEA	350	62	140	9	0.7/0.7
PEA	300	62	130	10	0.7/0.7
OEA	326	62	140	12	0.7/0.7
18:1 Lyso-2-GPCho[c]	184	184	325	0	0.7/0.7
18:1 Lyso-2-GPCho[c]	104	104	325	0	0.7/0.7

[a] AEA-d8 was used as an internal standard for the quantification of AEA, DHEA, Virodhamine, LEA, PEA, and OEA.
[b] 2-AG-d8 was used as an internal standard for the quantification of 2-AG, 2-AGE, and NADA.
[c] 18:1 Lyso-2-GPCho was used only for qualitative analysis.

the collision cell gas flow rate was adjusted to give an optimum high vacuum gauge reading of 2.9×10^{-5} Torr. Optimized values for each studied compounds for time segments, MRM transitions (*m*/*z*), fragmentor voltage (V), collision energy (V), and mass resolution (FWHM) values are presented in Table 1. The divert valve was programmed to allow eluent flow into the mass spectrometer from 4 to 15 min for each run, and an overlapping injection technique was programmed to start the injection cycle 14 min after the previous injection. Deuterated internal standards (AEA-d8 and 2-AG-d8) were used for quantification (Table 1), and peak area ratios of the analyte to the internal standard were calculated as a function of the concentration ratios of the analyte to the internal standard. Weighted least square linear regression was used for obtaining calibration curves with Agilent MassHunter software (Quantitative Analysis Version B.01.03).

2.5. Validation

This method was validated in terms of selectivity, linearity, precision, accuracy, recovery, and stability [54,64,65]. Because ECs are endogenous compounds and a sample matrix without analytes is not normally available, the *C. elegans fat-3* mutant and 4% (w/v) fatty acid free bovine serum albumin (BSA) in water were used as surrogate matrixes [54]. Homogenized rat brain tissues were also used as an authentic biological matrix. The whole rat brain was homogenized in MeOH, and samples were prepared according to Section 2.3. The homogenate sample resulted in 8.2 mg of rat brain tissue per sample (500 μl).

2.5.1. Selectivity and matrix effect

The selectivity of the method was assessed by analyzing reference standards, authentic matrices with and without analytes, buffers, and solvents for interfering peaks at the retention times of ECs. An in-source fragmentation experiment with 18:1 Lyso-2-GPCho was performed in order to study matrix effects by other lipid classes, such as glycerophosphocholines [53]. The above-mentioned instrument parameters (Section 2.4) were used with slight modifications: mobile phase flow was 100 μl/min in columns with a width of 1.0 mm and an injection volume of 1 μl. The combination of time segments and the change of run time were due to different retention times of compounds for the tested columns. One microliter of test solution containing 490, 260, 1400, and 15 μM of 18:1 Lyso-2-GPCho, 2-AG, AEA, and virodhamide was injected to the tested columns. The following reversed phase columns were tested during the in-source fragmentation studies: from Agilent Technologies Zorbax Eclipse XDB-C18 Rapid Resolution HT 2.1 mm × 50 mm, 1.8 μm; Zorbax SB-C18 Rapid Resolution HT 2.1 mm × 50 mm, 1.8 μm; Zorbax Eclipse XDB-C8 2.1 mm × 50 mm, 3.5 μm; Zorbax Eclipse XDB-C18 1.0 mm × 150 mm, 3.5 μm; Zorbax Eclipse plus C18 3.0 mm × 100 mm, 3.5 μm; and from Waters Corporation (Milford, MA, USA) Xterra MS C8 1.0 mm × 50 mm, 3.5 μm; XBridge C18 2.5 mm × 50 mm, 2.5 μm.

In addition, the standard addition method was used to study the selectivity and the matrix effect. The rat brain homogenate sample pool was divided into sub-samples of equal volumes, which were spiked with two concentrations of the EC standards (50 and 100 μl of QC level 3) [54]. Similar standard additions were also made to the methanol, and these reference samples were further extracted according to Section 2.3. Three replicate samples were prepared at each concentration/addition level and analyzed. The slopes of the standard addition curves were compared to the slopes of standard curves prepared without tissue matrix using analysis of variance (ANOVA) with GraphPad Prism® 4.03 for Windows (San Diego, CA, USA). The standard addition method was futher used for the calculations of the EC concentrations in unspiked rat brain homogenate, and these results were compared to those obtained with the internal standard method. The systematic bias was calculated with the following equation: $\text{bias}\% = 100(\bar{X} - \mu)/\mu$. In this equation, $\bar{X}$ represents the result from the standard addition method and μ represents the result from internal standard method.

A post-column infusion experiment was also performed to evaluate the ion suppression time window after injection. The infusion setup consisted of a syringe pump and a post-column T-piece as reported elsewhere [55]. A constant flow of EC stock solution was delivered via the T-piece to the mobile phase at a flow rate of 6 μl/min. The analyte signal was monitored after the injection of a *C. elegans* N2 sample, and after brain samples from both rats and humans.

Finally, selectivity was studied by diluting the rat brain homogenate 1:5, 1:10, 1:20, 1:30, 1:40 and 1:50 with HPLC mobile phase before injecting onto LC/MS/MS [51]. The measured concentrations of the dilution samples were plotted against 1/dilution factor using log scales and a linear regression analysis was performed. The relative error (RE%) of the undiluted concentration, and the relative standard deviation (RSD) among the recovered concen-

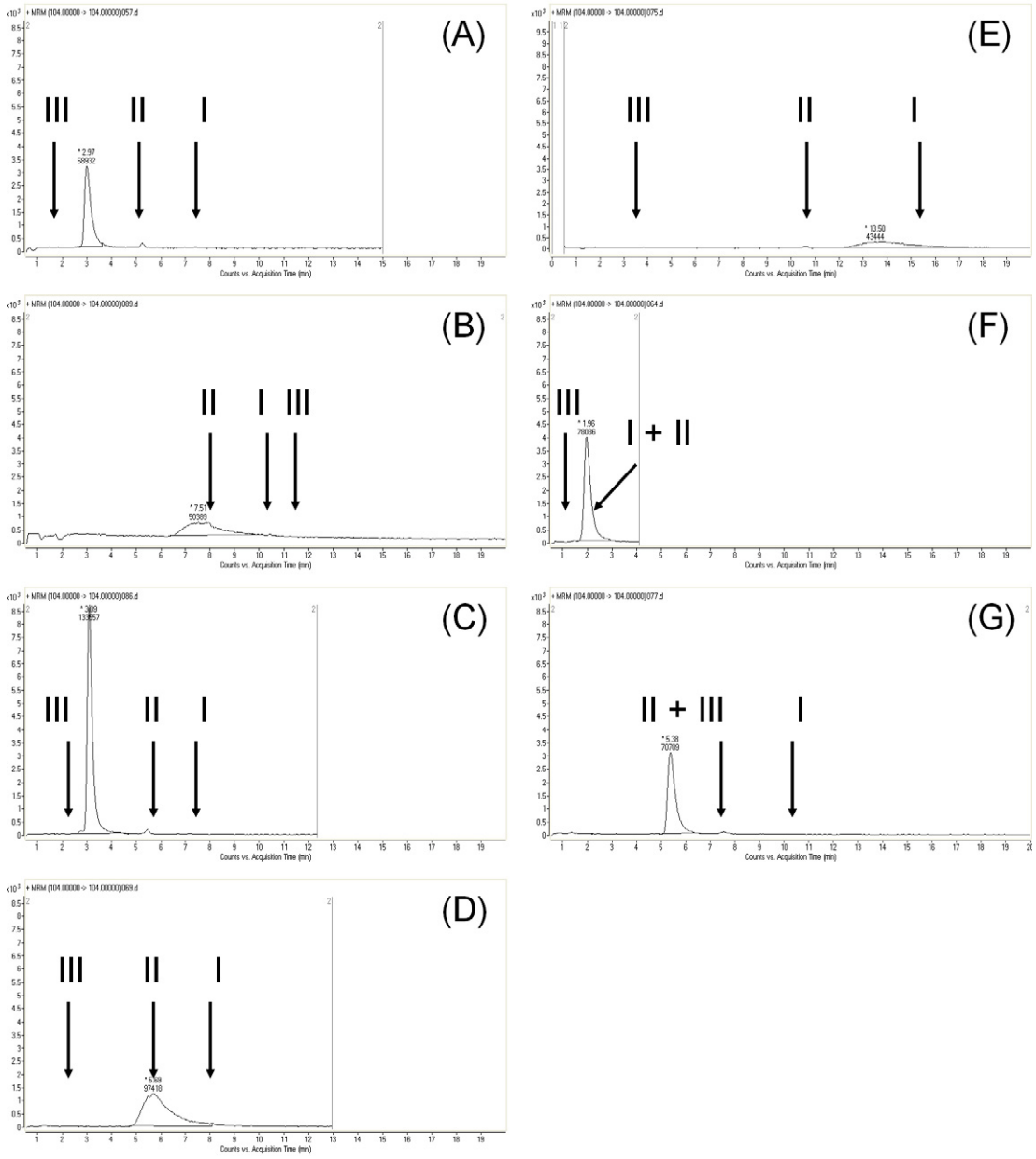

Fig. 1. Selection of an analytical reversed phase column and resolution studies of 18:1 Lyso-2-GPCho with the following ECs; 2-AG (**I**), AEA (**II**) and virodhamine (**III**). Followed IS-MRM transition was m/z 104 → 104 for Lyso-2-GPCho's. Retention of followed compounds with different reversed phase HPLC columns: (A) Agilent Zorbax Eclipse XDB-C18 Rapid Resolution HT 2.1 mm × 50 mm, 1.8 μm; (B) Agilent Zorbax SB-C18 Rapid Resolution HT 2.1 mm × 50 mm, 1.8 μm; (C) Agilent Zorbax Eclipse XDB-C8 2.1 mm × 50 mm, 3.5 μm; (D) Agilent Zorbax Eclipse XDB-C18 1.0 mm × 150 mm, 3.5 μm; (E) Agilent Zorbax Eclipse plus C18 3.0 mm × 100 mm, 3.5 μm; (F) Waters Xterra MS C8 1.0 mm × 50 mm, 3.5 μm; (G) Waters XBridge C18 2.5 mm × 50 mm, 2.5 μm (G). Instrumentation parameters are described in Sections 2.4 and 2.5.1. In the chromatograms, the retention times of compounds **I**, **II**, and **III** are indicated with an arrow.

trations at different dilutions of the test sample, were used to verify parallelism of the results.

2.5.2. Calibration

A calibration curve included a blank sample (solvents used in the method, processed without internal standard), a zero sample (solvents used in the method, processed with internal standard), and eight non-zero samples covering the expected concentration range in the study. The linearity of the assay for each of the analytes was assessed by analyzing the calibration curves from eight concentrations of calibration samples in triplicate covering the range of the method. A weighted ($1/x$) least square linear regression was used to make the calibration curve. The origin was independent of the calibration curve. The calibration curve equation $y = kx + b$ was used for least squares regression analysis, where y represents the EC to IS peak area ratio and x represents the concentration ratio of EC to IS, and k represents the slope of the curve. The selection of the curve and its weighting were justified by calculations for the sum of the absolute values of the relative error (ΣRE), scatter plots of the RE% vs. concentration, and the coefficient of determination (R^2). The acceptance criterion for RE% was set to ±20%. Different weighting factors ($1/x^0$, $1/x$, $1/x^2$, $1/y$, $1/y^2$, log, $1/\mathrm{sd}^2$) were studied. The lower limit of quantification (LLOQ) was determined by calculating the precision and accuracy of five LLOQ samples that were independent of the calibration curve.

The calibration was further investigated with the *C. elegans fat-3* mutant. Ten microliters of the *fat-3* mutant was transferred to a

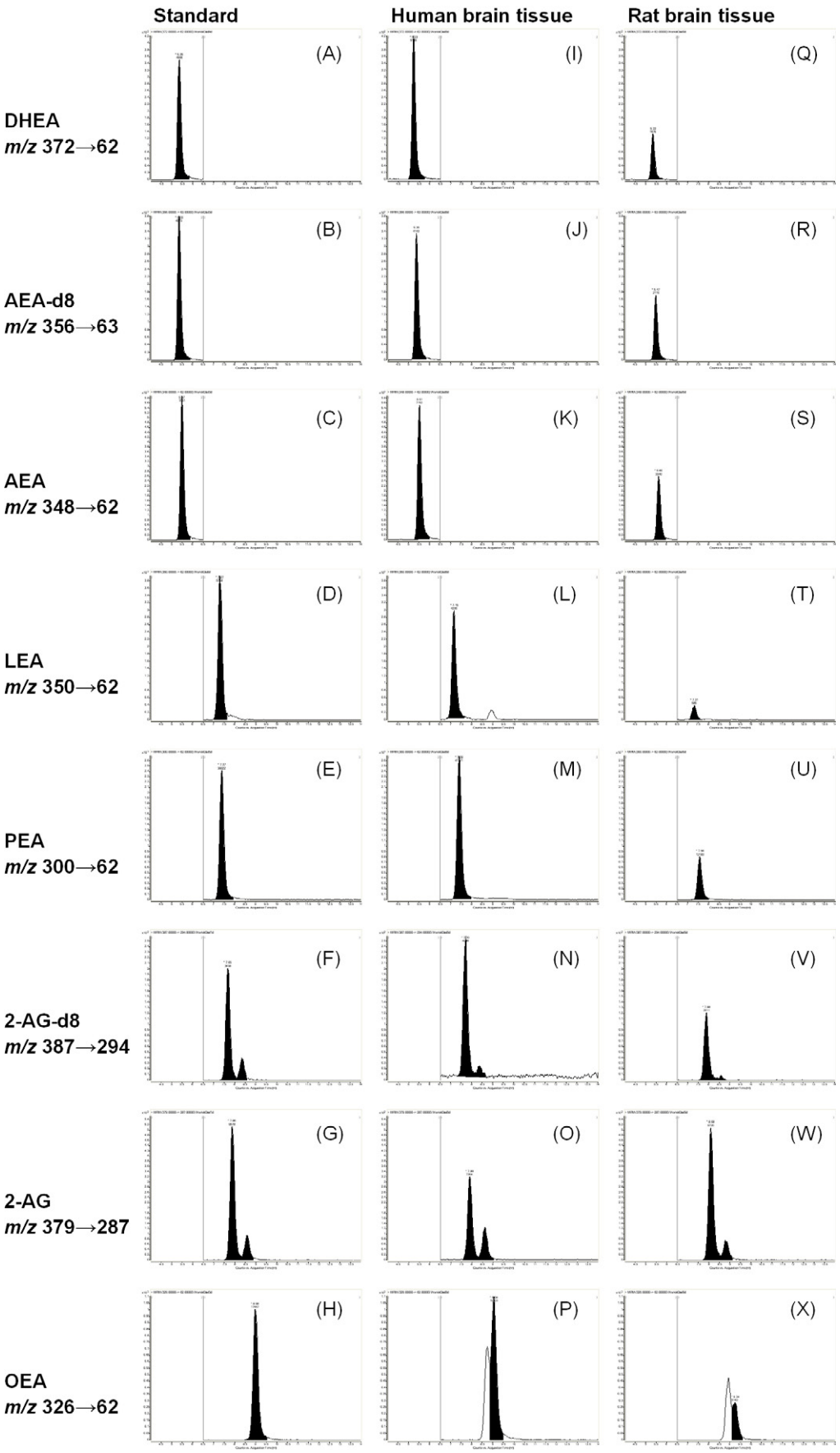

Fig. 2. Representative MRM chromatograms of (A) standard sample of DHEA (10 nM, retention time (RT) 5.35 min), (B) AEA-d8 (IS for NAEs, 40 nM, RT 5.33 min), (C) AEA (10 nM, RT 5.47 min), (D) LEA (2.6 nM, RT 7.27 min), (E) PEA (35 nM, RT 7.37 min), (F) 2-AG-d8 (IS for 2-AG and noladin, 460 nM, RT 7.65 min), (G) 2-AG (380 nM, RT 7.86 min), and (H) OEA (11 nM, RT 8.96 min). Endogenous levels of each analyte were measured from a rat brain homogenate and a human brain sample from the amygdala.

test tube. Calibration standards were added on top of the nematode sample, and the sample preparation was made according to Section 2.3. The slopes and intercepts of the normal standard curve were compared to the slopes of standard curves prepared on top of the *fat-3* mutant by using ANOVA.

2.5.3. Precision, accuracy and recovery

The intra-batch precision of the assay was assessed by calculating the RSD for the analysis at four different QC sample concentration levels in five replicates, and inter-batch precision was determined by the analysis of QC samples on three consecutive days. The precision of the method was further studied by analyzing rat brain homogenates in five replicates on three consecutive days. Precision is expressed as the RSD of the concentrations. The precision determined at each concentration level should not exceed 15% of the RSD except for the LLOQ, where it should not exceed 20% of the RSD.

Accuracy was calculated by comparing the mean experimental concentrations of assayed QC samples with their nominal values. Accuracy is expressed as the ratio between the experimental and the nominal values observed. The mean value of accuracy should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The 2-way ANOVA for comparing each analyte's accuracy results between days and QC levels was performed with GraphPad Prism® 4.03. The QC samples were also prepared in the presence of a surrogate matrix (i.e., 4% BSA and the *fat-3* mutant), and both intra- and inter-batch precision and accuracy were calculated as described above.

The recovery of the analytes was also calculated by using brain tissue homogenates where two concentrations of standards (i.e., 50 and 100 μl of QC level 3) were spiked, according to the following equation: recovery (%) = $100(S - U)/C$, where S represents the concentration of spiked sample, U represents the concentration of non-spiked sample and C represents the nominal concentration of the analyte.

Robustness of the method was tested by analyzing a set of *C. elegans* N2 samples ($n = 48$) at an elevated mobile phase flow rate (0.5 ml/min). Sample concentrations with elevated flow rate were compared to results obtained from a normal 0.15 ml/min flow rate method by the *t*-test with GraphPad Prism® 4.03.

2.5.4. Stability

Analyte stability in the samples was evaluated by determining short-term temperature stability, freeze–thaw stability, post-preparative stability, and long-term sample stability. Stock solution stability and working standard solution stability were also determined. The stability of the analytes was studied in triplicates using brain tissue homogenate samples. Stability is expressed as a percentage, and was calculated by dividing the sample concentration, at each study point, by the sample concentration at the outset of the study, and then multiplying the resulting value by 100. The predetermined limits for stability were set at 20% for variation and mean deviation. The freeze–thaw stability was determined after three freeze–thaw cycles. The short-term temperature stability was investigated by keeping the samples for 4 h at room temperature before sample preparation. The long-term stability was evaluated by analyzing samples which had been stored up to eleven weeks at −80 °C and up to two weeks at −20 °C. The long-term stability was further determinated from rat brain homogenates stored at −80 °C for 18 months. The stock solution stability was investigated by comparing freshly prepared standards to standards prepared from a stock that had been stored at −80 °C for 90 days and kept at room temperature for 6 h after thawing. For the post-preparative stability study, two sets of rat brain homogenates were prepared and analyzed on 2 consecutive days, i.e., one set immediately after the sample preparation and the other after 24 h storage in the instrument autosampler (10 °C).

3. Results

3.1. Method development

The molecular ions for the compounds of interest were followed in full-scan MS experiments over a mass range of m/z 50–500 by triple quadrupole mass spectrometry. The protonated molecules $[M+H]^+$ for AEA, AEA-d8, DHEA, LEA, virodhamide, PEA, and OEA were m/z 348, 356, 372, 350, 348, 326, and 300, respectively. The sodium adducts $[M+Na]^+$ were present in all of the full scan spectrums of NAEs except in the spectrum of virodhamine. The most intense fragment ion in the product ion spectrum was m/z 62 for NAEs and m/z 63 for AEA-d8 (IS for NAEs) (Supplementary Fig. 1). The $[M+H]^+$ ions for 2-AG, 2-AG-d8, noladin, and NADA were found to be m/z 379, 387, 365, and 440, respectively. The sodium adducts $[M+Na]^+$ were present in all of the full scan spectrums of 2-AG, 2-AG-d8, noladin, and NADA. Noladin had very intense sodium adduct ion of m/z 387. A loss of water $[M+H-H_2O]^+$ could be seen by m/z 361 for 2-AG. The most intense fragment ion in the product ion spectrum was m/z 287, 294, 273, and 137 for 2-AG, 2-AG-d8, noladin, and NADA, respectively (Supplementary Fig. 1). The $[M+H]^+$ ion for 18:1 Lyso-2-GPCho was m/z 522, which was measured with full-scan MS experiments over a mass range of m/z 100–700. In source fragmentation of 18:1 Lyso-2-GPCho was achieved by a high fragmentator voltage (325 V), and two main fragments were followed as IS-MRM channels m/z 184 → 184 for GPChos and 104 → 104 for Lyso-2-GPChos (Supplementary Fig. 1). The MRM channels from the CID for the $[M+H]^+$ ion and optimized values of the fragmentor voltage and collision energy for the studied compounds are summarized in Table 1. No cross-talk between MS/MS channels that were used for monitoring ECs and ISs, or any other problems with isotopic integrity of the stable isotope label during sample processing, were observed.

Seven commercially available reversed phase columns were tested, and representative IS-MRM chromatograms of 18:1 Lyso-2-GPCho are presented in Fig. 1, together with marked retention times for 2-AG, AEA, and virodhamide. The best resolution of 18:1 Lyso-2-GPCho and the studied compounds was achieved by a short narrow bore high resolution sub 2 μm stationary phase. This column was also able to separate AEA and virodhamide from each other. In many tested columns 18:1 Lyso-2-GPCho gave a broad chromatographic peak, and ECs coeluated with 18:1 Lyso-2-GPCho (Fig. 1). One of the tested columns used a hybrid particle technology for the stationary phase, which was unable to separate AEA and virodhamide from each other under these chromatographic conditions (Fig. 1). Representative IS-MRM chromatograms from rat and human brain tissues and whole *C. elegans* N2 samples are presented together with the two main ECs of interest (i.e., AEA and 2-AG) in Supplementary Fig. 2. In the IS-MRM channel m/z 104 → 104 for Lyso-2-GPChos two peaks were obtained in all tested biological matrices with retention times of 12.1 and 13.9 min (Supplementary Fig. 2), thus making the total run time of 15 min for this method.

Representative MRM chromatograms of a human brain sample for (I) DHEA (5.9 nM), (J) AEA-d8 (40 nM), (K) AEA (7.2 nM), (L) LEA (1.0 nM), (M) PEA (42 nM), (N) 2-AG-d8 (460 nM) (O) 2-AG (199 nM), and (P) OEA (9.9 nM). Representative MRM chromatograms of a rat brain homogenate for (Q) DHEA (3.7 nM), (R) AEA-d8 (40 nM), (S) AEA (6.8 nM), (T) LEA (0.3 nM), (U) PEA (23 nM), (V) 2-AG-d8 (460 nM), (W) 2-AG (458 nM), and (X) OEA (5.9 nM). All samples were prepared according to Sections 2.2 and 2.3. Instrumentation parameters were the same as described in Section 2.4. The chromatographic peak of each analyte is indicated with darker shading.

3.2. Selectivity and matrix effect

In order to determine the selectivity of the method, both standards and tissue samples were prepared and analyzed (Fig. 2). The solvents did not contribute any interfering peaks or background to any of the standard chromatograms. All other studied compounds, except noladin with a retention time of 10.14 min and NADA with a retention time of 7.54 min, were present in the biological matrices (Fig. 2). In *C. elegans* the content of DHEA was below the limit of detection. The selectivity of this method was investigated further with *C. elegans fat-3*, *fat-1* and *fat-4* mutants (Fig. 3).

When two concentrations of standards (50 and 100 μl of QC level 3) were added to the rat brain homogenate, and the slopes of the regression curves were compared to those curves prepared without tissue matrix, the slopes were found to be statistically equal for the studied ECs present in this tissue, except for PEA (Fig. 4). The systematic bias between concentration results of the standard addition and internal standard methods was less than 7% in all other analytes except PEA (30%). The dilution linearity further shows the absence of a matrix effect for DHEA, AEA, OEA, and 2-AG (Fig. 5). Linear regression with a curved line shows the lack of parallelism for LEA and PEA (Fig. 5). Furthermore, the RE% for the undiluted concentrations was less than 20% in all cases and the RSD values were less than 10% for all studied ECs, except for LEA (11%) (Supplementary Table 1). The post-column infusion experiments showed that the only other significant ion suppression was observed at the beginning of the analysis. The ion suppression time window was between 0.7 and 1.5 min, thus further indicating the absence of a sensitivity affecting matrix effect at the retention time of ECs and ISs (data not shown).

3.3. Calibration

The eight point calibration curves were highly linear over the range of the method for the followed NAEs and acyl glycerols (Table 2). The simplest model to describe the concentration–response relationship was achieved by using a weighted ($1/x$) least square linear regression (Table 2). The effectiveness of the various weighting schemes for reducing method error was determined by calculating the ΣRE, and is shown in Supplementary Table 2. The slope and intercept of the calibration curve with a 95% confidence interval, the ΣRE, and R^2 values are summarized in Table 2. Deviation of the calibration standards from their nominal concentrations was always less than 20% at the LLOQ concentration and less than 15% in other studied concentrations (Fig. 6), except in the cases of PEA, noladin, and NADA. In all other cases, except for NADA, the goodness of fit was greater than 0.99, as indicated by the coefficient of determination (R^2) (Table 2). The LLOQs are reported in Tables 2 and 3. The limit of detection (LOD) was further studied with five replicate samples and reported in Table 2.

In addition, calibration with the *fat-3* mutant was used to validate the calibration with pure standards. There were no statistical differences in calibrations between normal calibration and calibration prepared on top of the *fat-3* mutant for either 2-AG or AEA when their slopes ($p = 0.1715$ for 2-AG and $p = 0.1391$ for AEA) and intercepts ($p = 0.6544$ for 2-AG and $p = 0.9587$ for AEA) were compared. These two calibration curves were also used for calculating results from QC samples prepared on top of the *fat-3* mutant sample for 2-AG and AEA at two different concentration levels. There were no statistical differences for 2-AG QC results at 943 and 3774 nM ($p = 0.2752$ and 0.9972, respectively). Similar results were obtained for AEA at 5.1 and 21 nM ($p = 0.2067$ and 0.7452, respectively).

3.4. Accuracy, precision and recovery

The intra-day precision of all QC samples, except the lowest QC concentration of PEA, was within the predeterminated acceptance criteria (Table 3). The intra-day accuracy of all QC samples was also within the predeterminated acceptance criteria (Table 3). Only the highest QC concentration of noladin and two highest concentrations of PEA fulfilled the RSD values under 20% (RSD) for the inter-day precision test samples. Statictically significant differences between QC concentrations were observed for 2-AG (2-way ANOVA, $p = 0.0427$) and OEA (2-way ANOVA, $p < 0.0001$) results (Fig. 7). Despite this statistical difference, QC results for 2-AG and OEA fulfilled the predetermined limits for precision and accuracy, and the difference of OEA at QC level 1 was approx. 10%. The intra-day precision results for rat brain homogenates was ($n = 5$; average ± SD) 31.3 ± 0.4 pmol/g, 57.8 ± 0.9 pmol/g, 2.3 ± 0.1 pmol/g, 200 ± 14 pmol/g, 50.5 ± 1.5 pmol/g, and 3.91 ± 0.01 nmol/g for DHEA, AEA, LEA, PEA, OEA, and 2-AG, respectively. There were no statictical differences in concentration results between 3 days for DHEA, AEA, LEA, OEA, and 2-AG ($p > 0.05$). However, there was a statistically significant difference in PEA concentrations between analysis days ($p = 0.0253$). In addition, the method was accurate and precise at all studied concentrations for AEA and 2-AG when QC samples were determined in the presence of the surrogate sample matrixes (i.e., 4% BSA, w/v and *fat-3* mutants).

For the recovery studies, rat brain homogenates were spiked at QC level 3 in two volumes (50 and 100 μl) before sample processing. The recoveries for DHEA, AEA, LEA, PEA, 2-AG, OEA, and noladin were found to be ($n = 10$; average ± SD) 100 ± 5%, 99 ± 4%, 89 ± 2%, 74 ± 7%, 94 ± 5%, 98 ± 6, and 95 ± 7%, respectively. The recovery of 2-AG-d8 was 97 ± 8%, while the recovery of AEA-d8 was 96 ± 5% ($n = 10$; average ± SD).

Increasing the mobile flow rate from 0.15 ml/min to 0.5 ml/min increased the back pressure of the system from 65 bar to 215 bar. At the same time, the intensity of the peak measured by peak area decreased by a factor of 2.6 for both AEA and 2-AG, while peak height remained unchanged, and both ECs eluated from the column 3.3 times faster with the higher flow rate (Fig. 8). The intra-day precision ($n = 3$) of the method for AEA, expressed as RSD ($n = 5$), was 3.0, 4.3, 3.2, and 2.6%, for QC samples at concentrations of 1.0, 5.1, 21 and 41 nM, respectively. The inter-day precision ($n = 3$) for AEA was 5.1, 9.4, 4.7, and 2.3% (RSD, $n = 15$) at the above-mentioned QC concentrations. The intra-day precision of the method for 2-AG was 14, 11, 6.4 and 8.6%, for quality control samples at concentrations of 190, 940, 3770, and 5660 nM, respectively. The inter-day precision ($n = 3$) for 2-AG with this method was 11, 8.0, 7.1, and 12% (RSD, $n = 15$) at these respective QC concentrations. The accuracy of the method was 95, 95, 97 and 98% for AEA and 102, 98, 110 and 98% ($n = 5$) for 2-AG at these respective concentrations. The higher flow rate was also applied to a set of *C. elegans* samples ($n = 48$). There were no statistical differences in AEA and 2-AG concentrations obtained at these two flow rates (Fig. 8), according to a paired two-tailed t-test with $p < 0.05$.

3.5. Stability

There was no significant degradation of ECs after three freeze–thaw cycles, compared to determinations at the outset (98–100%). The short-term stability at room temperature for 4 h showed no degradation of ECs, and determinated stability values were between 95 and 102% for all ECs. There was also no degradation in samples that were stored for two weeks at −20 °C (97–99%). The EC concentrations in biological samples after eleven weeks of long-term storage at −80 °C were within the range of 95–103%. The concentrations of DHEA, AEA, LEA, PEA, 2-AG, and OEA changed

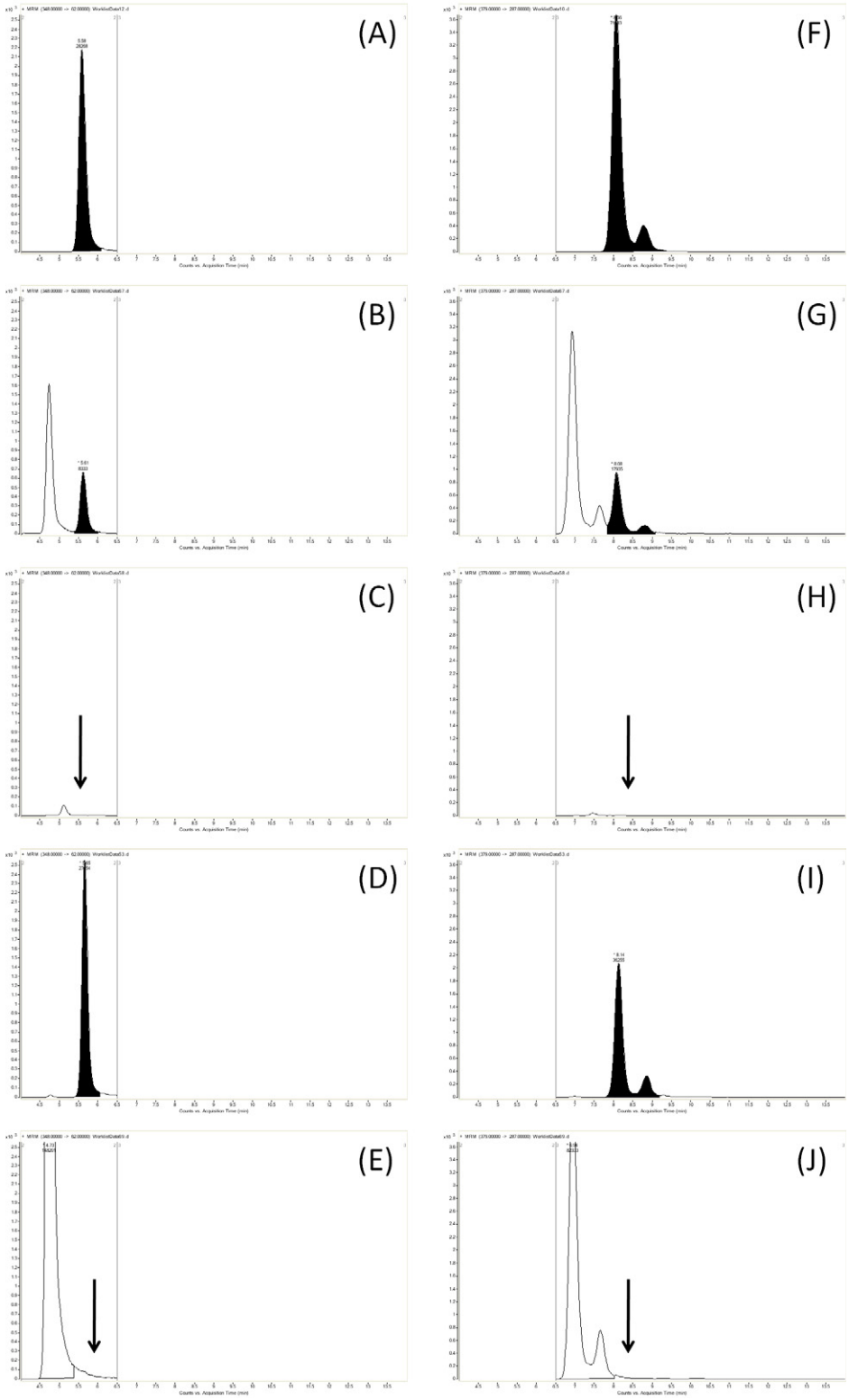

Fig. 3. The selectivity studies with *C. elegans* N2, *fat-3*, *fat-1*, and *fat-4* mutants. Representative MRM chromatograms of AEA in (A) standard sample (62 nM, retention time (RT) 5.58 min), (B) in *C. elegans* N2 (18 nM), (C) in *C. elegans fat-3* mutant (not detected), (D) in *C. elegans fat-1* mutant (60 nM), and (E) in *C. elegans fat-4* mutant (ω-6 isomer not detected, ω-3 isomer at retention time 4.73 min). Representative MRM chromatograms of 2-AG in (F) standard sample (3770 nM, retention time (RT) 8.05 min), (G) in *C. elegans* N2 (956 nM), (H) in *C. elegans fat-3* mutant (not detected), (I) in *C. elegans fat-1* mutant (1932 nM), and (J) in *C. elegans fat-4* mutant (ω-6 isomer not detected, ω-3 isomer at retention time 6.94 min). All the samples were prepared according to Sections 2.2 and 2.3. Instrumentation parameters were the same as described in Section 2.4. The chromatographic peaks of ω-6 isomers of AEA and 2-AG are indicated with darker shading, and arrows are used to indicate the times on chromatograms that did not contain any AEA or 2-AG.

during 18 months of −80 °C storage to 92, 95, 67, 78, 78, and 107% of the original value, respectively. The stock solutions of ECs were stable for 90 days when stored at −20 °C, after being kept at room temperature for 6 h after thawing. Post-preparative sample stability was found to be constant when stored for 24 h at 10 °C in the autosampler, however post-preparative stability increased migra-

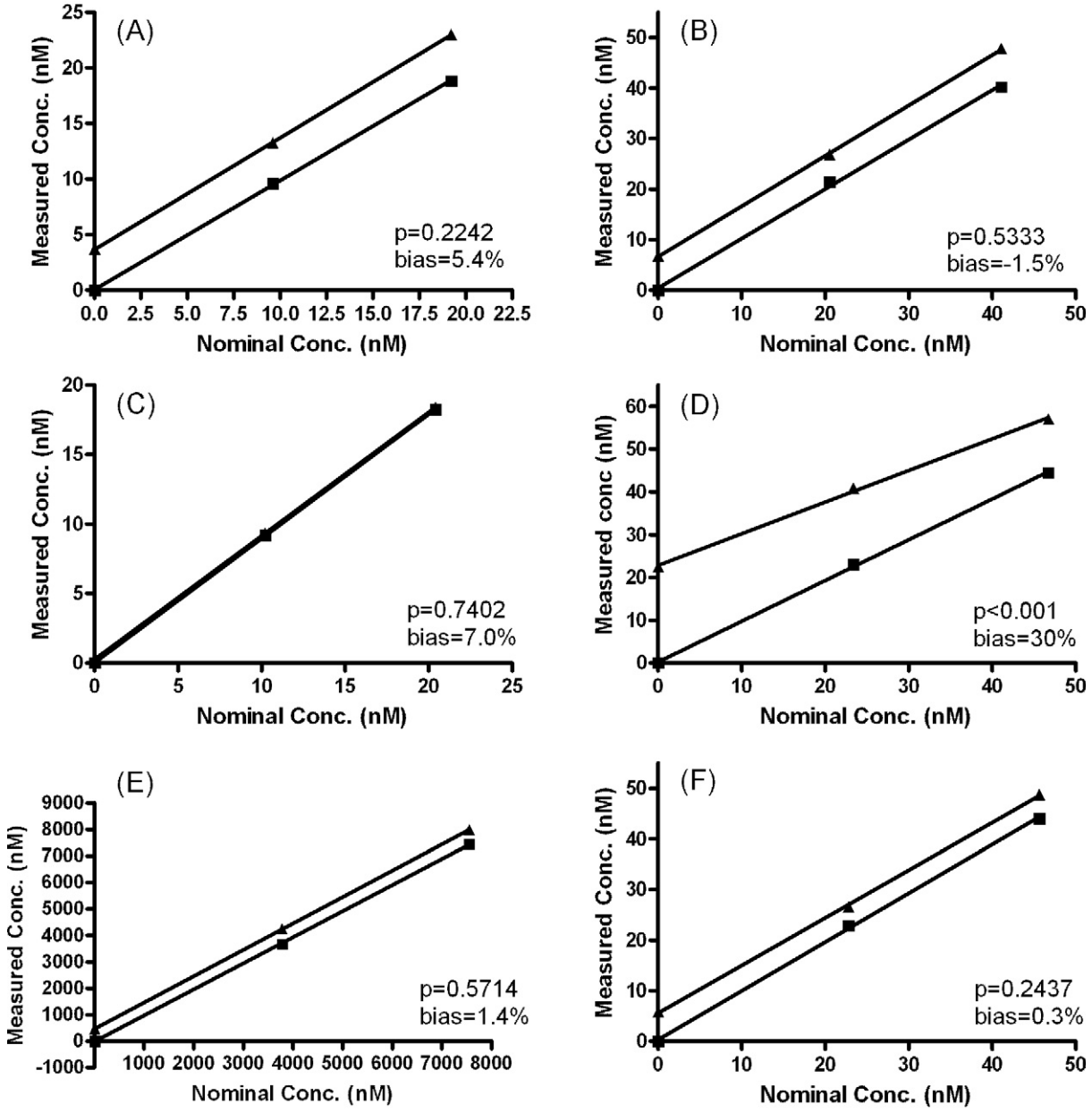

Fig. 4. The matrix effect was studied for (A) DHEA, (B) AEA, (C) LEA, (D) PEA, (E) 2-AG, and (F) OEA by the standard addition method. Two concentrations of standards (50 and 100 μl of QC level 3) were spiked into rat brain homogenate to obtain a regression curve (triangles). A standard curve using the same concentrations was prepared, without tissue matrix (squares). All samples were prepared according to Sections 2.3 and 2.5.1. Instrumentation parameters were the same as described in Section 2.4.

Table 2
The linear range, ΣRE, R^2, and calibration curve parameters with 95% confidence intervals of ECs ($n = 3$). All the samples were prepared and results calculated according to Sections 2.2, 2.3, and 2.5.2.

Compound	Linear range (nM)	ΣRE	R^2	Calibration curve parameters		LLOQ (nM)	LOD (nM)
				Slope ± 95% CI	Intercept ± 95% CI		
DHEA	0.1–29	157	0.9988	7.8264 ± 0.0144	−0.0040 ± 0.0007	0.5	0.2
AEA	0.2–62	107	0.9992	8.8096 ± 0.0195	−0.0084 ± 0.0022	1.0	0.4
LEA	0.1–31	103	0.9989	41.4134 ± 0.0733	−0.0223 ± 0.0040	0.5	0.2
PEA	0.2–70	172	0.9985	10.2067 ± 0.0332	0.1413 ± 0.0043	2.3	0.9
OEA	0.2–69	133	0.9991	16.5685 ± 0.0405	0.0502 ± 0.0050	1.1	0.5
2-AG	38–11,321	57	0.9984	4.4228 ± 0.0540	0.0055 ± 0.0951	189	75
Noladin	20–58,773	530	0.9917	0.2333 ± 0.0047	0.0085 ± 0.0043	98	39
NADA	4.1–244	9358	0.6460	0.5476 ± 0.1793	0.0303 ± 0.0395	n.d.	n.d.

n.d.: not determinated.

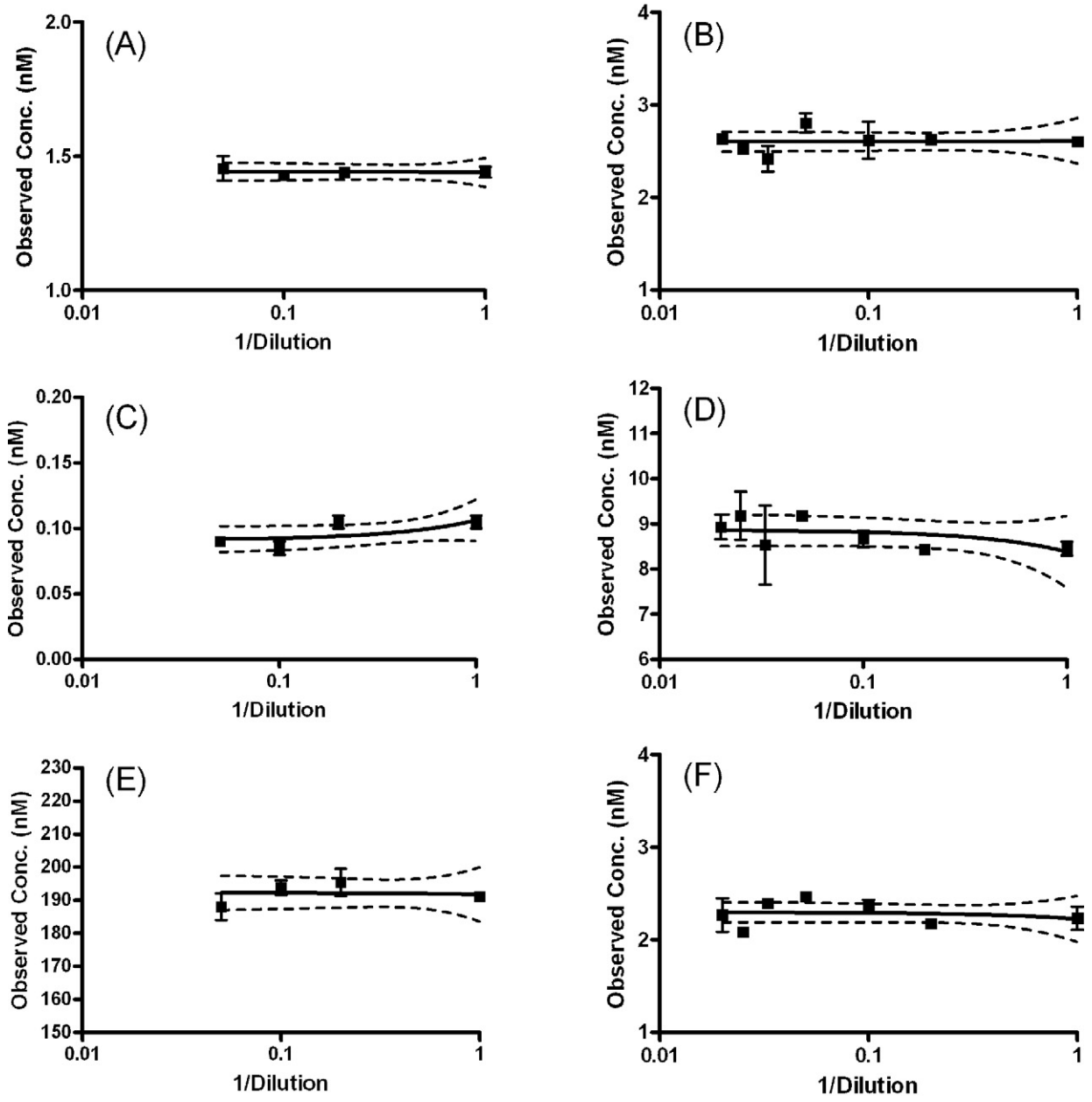

Fig. 5. Parallelism and selectivity of the method was studied by diluting the extracted tissue homogenate with HPLC mobile phase before injecting on to the LC/MS/MS. All samples were prepared according to Sections 2.3 and 2.5.1. Instrumentation parameters were the same as described in Section 2.4. The dashed line highlights the 95% confidence level scatter line of the linear regression curve. The dilution linearity shows no matrix effect for (A) DHEA (dilutions studied: 1:5, 1:10, and 1:20), (B) AEA (dilutions studied: 1:5, 1:10, 1:20, 1:30, 1:40 and 1:50), (E) 2-AG (dilutions studied: 1:5, 1:10, and 1:20), or (F) OEA (dilutions studied: 1:5, 1:10, 1:20, 1:30, 1:40 and 1:50). Linear regression shows lack of parallelism for (C) LEA (dilutions studied: 1:5, 1:10, and 1:20) and (D) PEA (dilutions studied: 1:5, 1:10, 1:20, 1:30, 1:40 and 1:50).

tion of the acyl group from *sn*-2 to the *sn*-1 position in the case of 2-AG (data not shown).

3.6. Applications

In the white matter of human brain tissues, the levels of 2-AG, PEA, and OEA were higher in all samples when compared to other brain regions, while levels of other NAEs (AEA, DHEA, and LEA) were lower [58]. These EC levels were measured from six different human brain regions. Table 4 summarizes the average results for each analyte, with the standard deviation (SD) given for each brain region from the non-alcoholic control group ($n = 10$). These results from non-alcoholics post-mortem brains showed that the 2-AG level in white matter was 3–9 times higher than in other studied brain regions. On the other hand, AEA and DHEA levels were 2–6 times lower in white matter than other studied brain regions.

4. Discussion

Considerable disagreement exists between the reported results of endocannabinoid (EC) measurements from different methods and instrumentation (e.g., LC–MS vs. GC–MS). This disagreement could arise from a wide variety of reasons that include differences in sample-dependent biological processes, sampling, sample preparation, calibration, and instrumentation [20], in addition to the lability and lipophilicity issues of the analytes. Various physiological and pathological states also affect the EC system and cause substan-

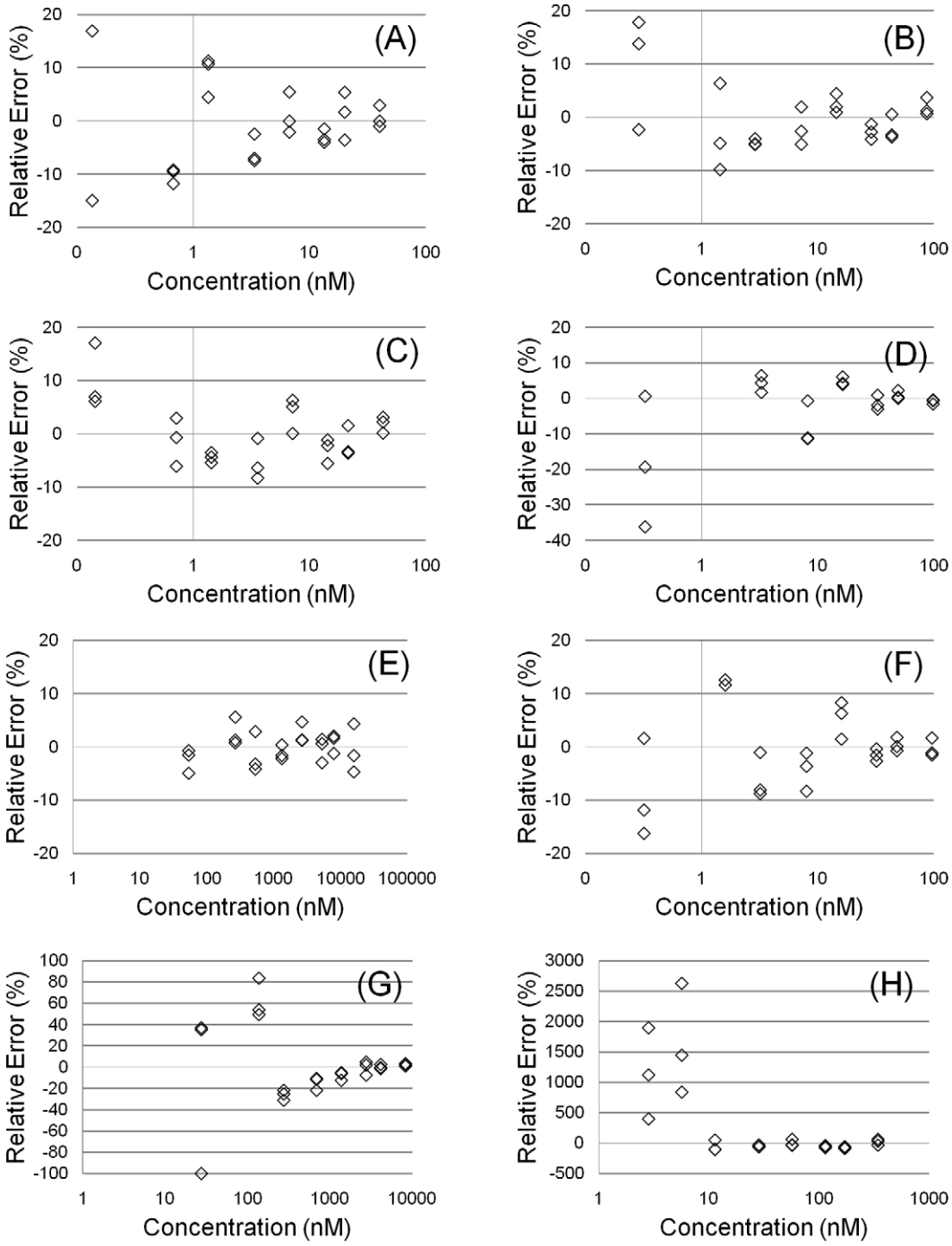

Fig. 6. A plot of the RE% vs. concentration obtained for (A) DHEA, (B) AEA, (C) LEA, (D) PEA, (E) 2-AG, (F) OEA (G) noladin, and (H) NADA. The calibration was prepared by a weighted ($1/x$) least square linear regression. The acceptance criterion for RE% was set to ±20%. All samples were prepared according to Sections 2.2 and 2.5.2. Instrumentation parameters were the same as described in Section 2.4.

tial differences between ECs levels [23,31,39,57,66–68]. The EC system has been implicated in several neurodegerative diseases [5,69,70] and neuropsychiatric disorders [58,71]. Also, some drugs have an effect on the EC system [27,36,46]. These methodological and biological effects should be carefully studied and taken into the account when planning research on the EC system or making analytical methods for EC analyses.

ECs are usually isolated from biological samples by standard liquid extraction techniques [40,42,49] with an additional chromatographic purification step with normal phase [23–25,28,38,40] or reversed phase [30–32,39] techniques. Unfortunately, major draw-backs of a multi-step sample preparation method are the amount of time required and subsequent difficulties in both standardization and automization. The lipid fraction of a tissue extract contains phospholipids, which are also precursors to ECs. Yang et al. [32] observed that the exposure of brain extracts to either basic or acidic conditions significantly enhanced the appearance of AEA. Acidification of the sample also leads to the artificial formation of lysophosphatidic acid (LPA) by acid-catalyzed hydrolysis of the more abundant lysophospholipids [72]. LPA is produced through several enzymatic pathways, mostly from lysophospholipids, such as lysophosphatidylcholine (Lyso-2-GPCho) or from

Table 3
Intra-day and inter-day precision and accuracy for DHEA, AEA, LEA, PEA, 2-AG, OEA, and noladin, and their nominal values at four QC levels. All samples were prepared and results calculated according to Sections 2.2, 2.3, and 2.5.3.

Compound	Nominal conc. (nM)	Intra-day precision and accuracy			Inter-day precision	
		Mean (n = 5) (nM)	RSD (%)	Mean accuracy (%)	Mean (n = 3 days) (nM)	RSD (%)
DHEA	0.5	0.5	2.1	95	0.5	7.5
	1.9	1.9	2.3	98	1.9	3.1
	10	10	1.8	102	10	2.5
	19	19	1.7	100	19	1.9
AEA	1.0	1.1	2.4	104	1.0	4.1
	4.1	4.2	2.2	102	4.2	4.1
	21	21	1.2	101	21	1.8
	41	41	1.5	103	41	1.5
LEA	0.5	0.5	3.9	102	0.5	5.2
	2.0	2.0	2.4	100	2.1	4.7
	10	10	1.5	101	10	2.4
	20	21	1.9	100	21	1.6
PEA	2.3	3.1	25	132	2.8	28
	9.3	8.7	15	93	8.1	23
	23	25	4.0	105	25	5.0
	47	46	1.1	98	48	4.5
2-AG	189	193	3.9	102	185	5.7
	755	786	1.3	104	771	4.4
	3774	3775	2.4	100	3818	4.2
	7548	7597	3.2	101	7537	2.7
OEA	1.1	1.2	6.9	107	1.3	7.9
	4.6	4.6	5.2	101	4.8	4.6
	23	23	3.2	99	23	3.2
	46	45	1.1	99	46	1.9
Noladin	98	100	12	103	92	23
	392	394	3.4	101	413	16
	1959	1828	4.3	93	1990	19
	3918	4217	3.7	108	4281	5.5

phosphatidic acid [73,74]. Both Lyso-2-GPChos and phosphatidic acids are directly or indirectly converted to diacylglycerols and then to monoacylglycerols (MAGs) in rat brain [75,76]. Sugiura et al. [75] also reported a rapid post-mortem increase in 2-AG and other MAGs in the rat brain, which occurs within the first 30 s after decapitation. In addition, Palkovits et al. [77] found a strong post-mortem effect during 6 h for ECs in micro-dissected human brains, where tissue levels of 2-AG rapidly declined while AEA increased continuously in a region-dependent manner. An influence of post-mortem effect on NAEs levels in mammalian [23] and rodent brains [31,78] have also been reported. In our extraction method, LPAs mostly remain in the water phase. We also found that LPAs significantly coat the small particle stationary phase of the narrow bore reversed phase column, and therefore significantly reduce column efficiency [79]. After brief homogenization of the tissue sample with sonification in ice-cold methanol, we found that a one-step extraction protocol (without the addition of any acid or base) was the most suitable isolation method for AEA and 2-AG [57]. The same sample preparation protocol was also found to be suitable to other studies of ECs and chemically related cannabimetic compounds, except NADA, as reported in this paper. Liquid–liquid extraction (LLE) was reported to be the most efficient extraction procedure when compared with protein precipitation and common SPE techniques for removing potential matrix effects during sample preparation [53,80]. This may at least be partly explained by LLE's ability to effectively remove nonvolative particles (e.g., salts) from these samples, which cause the matrix effect in electrospray ionization. In our hands, ECs were highly retentative in reversed phase columns, and early eluating nonvolatile materials such as salts were eluated during the first 2 min of the run, which was studied by a post-column infusion experiment. ECs are also reported to adsorb to glass and plastic surfaces [26,47]. During the preliminary method development we studied AEA and 2-AG behavior towards the quality of various glass-ware used for extraction and autosampler vial inserts. During the sample preparation and after 12 h of autosampler storage at 10 °C, no dramatic differences were detected between deactivated glass-ware and normal glass-ware. HPLC vial inserts made from polypropylene were also studied,

Table 4
The levels of ECs in human post-mortem brains [58]. These EC levels were measured from six different brain regions of non-alcoholic controls (n = 10). The average result of each analyte with the standard deviation for each brain region is presented.

Compound	White matter (n = 10)	NAcc[a] (n = 10)	Frontal cortex (n = 10)	pACC[b] (n = 10)	Amygdala (n = 10)	Hippocampus (n = 10)
2-AG (nmol/g)	360 ± 93	67 ± 39	55 ± 38	41 ± 36	120 ± 64	58 ± 36
AEA (pmol/g)	567 ± 199	1880 ± 638	1941 ± 619	1915 ± 362	1009 ± 306	1150 ± 278
DHEA (pmol/g)	285 ± 47	1417 ± 502	1704 ± 263	1348 ± 240	802 ± 140	888 ± 190
LEA (pmol/g)	91 ± 23	128 ± 42	165 ± 47	165 ± 49	107 ± 23	128 ± 33
PEA (pmol/g)	6443 ± 1852	4473 ± 471	4230 ± 1615	3939 ± 895	4687 ± 1638	5749 ± 888
OEA (pmol/g)	2217 ± 623	1585 ± 433	1659 ± 733	1701 ± 628	1541 ± 394	2142 ± 1104

[a] Nucleus accumbens.
[b] Perigenual anterior cingulate cortex.

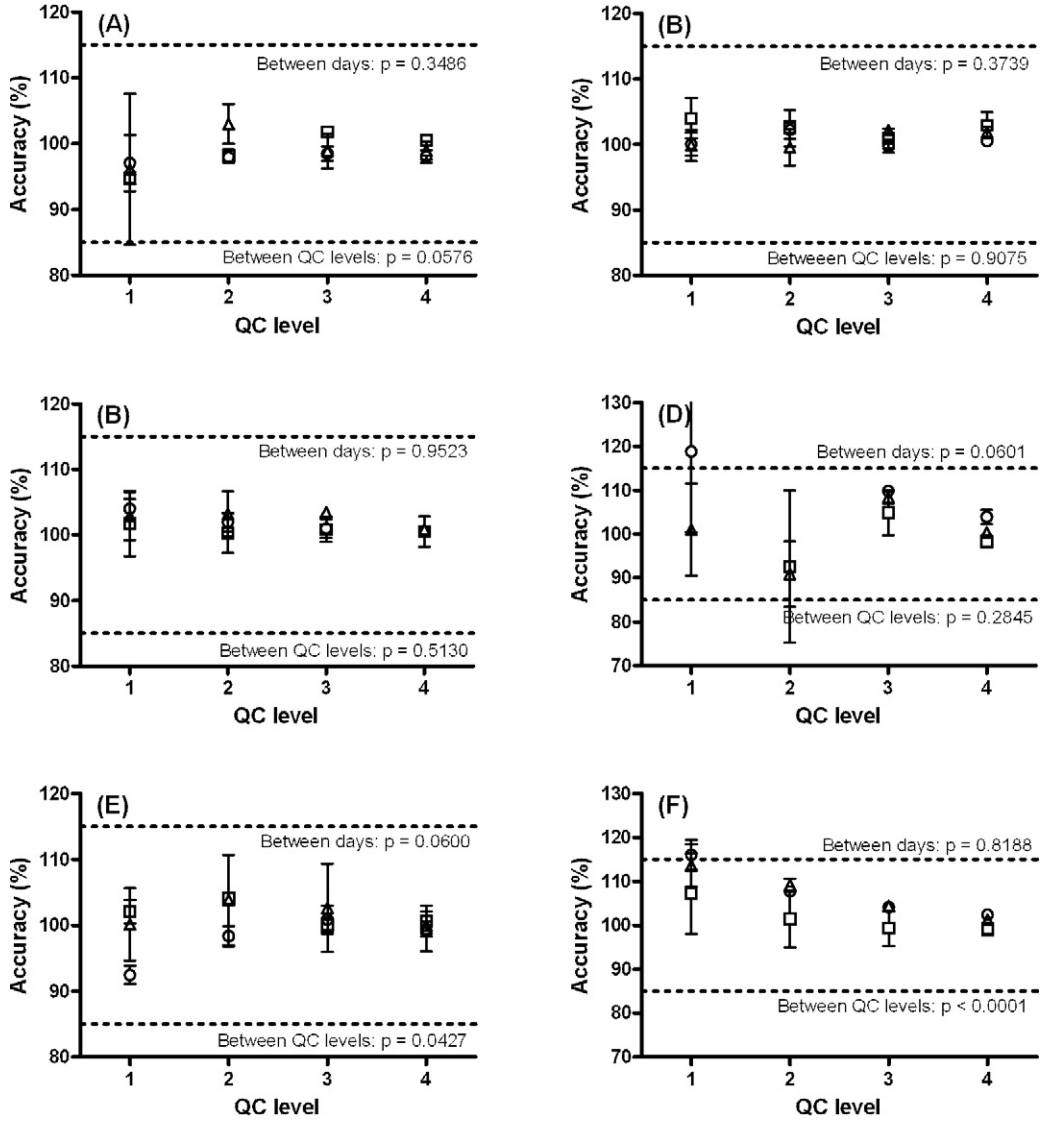

Fig. 7. A 2-way ANOVA was used for the test for comparing each analyte's accuracy results between days and QC levels. A plot of accuracy results from four different QC levels ($n = 5$) on three different days (day 1 = □; day 2 = △; and day 3 = ○) is presented for (A) DHEA, (B) AEA, (C) LEA, (D) PEA, (E) 2-AG, and (F) OEA. The figure shows the mean concentrations on 3 different days with error bars (95% confidence interval). All samples were prepared according to Sections 2.2, 2.3, and 2.5.3. Instrumentation parameters were the same as described in Section 2.4.

where signal background in IS-MRM transitions m/z 104 → 104 and 184 → 184 was found to be higher in plastic inserts when compared with normal glass inserts (data not shown). According to these results, no significant adsorption was observed for analytes to either glass or plastic surfaces, yet we decided to use normal glass-ware without deactivation.

In the tandem mass spectrometric methods, ESI ionization has been shown to be slightly more sensitive in terms of having lower LLOQ values than APCI [41,42]. ECs were also known to produce 4 times more intensity of ionization in positive ESI compared to negative ESI [35], which is why we used positive ESI (Supplementary Fig. 1), even though APCI could be used to avoid matrix effects [51,52]. Phospholipids are abundant in biological membranes and glycerophosphocholines (GPChos) are fundamental membrane components that provide one of the main sources of the matrix effect (e.g., ion suppression and/or ion enhancement) [1,52,53]. As zwitterions, GPChos can cause matrix effects in both positive and negative ESI during mass spectrometric detection [81]. This matrix effect results from ionization competition between the different species eluted from the column and most likely in ESI where high concentrations of nonvolatile materials are present in the spray with the analyte [82]. As a phenomenon, the matrix effect is not reproducible between tissue samples, as the sample weight may fluctuate considerably and thus could cause systematic and/or random errors in a quantitative methodology. For this reason the matrix effect had to be investigated and controlled, or at least minimized, by improved sample preparation and by using a more efficient chromatographic separation between analytes and interfering matrix compounds, and also by using deuterated analogues as internal standards [51,52]. To optimize the MS conditions, the effects of varying the fragmentor voltage (V), collision energy (V), and mass resolution values (FWHM) on analyte abundance were examined by direct replicate injections, without an analytical column for a fixed amount of studied compounds (Table 1). During method development, we further optimized the following parameters: electron multiplayer voltage (EMV), the amount of organic solvent in the mobile phase and by adjusting the flow rate. Decreasing the flow, increasing both the amount of organic solvent in the mobile phase, and increasing EMV strengthened the abundance of the followed ions. With these transitions and optimized detector

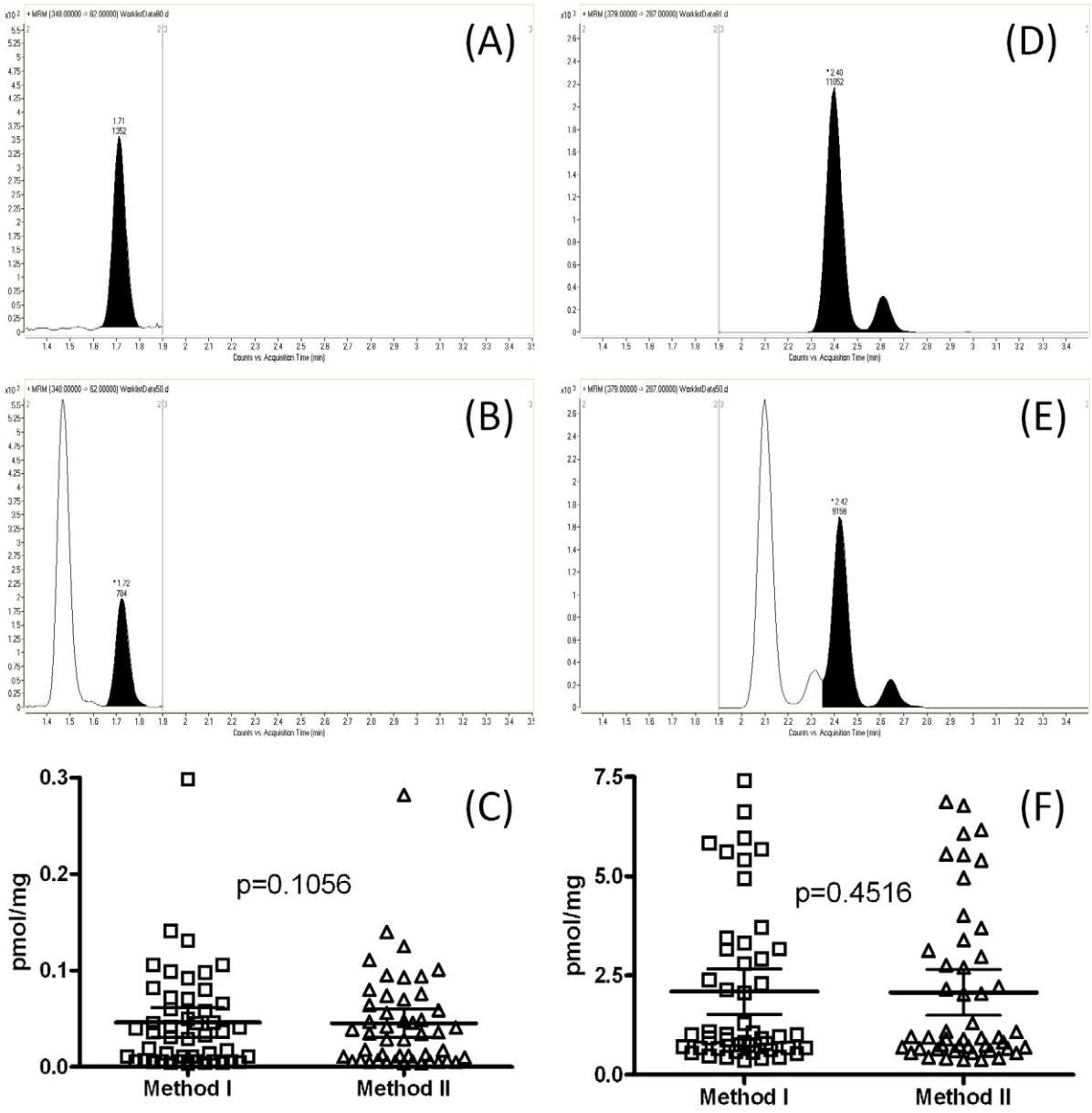

Fig. 8. Representative MRM chromatograms of (A) a standard sample of AEA (2.1 nM, RT 1.71 min), (B) AEA in *C. elegans* N2 (1.5 nM), (D) standard sample of 2-AG (892 nM, RT 2.40), and (E) 2-AG in *C. elegans* N2 (793 nM). The higher flow rate method was also applied to this set of *C. elegans* samples ($n = 48$). There were no statistical differences in (C) AEA and (F) 2-AG concentrations obtained with methods at flow rates of 0.15 ml/min (Method I) and 0.5 ml/min (Method II).

parameters, a robust and sensitive assay for the detection of ECs was developed. Stable deuterated analogues of 2-AG and AEA with high isotopic purity were selected as internal standards to ensure uniform behavior of the followed compounds and ISs (Table 1).

Lipids like ECs and GPChos are very hydrophobic species that require a significantly strong eluation media to completely pass through a reversed-phase column. Employing a variety of RP columns, with different diameters and different selectivities (i.e., stationary phases) made it possible to establish a stable and reliable chromatographic method for measuring ECs (Fig. 1). During method development, three column-related factors were manipulated: the selectivity of a column for the best resolution, efficiency was improved with increasing column length, decreasing particle size and extra column volume, and retention. Due to a highly selective extraction protocol, a high retention of both species (ECs and GPChos) with a RP column, and thus a similar sample retention range, an isocratic method was selected to develope the final HPLC method. For studying the retention of 18:1 Lyso-2-GPCho and ECs we used an IS-MRM technique [53]. In this technique a high fragmentator voltage is used to fragment GPChos and Lyso-2-GPChos inside the ionization source to ions m/z 104 and 184. Sphingomyelins also form a m/z 184 ion via in-source fragmentation [53]. The best separation of 18:1 Lyso-2-GPCho and the studied compounds was achieved by a short, narrow bore high resolution sub-2 μm stationary phase (Fig. 1; Supplementary Fig. 2). The column was also able to separate AEA and virodhamide from each other (Fig. 1). Those compounds have the same MRM transition (m/z 348 → 62), and therefore they must be chromatographically separated. In addition, a narrow bore column with a small particle size and ultra high pressure instrumentation enables the use of higher pressures and higher flow rates (Fig. 8). When compared to results from a set of nematode samples ($n = 48$) obtained with a 0.15 ml/min flow rate, no statictical difference could be found for either AEA or 2-AG at a higher flow rate of 0.5 ml/min. Due to slightly lower RSD values in precision tests, we decided to use 0.15 ml/min flow in our final LC/MS/MS method. We also found

it necessary to use an in-line filter to protect the narrow-bore, small-particle analytical column. Blockage of microbore columns is a particular problem because the small diameter of the column inlet frit leads to increased column back pressure and subsequent loss of efficiency for the column. We also noticed the precipitation from a highly lipidic fraction of sample extract after the addition of water in the last step of sample preparation. This back precipitation could shorten the lifetime of the analytical column when injecting samples with high concentrations of organic solvents or in gradient methods. We did not see any back precipitation problems with this isocratic method, and the method remained unchanged even after thousands of injections. GPChos are strongly retained in reversed-phase columns, where they form a steady-state background, which could be seen as an elevated signal background at m/z 184 → 184 [53]. In our method this signal essentially remained at steady-state after 170 injections on a new HPLC column.

In protic solvents, 2-AG undergoes a nonenzymatic acyl migration from *sn*-2 to the *sn*-1 position, resulting in a chromatographic peak corresponding to 1-arachidonylglycerol (1-AG) [30]. Schmid et al. [28] showed that this migration is also dependent on temperature. Kingsley and Marnett [42] found that the mass spectral response factor for Ag^+ complexes of 2-AG and 1-AG was not equal in ESI. Therefore, in the present work, we avoided the use of protic solvents and/or tried to keep sample contact time with such solvents as short as possible. Furthermore, we kept samples on ice during sample work-up and at 10 °C until the time they were injected for analysis. With these procedures we found that acyl migration of 2-AG was greatly reduced. However, we also observed that this acyl migration did take place during the autosampler stability tests. This migration did not have a significant effect on either the precision or accuracy determined from QC samples stored in the autosampler for 24 h and then compared with freshly prepared calibration standards. The 1-AG positional isomer is chromatographically separated from 2-AG (Figs. 2 and 3) and the peak areas of 1-AG and 2-AG were subsequently combined for all quantitative analyses reported below and are thus collectively termed "2-AG".

The objective of method validation is to quarantee the quality of results with authentic analytes in an authentic matrix [54]. Clearly, the development and validation of analytical methods for the accurate and precise determination of endogenous analytes like ECs in biological samples is extremely demanding. The lack of an analyte-free sample to study the biological matrix forces the researcher to use alternative strategies for method verification. In effect, two main strategies may be used for such verifications, i.e., the use of an authentic analyte in a surrogate matrix and the use of a surrogate analyte in an authentic matrix [54]. Endogenous analytes may be removed by a variety of procedures, such as charcoal stripping, high-temperature incubation, acid or alkaline hydrolysis, or affinity chromatography [83]. In addition, alternatives to matrix processing include the use of surrogate protein-containing buffers or even a more heterologous matrix (e.g., a related species or appropriate mutant of a species such as *C. elegans fat-3* mutant), which lacks the analyte(s) of interest or contains a less reactive homolog [54,83]. The nematode *C. elegans* is a good model for studying the physiological functions of polyunsaturated fatty acids (PUFAs), and we recently reported that nematodes produce AEA and 2-AG [56]. Mutants of *C. elegans* have been developed that are unable to synthesize specific PUFAs due to mutations in genes encoding fatty acid desaturases and elongases [84]. The two main ECs of interest in this study (AEA and 2-AG) were not identified in the *C. elegans fat-3* mutant (Fig. 3), and therefore this mutant provides an ideal analyte-free surrogate matrix for selectivity and calibration studies. The *fat-3* mutant lacks the necessary enzyme to produce arachidonic acid, the fatty acid precursor to both AEA and 2-AG. The absense of arachidonic acid was also comfirmed by gas chromatography mass spectrometric (GC–MS) analysis of the relative content of *C. elegans*' total fatty acids (data not shown). Furthermore *fat-1* and *fat-4* mutants are unable to produce *omega*-3 (ω-3) and *omega*-6 (ω-6) fatty acids, respectively (Fig. 3). Unlike all the other tested biological sources, wild type nematodes are able to produce both ω-3 and ω-6 analogues of AEA and 2-AG, which can be seen in chromatograms as two peaks at each MRM channel (Figs. 2 and 3). The ω-6 isomers of AEA and 2-AG have higher retention on reversed phase columns than the corresponding ω-3 isomers.

Selectivity and analytical bias can be evaluated by the method of standard addition, which is especially powerful when analyzing endogenous compounds [65]. When the calibration standards are not matrix-matched, it is then possible to evaluate the bias by comparing the slopes of the standard addition line and the calibration line. We found no matrix effect causing bias in our method for any of the studied compounds, except PEA (Fig. 4). In the case of PEA, a matrix effect due to overlapping material from the biological sample was clearly observed, and the internal standard method used for calibration subsequently underestimates the concentrations in the tissue by approximately 30% (Fig. 4). Dilution of post-sample processing was also found to be very useful for recognizing and subsequently eliminating matrix effects [51,83]. The study of parallelism also identified the matrix effect for PEA (Fig. 5 and Supplementary Table 1). In the case of LEA, the lack of parallelism was due to a small content of LEA in the biological matrix, which increased the deviation of the determinations. The lack of any major matrix effect was further studied by post column infusion, where the only other significant ion suppression was observed at the beginning of the analysis due to the presence of salts and various other non-retained components. These studies were used to ensure that there was no matrix effect or interference that could transfer systematic errors to the results. These selectivity studies provided the evidence that validated the use of the calibrator preparation described in Section 2.2. It has been suggested that OEA and PEA are present as contaminates from laboratory materials [33], which could partly explain the matrix effect found in our method. This matrix effect was also present with pure solvents and could be seen as out-of-specification QC values in the case of PEA. Therefore this method has to be considered as only semi-quantitative for PEA.

The eight point calibration curves were highly linear over the range of the method (Table 2). Exceptions to this were NADA and noladin. The poor stability of NADA was also easily seen in the calibration data, and noladin had a notably higher LLOQ than the other studied analytes. For minimizing the total error of the method, a calibration curve should be prepared in the same biological matrix as the samples, by spiking the matrix with known concentrations of the analyte [64]. In the case of endogenous analytes, a researcher should select the most appropriate strategy for calibrating the method or carefully study the effect of calibration with pure standards by these established strategies in order to demonstrate the method validity. In our method, we justified the calibration with pure standards by means of tests like standard addition (Fig. 4), parallelism (Fig. 5), and the use of the *C. elegans fat-3* mutants as a surrogate material for preparing the calibration standards. We found it also necessary to use weighting in our calibrations to obtain values that fullfill the predeterminated criteria for calibrators' RE% (Fig. 6 and Supplementary Table 2). In many bioanalytical methods with broad dynamic concentration ranges of three or more orders of magnitude, the RSD is roughly constant over a range of concentrations, yet the absolute error subsequently increases with the concentration of the analyte. A simple and effective way to counteract the heteroscedasticity is to use a weighted least square linear regression [85,86]. In our hands, the effect of the weighting process was clear, and the accuracy of the method was highly improved by this strategy at the lower end of the calibration curve (Table 2 and Fig. 6). The preparation of calibration samples on top of surrogate

materials was also studied. We did not find any statistical differences in calibrations of AEA and 2-AG between pure standards and standards added to the *fat-3* mutant samples. This finding, together with previously discussed results from selectivity studies, validates the preparation of calibration curves from pure standard solutions as described in Section 2.2.

Whenever possible, the actual validation samples should be aliquots of the authentic matrix, both unspiked and spiked, with known amounts of the authentic analyte [54]. The QC samples were prepared from pure standard solutions, and were prepared at four different QC concentrations, and from two different surrogate matrixes, i.e., BSA and the *C. elegans fat-3* mutant. The precision of this method was further studied with rat brain homogenates, which were also used for stability tests. We found that these results were highly accurate and precise (Table 3 and Fig. 7). We also found high recovery values with our method, which were close to 100%. The recovery was measured by the standard addition method. Our results seem to be higher than those in previously published methods [30,37,42], which may be due to slightly different sample preparation protocols. On the other hand, recovery values from biological samples as higher than 90% have been reported [40,41,43]. ECs are reported to be chemically stable when stored frozen, and this was also observed in our stability tests. Palkovits et al. [77] reported that levels of ECs in brain tissue samples that had been stored for 2–8 months did not differ significantly from samples that had been frozen for 13 years, which indicates that ECs are chemically stable when stored frozen, even over very long periods of time.

Our LC/MS/MS method was able to follow the four most common ECs (i.e., 2-AG, AEA, LEA, and DHEA) and two other cannabimimetic compounds (PEA and OEA) in biological samples that ranged from rats, to humans and nematodes (Figs. 2 and 3). An additional peak in the MRM channels of OEA was observed in all studied biological samples. This extra peak could be due to *N*-(*cis*-7-octadecenoyl)ethanolamine, which is the positional isomer of OEA (*N*-(*cis*-9-octadecenoyl)ethanolamine). In the GC–MS analysis of *C. elegans* total fatty acid profile we observed a peak for vaccenic acid methyl ester (C18:1, n-7) (data not shown). This extra peak in the MRM channel of OEA was not baseline separated from OEA (Fig. 2) and with gradient methods it could coeluate with OEA. In addition, a similar phenomenon exists in the case of OEA as an extra peak observed by a GC/MS/MS technique used for plasma analysis [33]. Due to this unacceptable resolution, the current method must also be considered as semi-quantitative for OEA. The measured concentrations of ECs in all samples were above the LLOQ of the method, and only a couple of samples had concentrations outside the range of the calibration curve. The low affinity CB1 agonists noladin and NADA were not detected by this method in these biological samples. Noladin [16,87] and NADA [18,39,88] were previously demonstrated to occur at levels that could be detected with MS techniques. In case of acyl dopamines, dopamine itself is quite a labile compound and, according our results, it was unstable in the described sample preparation protocol. It also seems that a sample preparation method that included homogenization and SPE extraction, in addition to small amounts of triethylamide with trifluoroacetic acid in the chloroform, stabilizes the sample for NADA analysis [39,40]. In addition, high recovery values from plasma for NADA and noladin have been reported after protein precipitation with ACN and further sample clean up with SPE [47].

Although preliminary and from diagnostic groups having relatively small numbers of subjects, levels of 2-AG, PEA, and OEA in human brain white matter were observed to be relatively high in all non-alcoholic control samples when compared to other brain regions, while levels of other NAEs (AEA, DHEA, and LEA) were relatively low (Table 4). This finding seems to correlate with results from a previous study of post-mortem changes of endocannabinoids levels in human micro-dissected brains [77]. This finding may be at least partly explained by differences in white matter post-mortem activity and brain region-dependent differences in the distribution of EC degrading enzymes [89,90]. It has also been suggested that the accumulation of AEA, but interestingly not PEA and OEA, is due to a nonenzymatic process, via aminolysis of the arachidonate-rich phospholipid or triglyceride by ethanolamine [31,78]. Maccarrone et al. [29] used perilesional white matter surrounding the tumour area to compare levels of ECs in meningioma and compared it with the surrounding healthy tissue (white matter). In that study, the meningioma showed an approximate six-fold smaller amount of all EC-like compounds except 2-AG. These reports suggest the possibility to use white matter as an *in situ* comparative reference sample to compensate or perhaps even control for post-mortem effects when measuring ECs.

5. Conclusion

A method for the targeted analysis of the four main ECs (2-AG, AEA, DHEA, and LEA) and two other cannabimimetic compounds (PEA and OEA) was developed and validated. The method was found to be highly selective, linear, accurate, and precise for ECs. In case of PEA and OEA the method can only be considered semi-quantitative. Single step liquid–liquid extraction ensured high recovery of the studied analytes. ECs are endogenous compounds and an analyte-free sample matrix was not readily available, which made the validation of the method demanding. Multiple tests were used for the selectivity and calibration studies, including surrogate matrix tests, in-source MRM studies, standard addition studies, on-line infusion studies and parallelism studies. To our knowledge, this is the first report of using the *C. elegans fat-3* mutant as a surrogate biological material for method development and validation. This mutant lacks the necessary enzyme to produce arachidonic acid (C20:4) and therefore does not produce either of the two main ECs; AEA and 2-AG. This mutant was successfully used during the selectivity and calibration studies that led to a LC/MS/MS method for the quantitative analysis of ECs in both nematodes and post-mortem human brain samples. For this reason, the *C. elegans fat-3* mutant has use in method development for the quantitative analyses of eicosanoids in general.

The method described in this report has been successfully used to quantify the levels of ECs from a wide variety of matrices, such as human and rat brain tissues, nematodes, human adipocytes, human skeletal muscle cells, and cell culture media. The sub 2 μm particle size column and ultra high pressure instrumentation provides the possibility to decrease the analysis time to 3.5 min for these analytes. No column degradation was observed and the system suitability tests, as well as the QC samples analyzed within each sequence, indicated reliable performance of the method even after thousands of biological sample injections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.02.004.

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