Chapter 5

Analysis of Omega-3 Fatty Acid-Derived *N*-Acylethanolamines in Biological Matrices

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Abstract

The adequate quantification of endocannabinoids and related *N*-acylethanolamines can be complex due to their low endogenous levels, structural diversity, and metabolism. Therefore, advanced analytical approaches, involving LC–MS, are required to quantify these molecules in plasma, tissues, and other matrices. It has been shown that endocannabinoid congeners synthesized from n-3 poly-unsaturated fatty acids (n-3 PUFAs), such as docosahexaenoylethanolamide (DHEA) and eicosapentaenoylethanolamide (EPEA), have interesting immunomodulatory and tumor-inhibiting properties. Recent work has shown that DHEA and EPEA can be further enzymatically metabolized by cyclo-oxygenase 2 (COX-2), forming oxygenated metabolites. Here, an LC–MS-based method for the quantification of the n-3 PUFA-derived endocannabinoid congeners DHEA and EPEA is described, which is also suited to measure a wider spectrum of endocannabinoids. The chapter contains a step-by-step protocol for the analysis of (n-3) endocannabinoids in plasma, including sample collection and solid phase extraction, LC–MS analysis, and data processing. In addition, protocol modifications are provided to allow quantification of n-3 PUFA-derived endocannabinoids and their COX-2 metabolites in tissues and cell culture media. Finally, conditions that alter endocannabinoid concentrations are briefly discussed.

Key words Endocannabinoids, n-3 fatty acid, Docosahexaenoylethanolamide, Eicosapentaenoylethanolamide, Solid phase extraction, LC–MS

1 Introduction

According to the current (IUPHAR) classification system, endocannabinoids constitute a relatively small group of fatty acid-derived endogenous ligands of the cannabinoid receptors CB_1 and CB_2 [1, 2]. From a biochemical perspective, these endocannabinoids are part of a large family of structurally related amides, esters, and ethers of fatty acids, which are continuously formed and degraded in a dynamic equilibrium. The sofar best-studied group of endocannabinoids is the class of *N*-acylethanolamines (NAEs), which comprises conjugates of fatty acids to ethanolamine, such as arachidonoylethanolamide (AEA, anandamide) [1]. Other

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examples of endocannabinoids include glycerol esters, such as 2-arachidonoylglycerol (2-AG), and the dopamine conjugate *N*-arachidonoyldopamine (NADA) [1].

Next to AEA, ethanolamine conjugates have been described for palmitoic acid, stearoic acid, and the n-3 fatty acids docosahexaenoic acid and eicosapentaenoic acid, from which some have direct or indirect endocannabinoid activity [3–7]. It has been shown that cells are able to "combine" different fatty acids and biogenic amines, thus producing several possible permutations of fatty acid amides [8, 9]. Studies have also demonstrated that the local relative availability of their fatty acid precursors, which is, among others, determined by diet, plays an important role in determining the pattern of amide conjugates formed [3–5, 10].

The high diversity and dynamics of endocannabinoids and related compounds in tissues demand for adequate methods for their quantification. Most published reports are using advanced analytical chemical techniques, such as gas chromatography coupled to mass spectrometry (GC–MS) or liquid chromatography coupled to mass spectrometry (LC–MS), to meet this challenge [11]. MS-based methods use the molecular mass of a compound to detect it, which allows to simultaneously measure a broad range of compounds in a single sample. The molecular mass of a compound is more specific than, for example, its ultraviolet (UV) absorbance characteristics, and therefore, MS analysis results in data with higher specificity. Another advantage of MS over optical detector is that similar molecules of different molecular weight can be measured, even when there is no chromatographic separation.

Typically, published methods on the quantification of "classical" endocannabinoids and related compounds are focusing on a rather limited number of molecules derived from selected precursor fatty acids such as AEA, 2-AG, palmitoylethanolamine (PEA), and oleoylethanolamine (OEA). However, increasing data underline the (patho-)physiological relevance of compounds derived from other biologically important fatty acids. For instance, n-3 fatty acids are abundantly present in the brain and important for brain functioning. We and others have also demonstrated the presence of n-3 fatty acid-derived endocannabinoids such as docosahexaenoylethanolamine (DHEA) and eicosapentaenoylethanolamine (EPEA) in mammalian plasma and other tissues, at concentrations similar to those of the "classical endocannabinoids" (*see* Fig. 1 for molecular structures) [5, 6, 12]. Studies have shown that these endocannabinoid-like compounds have biological effects on immune cells [13, 14], cancer cells [15, 16], and hippocampal cells [17]. Based on their affinity for CB_1 and CB_2, DHEA and EPEA could be classified as "true" endocannabinoids, although much of their bioactivity appears to be not directly related to cannabinoid receptors alone [2, 7, 14, 15]. Over the last years, it has also been demonstrated that various endocannabinoids,

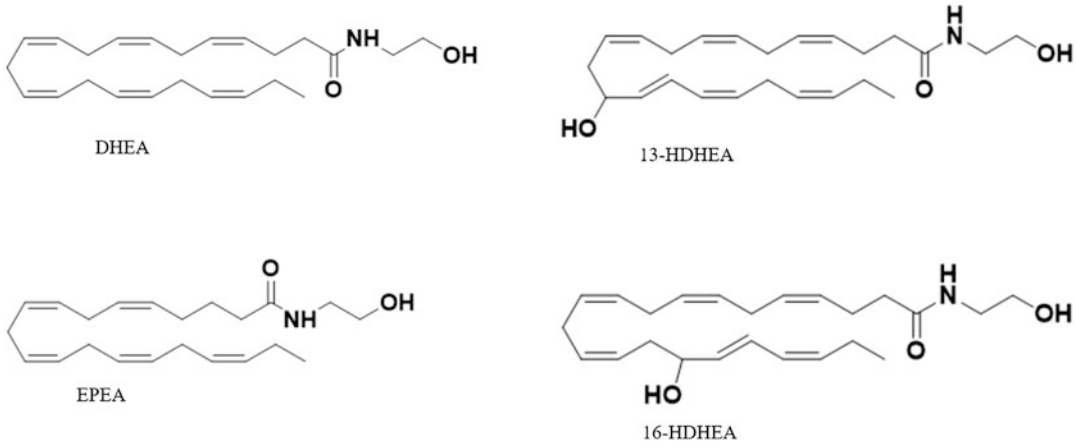

Fig. 1 Molecular structures for DHEA and EPEA and their hydroxy derivatives

including DHEA, are substrates for oxygenases such as cyclo-oxygenase 2 (COX-2), lipo-oxygenases (LOXs), and cytochrome P450 (CYP450) enzymes [18]. For instance, work in our labs has shown that DHEA can be metabolized by COX-2 into 13- and 16-hydroxy-DHEA (13-HDHEA and 16-HDHEA, respectively; *see* Fig. 1), which have different immunomodulatory properties than their parent DHEA [19, 20]. This further underlines the concept that the "prototypical" endocannabinoids like AEA and 2-AG, as well as their n-3 congeners DHEA and EPEA, are part of dynamic biochemical pathways, where metabolites are constantly formed, converted, or broken down. This demands for methodologies that include the analysis of oxygenated metabolites in biological matrices, in particular, in situations where high activity of, for example, the COX-2 enzyme can be expected.

In this chapter, we describe a protocol for the quantification of the n-3 fatty acid-derived endocannabinoids DHEA and EPEA in (human) plasma using LC–MS. This method allows to determine a wider spectrum of endocannabinoids, including AEA, 2-AG, and NADA, which we will also address briefly where relevant. Depending on the sensitivity of the LC–MS system, 0.1–1.0 mL of plasma is sufficient to obtain adequate peaks. Plasma is collected from blood, and a plasma extract is made using acetonitrile (ACN), which is subsequently washed and concentrated using solid phase extraction (SPE). The eluate from the SPE step is evaporated to dryness, reconstituted, and subsequently analyzed using LC–MS. Proper data analysis is also crucial. In addition to the standard plasma protocol, we will also present modifications that are aimed at quantifying the COX-2 metabolites of DHEA (13- and 16-HDHEA) and adaptations to extract n-3 endocannabinoids from other matrices such as tissues and cell culture medium.

2 Materials

Water and solvents should be of high purity. Different suppliers might use different terminology, such as "LC–MS grade" or "ULC grade." Fresh, ultrapure deionized water is preferred, as it is present in most labs. MQ water of >18 MΩ at 25 °C is a good option.

Polypropylene-based plastics show good recoveries during the sample preparation; glass should be avoided, in particular for the vacuum concentration step [21].

2.1 Analytical Standard Solutions

1. Calibration curve solutions.

   Prepare a set of calibration solutions in ACN containing the concentrations described in Table 1. Store at −80 °C (*see* **Note 1**). All calibrators have the same concentration of deuterated analogs. During LC−MS analysis, both the intensity of the natural compound (e.g., AEA) and its deuterated analog (e.g., AEA-d8) will be measured. The AEA:AEA-d8 ratio will be proportional to the concentration. Deuterated standards will also be spiked to the plasma samples in exactly the same amounts as present in the calibrators (*see* **Note 2**).

2. Deuterated standard spiking stock solution.

Table 1
Overview of concentrations in calibration curve solutions

Concentration (ng/ml)	cal8	cal7	cal6	cal5	cal4	cal3	cal2	cal1
AEA	406.50	135.50	45.17	15.06	5.02	1.67	0.56	0.19
2-AG	5617.00	1872.33	624.11	208.04	69.35	23.12	7.71	2.57
DLE	140.40	46.80	15.60	5.20	1.73	0.58	0.19	0.064
OEA	140.40	46.80	15.60	5.20	1.73	0.58	0.19	0.064
PEA	140.40	46.80	15.60	5.20	1.73	0.58	0.19	0.064
SEA	69.50	23.17	7.72	2.57	0.86	0.29	0.10	0.032
DHEA	140.40	46.80	15.60	5.20	1.73	0.58	0.19	0.064
EPEA	500.00	166.67	55.56	18.52	6.17	2.06	0.69	0.23
AEA-d_8	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25
2-AG-d_8	750	750	750	750	750	750	750	750
PEA-d_4	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
OEA-d_4	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25

These are values that are normally used in our lab. Typically, our lower limit of quantification (LLOQ) is in the range of cal1 or cal2

Prepare the spiking solution containing 312.5 ng/mL AEA-d8, 7500 ng/mL 2-AG-d8, and 312.5 ng/mL OEA-d8 in ACN (*see* **Note 2**). Store at −80 °C.

3. Reconstitution solution.

   Prepare the reconstitution solution containing 12.5 ng/ml PEA-d4 in ACN. Store at −80 °C. It is recommended to make amounts sufficient to run hundreds of samples.

2.2 Solutions and Reagents

1. 100 mM phenylmethanesulphonyl fluoride (PMSF) solution in isopropanol. Store at 4–8 °C. A volume of 10 mL 100 mM PMSF solution is sufficient to collect hundreds of samples.

   PMSF is an enzyme inhibitor which also inhibits fatty acid amide hydrolase (FAAH), the principal NAE-degrading enzyme (*see* **Note 3**).

2. Deuterated standard spiking work solution containing 100 μM PMSF.

   Make a tenfold dilution of the deuterated standard spiking stock solution in acetonitrile and add 100 mM PMSF to a final concentration of 100 μM. Make this solution fresh for every batch. Ensure that the stock solution has reached room temperature before opening the vial.

3. MQ water with 0.133% trifluoroacetic acid (TFA). Store at room temperature. Prepare fresh solution for each batch. A bottle containing 1 L of MQ-0.133% TFA is sufficient for approximately 60 plasma samples (15 mL/sample).

4. 20% ACN in MQ + 0.1% TFA. Store at room temperature. Prepare fresh solution for each batch. A bottle containing 100 mL is sufficient for 50 samples (2 mL/sample).

5. 80% ACN in MQ + 0.1% TFA. Store at room temperature. Prepare fresh solution for each batch. A bottle containing 100 mL is sufficient for 50 samples (2 mL/sample).

6. LC eluents A: 40% MQ water: 40% methanol: 20% ACN + 0.1% formic acid (FA).

7. LC eluents B: 70% methanol: 30% ACN + 0.1% FA.

2.3 Synthesis of 13- and 16-HDHEA Standards (Optional)

Since 13- and 16-HDHEA are not commercially available, these compounds have to be synthesized in the lab from 13- or 16-hydroxy-docosahexaenoic acid (13-HDHA or 16-HDHA, respectively), which are commercially available. Synthesis starts by first evaporating the EtOH that is used as a solvent for 13- and 16-HDHA. Then 100 μg of 13- or 16-HDHA (1 eq., 0.29 μmol) are dissolved in 2 mL dry DCM under argon atmosphere. Then, 100 μL of a freshly prepared solution of 59.3 μL NEt_3 in 10 mL dry dichloromethane (DCM) is added (14 eq., 4.25 μmol), followed by the addition of 100 μL of a freshly prepared solution of 47.3 μL isobutyl chloroformate in 10 mL dry DCM (12 eq., 3.65 μmol).

The solution is stirred at room temperature for 1 h under argon atmosphere to allow formation of a mixed anhydride, before cooling the reaction on ice. To the mixed anhydride 100 μL of a freshly prepared solution of 46.6 μL distilled NEt_3 (11 eq., 3.34 μmol) and 20.2 μL ethanolamine or ethanolamine-d_4 (11 eq., 3.34 μmol; also *see* **Note 4**) in 10 mL dry DCM is added. Overnight stirring on ice results in the formation 13- and 16-HDHEA. Purification of the products is performed using preparative HPLC, for example, using an Agilent 1260 Preparative HPLC with DAD and MSD from Agilent Technologies B.V. (Amstelveen, the Netherlands). An isocratic run of 30:70 ultrapure water:ACN containing 0.1% FA using a flow rate of 4 mL/min on a Zorbax Eclipse XDB-C18 5 μ column of 9.4 × 250 mm from Agilent Technologies B.V. (Amstelveen, the Netherlands) is successfully used to purify the compounds. After evaporating to dryness, the samples are dissolved in EtOH and are quantified at 240 nm absorbance using a quantitative HPLC run calibrated with 13-HDHA or 16-HDHA.The yield after synthesis and purification of the standards typically varies between 10% and 50%.

2.4 Consumables

For each plasma sample, the following is required:

1. One plasma collection tube containing ethylenediaminetetraacetic acid (EDTA), min. volume 5 mL and a 1.5-mL Eppendorf cup; 1 mL of plasma or less is required (*see* **Note 5**).
2. One 15-mL tube with screw cap (polypropylene plastic)
3. One 50-mL tube with screw cap (polypropylene plastic)
4. One C8 SPE column (Agilent, Bond Elut C8, 200 mg, 3-mL cartridge)
    4.1. Optional for 13/16-HDHEA analysis: one Oasis HLB column
5. One 2-mL Eppendorf cup (polypropylene plastic)
6. One LC injection vial (amber, screw cap) with insert. Most LC systems will accept 12 mm × 32 mm vials (*see* **Note 6**).

Furthermore, for the synthesis of 13- and 16-HDHEA, the following consumables are required:

1. Borosilicate glass vials
2. Amberized borosilicate glass vials

2.5 Equipment

1. Fume hood
2. Pipettes, including a large-volume dispenser
3. Vortex
4. Centrifuge suited for 15-mL volume tubes
5. SPE high-volume cartridges + adapter

6. SPE manifold, including vacuum pump and stopcocks
7. Vacuum concentrator
8. Chromatography column suited for MS analysis, C8 (or C18)
   8.1. optional for 13/16-HDHEA analysis: Zorbax Eclipse plus C18, 1.8 μm, 2.1 × 150 mm
9. LC–MS/MS system equipped with an electrospray ionisation (ESI) source (*see* **Note 7**)

For the synthesis of 13- and 16-HDHEA, the following equipment is required:

1. Hamilton syringes
2. HPLC with diode array detector (DAD)
3. Preparative HPLC with DAD and coupled MSD
4. Small magnetic stirring bar
5. Argon (gas)

3 Methods

3.1 Sample Collection

1. Prior to the collection of blood from the subject, precool the centrifuge to 4 °C.
2. Aliquot the PMSF solution; the 100 mM PMSF stock solution needs to be diluted in plasma to a final concentration of 100 μM in isopropanol. Aliquot 1.5 μL of the 100 mM PMSF solution in each 1.5-mL Eppendorf tube that will be used to store the plasma in after collection and centrifugation. Once 1.5 mL plasma is added (*see* **step 5**, below), the final concentration will be 100 μM PMSF.
3. Collect venous blood, for example, from the antecubital vein, in the EDTA tube (*see* **Note 5**). Measures to limit hemolysis are recommended as this might release degrading enzymes or intercellular endocannabinoids.
4. Immediately after blood collection, spin down the tubes in a centrifuge for 10 min at 2000 × *g* and 4 °C. Tubes that cannot be immediately centrifuged should be stored on ice but should always be centrifuged within 20 min after collection. Especially when working with a high number of samples, this might lead to some variation in time between blood collection and centrifugation. In our experience, this has never led to any obvious artefactual changes in measured endocannabinoid levels.
5. Immediately after centrifugation, collect the plasma and transfer 1.5 mL to the Eppendorf tube containing the PMSF. Store the sample at −80 °C (*see* **Notes 8** and **9**).

3.2 Sample Extraction

Organic solvents and acids are used during sample extraction. Take all necessary precautions to create a safe working environment. This includes performing all steps with organic solvents and acids in a fume hood and wearing gloves and safety glasses at all times.

It is recommended to include quality control (QC) samples in each analytical batch. These may be plasma samples with a known concentration of analytes, or a plasma sample that is spiked with a known amount of analytes. QC samples are helpful to monitor the accuracy and precision of each analytical batch.

1. Thaw the plasma samples. Ensure that all samples have reached room temperature before proceeding to the ACN extraction step. This may take 30–60 min.
2. While the plasma samples are thawing, prepare the deuterated standard spiking work solution.
3. Vortex the thawed plasma sample and transfer 1.0 mL of plasma to a clean 15-mL tube.
4. Add 4.0 mL of the deuterated standard spiking work solution to the 1.0 mL plasma. The ACN will precipitate all proteins and highly lipophilic compounds, while keeping the endocannabinoids in solution. The ACN will completely mix with the plasma. While adding this solution to the plasma, keep the tube on a vortexer running at a gentle pace. Vortexing the sample will improve the reproducibility of the precipitation.
5. Centrifuge the 15-mL tubes at 3000 $\times$ *g* for 5 min at room temperature.
6. After centrifugation, collect the supernatant and transfer to a clean 50-mL tube. To each 50-mL tube, add 15 mL of the 0.133% TFA solution. This will dilute the ACN from step 4 again to 20%, which is adequate for the subsequent SPE cleanup.
7. Prepare the SPE manifold; the vacuum pump is connected to the glass manifold, and the SPE columns are connected on the manifold through plastic stopcocks that allow to control the flow rate of fluids through the column.
8. Activate the C8 SPE column by applying 1 mL of methanol on the column's filter bed. Allow the methanol to run through the filter by gravitational pull; the stopcock has to be in the "open" position. The stopcock should be closed just before the bottom of the meniscus reaches the surface of the filter; the filter should remain wet (*see* **Note 10**).
9. Wash the C8 SPE column by applying 1 mL of MQ water on the filter. Allow the water to run through the filter by using the vacuum pump that creates a mild vacuum (600–700 mBar) in the manifold. The stopcock has to be in the "open" position and should be closed just before the bottom of the meniscus

reaches the surface of the filter. The vacuum pump can be turned off when all columns are washed. Again, the filter should remain wet. There may be some variation in the time needed to clear the water through the filter. It is advised to wash the columns a few at a time, to prevent the columns from running dry (*see* **Note 11**).

10. Load the 20 mL sample (from **step 6** in this subheading) to the SPE column. First, fill the SPE column with 2 mL of sample, then connect the high-volume cartridge and adapter on the SPE column, and add the remaining 18 mL. Open the stopcock and turn on the vacuum pump again. It might take a few minutes before all samples have run through the SPE column's filter; once completed, close the stopcock again. Loading the sample in the 20% ACN in MQ water + 0.1% TFA solution is critical for binding of the endocannabinoids to the filter. A higher organic solvent content may prevent the endocannabinoids to bind to the column.
11. Open the stopcocks again and wash the column with 2 mL % ACN in MQ water + 0.1% TFA solution. This will wash away any unbound residue that remained in the filter; the endocannabinoids will remain bound to the filter. Close the stopcocks once the wash has completed.
12. Prepare the manifold for eluting the SPE columns. Turn off the vacuum pump and gently release the vacuum from the manifold. Open the manifold, discard the fluid that has accumulated in the manifold; this contains organic solvents and TFA. Adjust the collection vessel rack to accommodate the 2-mL sample collection tubes. Ideally, the outlet tip of the gasket's tube connector should be positioned just over the 2-mL tube. Close the manifold again. Ascertain that all outlet tips are still positioned over the 2-mL collection tubes.
13. Elute the SPE columns by adding 2 mL of 80% ACN in MQ + 0.1% TFA on the SPE columns. Open the stopcocks and activate the vacuum pump again (600–700 mBar); the eluent should drip in the 2-mL collection tube. Close the stopcock immediately when the column is completely eluted to prevent air from violently blowing in the sample and spilling it. It is advised to elute the columns a few at a time to prevent this.
14. When all samples are eluted, switch off the vacuum pump and gently release the vacuum. Transfer the 2-mL collection tubes to a vacuum concentrator and evaporate the sample to full dryness. It may take a few hours before the samples are completely dry. Dried extracts can be stored in −80 °C until LC–MS analysis or immediately analyzed (*see* **Note 12**).

3.3 LC–MS Analysis

Before LC–MS analyses can be performed, a method needs to be programmed. Therefore, the different aspects of the method, such as chromatography, ion optics settings, and mass spectrometer scan events, must be (experimentally) optimized for the LC–MS system. Operating LC–MS systems requires specific training and expertise and is usually delegated to specialized technicians. Published reports provide a good starting point for optimizing the LC–MS settings, but differences between hardware from different manufacturers may exist. It is therefore difficult to provide a general step-by-step guide on how to perform LC–MS analyses, but some general points of attention will be discussed in this subheading.

1. The chromatography should be optimized to yield symmetric peaks and minimize ion suppression. We have good experience with gradient elution using eluents that contain water, methanol, and acetonitrile (eluents A and B, *see* Subheading 2). Due to the lipophilic nature of the extract, ion suppression may negatively influence peak heights. Ion suppression can be prevented by modifying the chromatography, hence increasing peak height but usually also analysis time. Special attention should be paid when monoacylglycerol esters such as 2-AG are to be determined; 2-AG undergoes isomerization to 1-AG in aqueous media, which may have to be resolved chromatographically depending on the research question.
2. We have good experiences with various C8 columns for "conventional" high-performance liquid chromatography (HPLC) analysis (e.g., Waters Xterra MS C8) or ultra-performance liquid chromatography (UPLC) analysis (e.g., Waters Acquity C8 BEH UPLC). Generally, UPLC columns can be operated against higher back pressures, allowing higher flow rates, improved separation and shorter run times. In our hands, a 2.1 × 100 mm UPLC C8 column and a 12-min gradient elution protocol with eluents A and B (*see* Subheading 2.2) resulted in adequate peak shape and recoveries [12]. Never use salt-containing eluents for MS analysis! The chromatography, together with the sensitivity of the MS, will determine the injection volume of the autosampler and will determine whether sample preconcentration is required. Too high sample volumes may deteriorate peak shape. Typically, depending on the available hardware (and sample loops), the injection volume will range between 2 and 10 μl.
3. All individual compounds should be infused directly into the system in order to tune the MS for the different endocannabinoids. In our hands, the parent of DHEA is visible at *m/z* 372 and EPEA at *m/z* 346, which correspond to their protonated forms. Generally, all ethanolamides, including DHEA and EPEA, yield a dominant *m/z* 62 fragment when subjected

to collision-induced dissociation (CID). This fragment corresponds to the ethanolamine moiety. Different classes of endocannabinoids, such as monoacylglycerols or acyldopamines, yield different fragmentation patterns. Too high CID energies may lead to further fragmentation of the dominant fragment, thus resulting in reduced signal intensities. It is therefore important to select the appropriate optimal CID energy, which has to be determined through experimentation on each MS instrument.

4. The performance of an LC–MS method depends on the quality of the sample, the sample pretreatment, the chromatographic separation, and the optimization of MS settings. It is highly recommended to perform a validation to establish the system performance. Items include inter- and intraday accuracy and precision, linearity, limit of quantification/detection (LOQ/-LOD), freeze–thaw stability, ion suppression, and recovery.

3.4 Data Processing and Results

Chromatograms have to be critically reviewed and adjusted if necessary to ensure that high-quality data are reported for further (statistical) processing. Attention has to be paid to a few aspects, such as retention time shifts, results from QC samples, and fit of calibration curves.

1. The accuracy of the calibration curve is essential as it influences the reported concentration for each sample. Usually, each calibration curve is analyzed in duplicate or triplicate for each run, and a single regression equation is generated. The curve fit can be optimized to achieve good accuracy in the concentration range relevant to the "unknown" study samples, for example, by modifying the curve type or "weighing." The quantification software provides several options to achieve this. In addition, calibration points at concentrations that are not relevant for the study samples may be unselected, to ensure that the calibration curve has optimal fit in the relevant concentration range.
2. Although most software suites allow for automatic peak processing, it is recommended to check all integrations manually. Especially in matrices where low concentrations are expected, such as in plasma, small background interferences ("noise") may interfere with peak integration and may severely influence the results. It is generally recommended to prefer working with "peak areas" over "peak height."
3. Correct for the concentration factor that occurs during sample preparation. The software reports concentrations as they were found in the extract. In the case of plasma, 100 μL extracts contain the endocannabinoids from 1 mL of original material, meaning that the reported concentrations are to be divided by 10.

4. It is difficult to give reference values for endocannabinoids in plasma because their levels are modified by a variety of factors (*see* Subheading 3.7). We have found human plasma concentrations for most endocannabinoids in the low ng/mL range [12, 21]. DHEA is present in human plasma whereas EPEA was undetectable [6]. However, EPEA is found in plasma and tissues of animals fed an n-3 fatty acid-rich diet (*see* Subheading 3.7) [5].

3.5 Modifications to the Sample Preparation Protocol for Matrices Other Than Plasma

We have applied the sample preparation protocol for a variety of matrices, such as adipose tissue, liver, intestinal tissue [5], and cell culture media [6]. Here, we briefly describe modifications to the standard protocol for a selection of matrices other than plasma.

Like most tissues, adipose tissue contains higher concentrations of endocannabinoids compared to plasma. In our experience, adipose tissue did not require SPE sample pretreatment. A simple extraction step using ACN followed by sonication was sufficient to accurately determine endocannabinoid levels. To 50–100 mg of adipose tissue, 1 mL of ACN containing deuterated standards is added, the sample is sonicated for 5–10 s and centrifuged for 5 min at 12,000 rpm on a table-top centrifuge. The supernatant is transferred to a clean 2-mL Eppendorf tube. To the tissue, again 1 mL ACN is added, sonicated, and centrifuged, and the supernatant is pooled. The 2 mL ACN extract is evaporated to full dryness and subjected to LC–MS analysis as described.

Liver and gut tissue contain relatively high concentrations of endocannabinoids. In our experience, 100 mg of freeze-dried liver and 50 mg of freeze-dried gut tissue contain quantifiable amounts of n-3 fatty acid-derived endocannabinoids. Liver or gut tissue is processed similarly to plasma, using SPE. First, the tissue is extracted using 2 × 1 mL ACN and sonication. The 2 mL supernatant is diluted with 8 mL of MQ + 0.1% TFA and subjected to SPE cleanup similar to plasma.

Cell culture medium may also contain endocannabinoids. In our experience, using 3T3-L1 adipocytes, 2 mL of medium contained quantifiable endocannabinoid levels. To 2 mL of medium, 2 μL of TFA and the deuterated standards are added, and the acidified medium is directly applied onto activated C8 SPE columns and processed as described for plasma. Different strategies may be used to improve endocannabinoid yield from cultured cells, such as increasing cell numbers, prolonging medium incubation time, or with ionomycin stimulation to immediate release of endocannabinoids in the medium.

3.6 Modifications to the Sample Preparation Protocol to Analyse COX-2 Metabolites of DHEA

An advantage of LC–MS is that it allows to quantify many target analytes from a single sample. As outlined in the introduction, several endocannabinoids, including DHEA, may be metabolized by COX-2 to form oxygenated products with different biological properties. It is important to realize that DHEA may be prone to auto-oxidation as well (*see* **Note 13**). We have successfully quantified 13- and 16-HDHEA from lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cell cultures using a modified protocol [19]. Since 13- and 16-HDHEA are not commercially available, they were synthesized (*see* Subheading 2.3 and **Note 4**) to make the respective calibration curves. The calibration range of 13- and 16-HDHEA is comparable to DHEA starting from 140 ng/mL for the highest calibrator, but this level could also be increased to about 1000 μg/mL if necessary. Sample extraction is performed on washed cell pellets, which are extracted with 2 × 1 mL of MeOH containing 13-HDHEA-d_4 to obtain a final 13-HDHEA-d_4 concentration of 16.125 ng/mL in the sample. For 13- and 16-HDHEA, SPE is subsequently performed using HLB Oasis columns, which is also compatible with AEA, EPEA, and DHEA extraction. Prior to application on the SPE column, 8 mL of MQ water containing 0.125% FA is added to the 2 mL MeOH extract. Additionally, ACN in the SPE extraction buffers is replaced by MeOH and TFA by FA. SPE columns are first activated with 2 mL of MeOH, washed with 2 mL of MQ water containing 0.1% FA, before applying the sample. The columns are then washed with 2 mL of 20% MeOH in MQ + 0.1% FA. Sample elution is performed with 1 mL MeOH + 0.1% FA, which is collected in borosilicate glass vials containing 20 μL of 500 μM BHT and 10% glycerol in EtOH to limit auto-oxidation of the metabolites. After evaporation, the sample is dissolved in 50–100 μL EtOH, which is then ready for LC–MS injection and analysis.

MS identification of the HDHEA metabolites can best be performed using the *m/z* 370 --> 62 transition, which corresponds to an $[M+H-H_2O]^+$ parent and the ethanolamine fragment, which give the highest signals for those metabolites. LC separation of 13- and 16-HDHEA can be performed on an Agilent Zorbax Eclipse Plus C18, 1.8 μ 2.1 × 150 mm column or on the previously described Waters Acquity C8 BEH column. Both columns allow for baseline separation of the two DHEA metabolites, where the Zorbax column has improved separation. The chromatographic methodology was further modified by using eluent A consisting of ultrapure water/ACN (95/5) with 0.1% FA and eluent B consisting of 100% ACN with 0.1% FA. Gradient elution was performed as follows; initial conditions 5% B followed by a linear increase to 50% B, which was achieved at min 5 and continued until min 8. This was followed by a linear increase toward 100% B, which was achieved at min 13 and maintained until min 16, after which the column was equilibrated to 5% B which was maintained until min 21 performed. During the run, a flow rate of 0.5 mL/min and a column temperature of 50 °C was set.

3.7 Factors that Influence Levels of n-3 PUFA-Derived Endocannabinoids and Their Metabolites

In general, endocannabinoid levels depend on a variety of factors, including dietary composition of fatty acids, the presence of inflammation, and postprandial status. DHEA is normally found in human and mouse plasma, but we have not been able to detect EPEA in human plasma [5, 6, 21]. Experience from animal studies demonstrated that EPEA is only detectable in plasma when the animals were fed a diet rich in n-3 fatty acids for 6 weeks [5]. Whether EPEA is present in human plasma after eating n-3 PUFA-rich diets is not known at present. Both DHEA and EPEA have been consistently found in mouse tissues such as adipose tissue, liver, and gut. Generally, with increased n-3 fatty acid content in the diet, or during inflammation, DHEA and EPEA levels were increased [5, 22]. Plasma endocannabinoid levels are also higher in the fasted state compared to the postprandial state [23]. Also cultured 3T3-L1 adipocytes released quantifiable amounts of DHEA and EPEA in the medium [6]. The COX-2 metabolites of DHEA, 13- and 16-HDHEA, were not detected in (human) plasma (unpublished results). To the best of our knowledge, 13- and 16-HDHEA were thus far only detected in LPS-stimulated RAW264.7 macrophages that were preincubated with DHEA; without DHEA or LPS, 13- and 16-HDHEA could not be detected [19]. This indicates that both presence of the parent (DHEA) as well as significant COX-2 expression (after LPS stimulation) are required to form 13- and 16-HDHEA.

3.8 Choice of Target Analytes

The method described in this chapter is suited to quantify the levels of DHEA and EPEA in plasma and other matrices. As mentioned above, using exactly the same sample preparation steps, a wider spectrum of endocannabinoids can be measured, including the classical endocannabinoids AEA and 2-AG. These molecules can be quantified parallel to DHEA and EPEA in the same sample, which requires adding the desired compounds to the calibration curve and MS settings. Most modern triple quadrupole MS instruments can easily scan 10–100 compounds in one sample. In our experience, quantifying DHEA and EPEA together with the other endocannabinoids provided additional information that helped to understand the dynamic context in which n-3 PUFA-derived endocannabinoids are present. For instance, with our comprehensive analytical approach, we were able to demonstrate that specific fatty acid-enriched diets cause a shift in multiple endocannabinoids, rather than affecting only one or two endocannabinoids [5]. With our modified protocol, we were able to demonstrate that DHEA is converted by COX-2 in, for example, LPS-stimulated RAW264.7 macrophages, which can be blocked with a COX-2 inhibitor [19]. It thus seems that establishing a comprehensive "endocannabinoid profile" can reveal changes that would not be detected using methodology that only focuses on a limited number of endocannabinoids.

4 Notes

1. It is needless to say that stock solutions and calibrators have to be prepared precisely. The concentrations in Table 1 depict the ranges that we routinely measure, but this depends on the sensitivity of the MS available in the lab. Therefore, depending on the sensitivity of the available equipment, different concentration ranges may apply in other labs.
2. During LC–MS analysis, the ratio of an endocannabinoid to its deuterated analog (= isotope ratio) is measured. The ratio is proportional to the concentration in the sample; therefore, it is crucial that the amount of deuterated compound that is spiked to the sample is identical to the amount present in the calibrators. Deuterated standards are normally not present in biological materials and are therefore added to the sample during sample preparation. Measuring isotope ratios has several advantages, because it intrinsically corrects for any sample spill during the sample preparation or any other factors that may vary between samples. It also corrects for so-called ion suppression. Ion suppression is the phenomenon where ionization of the compound of interest is suppressed due to interference of other molecules in the sample, leading to apparently lower peaks. With a carefully selected deuterated analog that elutes from the LC column at the same time, both molecules are equally affected by ion suppression, thus the isotope ratio remains stable. Different deuterated standards can be used. We have good experiences with using deuterated AEA (AEA-d8) for the quantification of DHEA and EPEA. In the protocol presented here, deuterated 2-AG and NADA are also added to allow for the quantification of a wider range of endocannabinoids.
3. Endocannabinoids are degraded by a variety of enzymes such as FAAH and monoacylglycerol lipase (MGL). Although FAAH is normally present within the cell, it may also be present in the plasma when cells are lysed, for instance, due to mechanical stress during the venapunction. Therefore, as a precaution, it is recommended to store plasma in the presence of FAAH inhibitors such as PMSF.
4. For the synthesis of deuterated HDHEA standard, it is of crucial importance to use 100% isotopically enriched ethanolamine to prevent interference of the internal standard with the naturally occurring HDHEA levels. It is advised to test potential interference of the internal standard before sample analysis.
5. Different anticoagulants are available to prevent ex vivo blood clotting, such as citrate, heparin, and EDTA. Generally, it is known that the anticoagulant can affect the performance of

LC–MS. Anticoagulants may cause interfering peaks in the chromatogram, and it is therefore recommended to investigate the presence of interfering peaks. In our hands, we did not encounter any interferences with EDTA.

6. Different LC systems are available, with either binary or quaternary pumps, autosamplers that accept different types of vials or plates, and operate at different pressures. When using only two eluents for the chromatography, a binary LC system is adequate. In the protocol presented here, the dried extract is reconstituted in 100 μL ACN; for most autosamplers, this means that vials with inserts have to be used. The right type of vial and the appropriate injection needle height have to be selected in the LC systems settings.
7. Mass spectrometry is an advanced analytical technique that allows to characterize and/or quantify levels of molecules in a certain matrix. Different types of MS machines are available, which all have their particular strengths, weaknesses, and area of application. For accurate and sensitive quantification of multiple compounds, a so-called triple quadrupole or tandem MS (annotated as MS/MS) is usually the first choice. A tandem MS system consists of three mass filters ("quadrupoles") that are connected in series. The second quadrupole acts as a collision cell. The detector is positioned after the third quadrupole. Intact ions of interest, "parent ions," are selected in the first quadrupole and move on to the collision cell, where they are subjected to collision with inert gas such as argon. As a result, the parent ions will dissociate into fragments, known as "daughter ions." The daughter ions will continue their flight to the third quadrupole, where only selected daughter ions of one specific mass-over-charge (m/z) ratio will be passed on to the detector. Thus, an ion that hits the detector is a selected fragment from a selected parent ion. Through this "dual selection," a high level of specificity can be achieved.
8. Information on the long-term stability of endocannabinoids in plasma at −80 °C is scarce. However, considering the potential absorption of endocannabinoids to plastics and the presence of degrading enzymes in the biological matrix, endocannabinoids are generally considered unstable [11]. Therefore, we recommend to analyze the endocannabinoid levels at the earliest convenience.
9. It is needless to say that blood and plasma should be treated as potentially infectious materials. Therefore, wear gloves when handling blood or plasma. It is common to take additional precautions, such as (compulsory) Hepatitis B vaccination for all personal handling human materials.

10. The methanol unfolds the C8 aliphatic chains in the filter bed, allowing the binding of lipophilic substances to the filter. Hence, this step is known as "activation."
11. This washing step is needed to wash away any overabundant methanol that may prevent compound binding in the sample loading step.
12. The vacuum concentration step is critical for good recoveries. We have compared different techniques to dry the SPE extract, including evaporation under nitrogen flow and freeze drying. In our hands, we obtained the most robust results and highest recoveries by drying the samples in a vacuum concentrator centrifuge. Please be aware that the vacuum concentrator has to be compatible to work with organic solvents and acids.
13. Apart from the points mentioned in **Note 8**, DHEA is also prone to auto-oxidation. This not only affects the concentration of DHEA but could also lead to increased background levels of 13- and 16-HDHEA. Apart from the general precautions to prevent oxidation described in this methodology, it is advised to (i) always use fresh DHEA standards, (ii) store the calibrator and extraction solutions in amber vials, (iii) properly flush the solutions with N_2 or Ar gas, and (iv) use sample tray cooling during the run. Most ideally, the calibrator and extraction solutions are prepared freshly and used directly on the day of the experiment. It is important to prevent long-time storage of the solutions and samples and to perform the measurements directly after sample preparation. It is advised to always check the level of auto-oxidation in the solutions before measuring the samples.

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