Chapter 4

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

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Abstract

The adequate quantification of endocannabinoids can be complex due to their low endogenous levels and structural diversity. Therefore, advanced analytical approaches, such as LC-MS, are used to measure endocannabinoids in plasma, tissues, and other matrices. Recent work has shown that endocannabinoids that are synthesized from n-3 fatty acids, such as docosahexaenoylethanolamide (DHEA) and eicosapentaenoylethanolamide (EPEA), have anti-inflammatory and anti-tumorigenic properties and stimulate synapse formation in neurites. Here, an LC-MS based method for the quantification of n-3 endocannabinoids DHEA and EPEA which is also suited to measure a wider spectrum of endocannabinoids is described. 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. Modifications to the protocol that allow quantifying n-3 endocannabinoids in tissues and cell culture media will also be discussed. Finally, conditions that alter endocannabinoid concentrations are briefly discussed.

Key words Endocannabinoids, n-3 Fatty acids, 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]. 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 so-far 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 examples of endocannabinoids include glycerol esters, such as 2-arachidonoylglycerol (2-AG), and the dopamine conjugate *N*-arachidonoyldopamine (NADA) [1].

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Next to AEA, ethanolamine conjugates have been described for palmitic acid, stearic acid, and the n-3 fatty acids docosahexaenoic acid and eicosapentaenoic acid [2–5]. 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 [6, 7]. Studies have also demonstrated that the local relative availability of their fatty acid precursors, which is amongst others determined by diet, plays an important role in determining the pattern of amide conjugates formed [2, 3].

The high diversity and dynamics of endocannabinoids and related compounds in tissues demands for adequate methods for the quantification. Recently published reports are all 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 [8]. MS-based methods use the molecular mass of a compound to detect it, which allows simultaneously measuring a broad range of compounds in a single sample. The molecular mass of a compound is more specific than, e.g., its ultraviolet (UV) absorbance characteristics, and therefore MS analysis results in higher specificity. Another advantage of MS over optical detectors 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 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 brain and important for brain functioning. We have also shown, using LC-MS, that n-3 fatty acid-derived endocannabinoids such as docosahexaenoylethanolamine (DHEA) and eicosapentaenoylethanolamine (EPEA) are present in mammalian plasma and other tissues, in concentrations similar to that of the "classical endocannabinoids" (*see* Fig. 1 for molecular structures) [4, 5, 9]. Work from our lab and others has also shown that DHEA and EPEA have biological effects on immune cells [10, 11], cancer cells [12, 13], and hippocampal cells [14]. Based on their affinity for CB_1 and CB_2, DHEA and EPEA could be classified as "true" endocannabinoids, although much of their bio-activity appears to be not directly related to cannabinoid receptors alone [11, 12].

In this chapter, we describe a protocol for the quantification of the n-3 fatty acid-derived endocannabinoids DHEA and EPEA in plasma using LC-MS. This method allows determining a wider spectrum of endocannabinoids, including AEA, 2-AG, and NADA, which we will also address briefly where relevant. Depending on

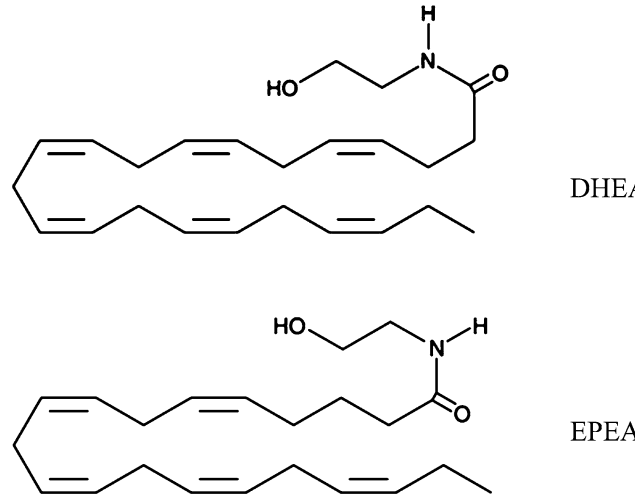

Fig. 1 Molecular structures for DHEA and EPEA

the sensitivity of the LC-MS system, 0.1–1 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. Modifications to determine endocannabinoid levels in other matrices will also be discussed.

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 [15].

2.1 Analytical Standard Solutions

2.1.1 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 analogues. During LC-MS analysis, both the intensity of the natural compound (e.g., AEA) and its deuterated analogue (e.g., AEA-d8) will be measured. The AEA:AEA-d8 ration 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**).

Table 1
Overview of concentrations in calibration curve solutions

	cal8	cal7	cal6	cal5	cal4	cal3	cal2	cal1
Concentration (ng/ml)								
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-d8	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25
2-AG-d8	750	750	750	750	750	750	750	750
PEA-d4	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
OEA-d4	31.25	31.25	31.25	31.25	31.25	31.25	31.25	31.25

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

2.1.2 Deuterated Standard Spiking Stock Solution

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.

2.1.3 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 phenylmethanesulfonyl fluoride (PMSF) solution in isopropanol. Store at 4–8 °C. A volume of 10 ml of 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 Consumables

For each plasma sample, the following is required:

1. One plasma collection tube containing ethylenediaminetetraacetic acid (EDTA), minimum volume 5 ml, and a 1.5 ml Eppendorf cup; 1 ml of plasma or less is required (*see* **Note 4**).
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).
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 5**).

2.4 Equipment

1. Fume hood.
2. Pipettes, including a large-volume dispenser.
3. Vortexer.
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.
9. LC-MS/MS system equipped with an electrospray ionization (ESI) source (*see* **Note 6**).

3 Methods

3.1 Sample Collection

1. Prior to the collection of blood from the subject, pre-cool 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 (**step 5**, below), the final concentration will be 100 μM PMSF.
3. Collect venous blood, e.g., from the antecubital vein, in the EDTA tube (*see* **Note 4**). 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 artifactual 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 7** and **8**).

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.

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. 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 × *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 clean-up.

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 controlling 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 9**).
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 the time, to prevent columns running dry (*see* **Note 10**).
10. Load the 20 ml of sample (from **step 6**) 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 violently blowing in the sample and spilling it. It is advised to elute the columns a few at the 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 11**).

3.3 LC-MS Analysis

Before LC-MS analyses can be performed, a method has to be programmed. Therefore, the different aspects of the method, such as chromatography, ion optics settings, and mass spectrometer scan events, have to 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 section.

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 backpressures, 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 [9]. 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. 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 the 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.
4. The performance of an LC-MS method depends on the quality of the sample, 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 Experimental 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, quality control (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, e.g., 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. We have good experience with using peak areas.

3. Correct for the concentration factor that occurs during sample preparation. The software reports concentrations as they were found in the extract. In case of plasma, 100 µL extracts contains 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 3.6). We have found human plasma concentrations for most endocannabinoids in the low ng/ml range [9, 15]. DHEA is present in human plasma whereas EPEA was undetectable [5]. However, EPEA is found in tissues at concentrations in the ng/g range of animals fed a n-3 fatty rich diet [4].

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, and cell culture media. 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 pre-treatment. 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 × *g* on a table-top centrifuge. The supernatant is transferred to a clean 2 ml Eppendorf tube. To the tissue, again 1 ml of ACN is added, sonicated, and centrifuged, and the supernatant is pooled. The 2 ml of ACN extract is evaporated to full dryness, and subjected to LC-MS analysis as described.

Liver and gut tissue contains high concentrations of endocannabinoids. In our experience, 100 mg freeze-dried liver and 50 mg freeze-dried gut tissue contain quantifiable amounts of n-3 fatty acid-derived endocannabinoids. Liver or gut tissue is processed similar to plasma, using SPE. First, the tissue is extracted using 2 × 1 ml of ACN and sonication. The 2 ml of supernatant is diluted with 8 ml of MQ + 0.1 % TFA, and subjected to SPE clean-up 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 stimulation with ionomycin to stimulate immediate release of endocannabinoids in the medium.

3.6 Factors That Influence Levels of n-3 Endocannabinoids

Generally, 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 [4, 5, 15]. 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 [4]. Whether EPEA is present in human plasma after eating n-3 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 [4, 16]. Also cultured 3 T3-L1 adipocytes released quantifiable amounts of DHEA and EPEA in the medium [5].

3.7 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 MS triple quadrupole equipment can easily scan 10–20 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 endocannabinoids are present. For instance, with our more 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 [4]. It thus seems that establishing a comprehensive "endocannabinoid profile" reveals changes that were not 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 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 analogue (=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 analogue 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. 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.
5. 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 2 eluents for the chromatography, a binary LC system is adequate. In the protocol presented here, the dried extract is reconstituted in 100 μl of 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.

6. Mass spectrometry is an advanced analytical technique that allows characterizing and/or quantifying 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 ion 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.
7. 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 [8]. Therefore, we recommend analyzing the endocannabinoid levels at the earliest convenience.
8. 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.
9. 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."
10. This washing step is needed to wash away any overabundant methanol that may prevent compound binding in the sample loading step.
11. 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.

References

1. Pertwee RG, Howlett AC, Abood ME et al (2010) International union of basic and clinical pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB1 and CB2. Pharmacol Rev 62:588–631
2. Artmann A, Petersen G, Hellgren LI et al (2008) Influence of dietary fatty acids on endocannabinoid and *N*-acylethanolamine levels in rat brain, liver and small intestine. Biochim Biophys Acta 1781:200–212
3. Matias I, Carta G, Murru E et al (2008) Effect of polyunsaturated fatty acids on endocannabinoid and *N*-acyl-ethanolamine levels in mouse adipocytes. Biochim Biophys Acta 1781:52–60
4. Balvers M, Verhoeckx K, Bijlsma S et al (2012) Fish oil and inflammatory status alter the n-3 to n-6 balance of the endocannabinoid and oxylipin metabolomes in mouse plasma and tissues. Metabolomics 8:1130–1147
5. Balvers MGJ, Verhoeckx KCM, Plastina P et al (2010) Docosahexaenoic acid and eicosapentaenoic acid are converted by 3T3-L1 adipocytes to *N*-acyl ethanolamines with anti-inflammatory properties. Biochim Biophys Acta 1801:1107–1114
6. Bradshaw HB, Raboune S, Hollis JL (2013) Opportunistic activation of TRP receptors by endogenous lipids: Exploiting lipidomics to understand TRP receptor cellular communication. Life Sci 92:404–409
7. Di Marzo V, Bisogno T, De Petrocellis L (2007) Endocannabinoids and related compounds: walking back and forth between plant natural products and animal physiology. Chem Biol 14:741–756
8. Zoerner AA, Gutzki F-M, Batkai S et al (2011) Quantification of endocannabinoids in biological systems by chromatography and mass spectrometry: a comprehensive review from an analytical and biological perspective. Biochim Biophys Acta 1811:706–723
9. Balvers MGJ, Wortelboer HM, Witkamp RF et al (2013) Liquid chromatography–tandem mass spectrometry analysis of free and esterified fatty acid N-acyl ethanolamines in plasma and blood cells. Anal Biochem 434:275–283
10. Meijerink J, Plastina P, Vincken J-P et al (2011) The ethanolamide metabolite of DHA, docosahexaenoylethanolamine, shows immunomodulating effects in mouse peritoneal and RAW264.7 macrophages: evidence for a new link between fish oil and inflammation. Br J Nutr 105:1789–1807
11. Meijerink J, Poland M, Balvers MGJ et al (2015) Inhibition of COX-2-mediated eicosanoid production plays a major role in the anti-inflammatory effects of the endocannabinoid *N*-docosahexaenoylethanolamine (DHEA) in macrophages. Br J Pharmacol 172:24–37
12. Brown I, Cascio MG, Wahle KWJ et al (2010) Cannabinoid receptor-dependent and -independent anti-proliferative effects of omega-3 ethanolamides in androgen receptor-positive and -negative prostate cancer cell lines. Carcinogenesis 31:1584–1591
13. Rovito D, Giordano C, Vizza D et al (2013) Omega-3 PUFA ethanolamides DHEA and EPEA induce autophagy through PPARγ activation in MCF-7 breast cancer cells. J Cell Physiol 228:1314–1322
14. Kim HY, Spector AA, Xiong ZM (2011) A synaptogenic amide *N*-docosahexaenoylethanolamide promotes hippocampal development. Development 96:114–120
15. Balvers MGJ, Verhoeckx KCM, Witkamp RF (2009) Development and validation of a quantitative method for the determination of 12 endocannabinoids and related compounds in human plasma using liquid chromatography-tandem mass spectrometry. J Chromatogr B 877:1583–1590
16. Balvers MGJ, Verhoeckx KCM, Meijerink J et al (2012) Time-dependent effect of in vivo inflammation on eicosanoid and endocannabinoid levels in plasma, liver, ileum and adipose tissue in C57BL/6 mice fed a fish-oil diet. Int Immunopharmacol 13:204–214