Chapter 2

Extraction and Simultaneous Quantification of Endocannabinoids and Endocannabinoid-Like Lipids in Biological Tissues

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Abstract

Extraction and quantification of endocannabinoids (eCBs) from biological tissues are essential to unravel their changes in physiological and pathophysiological conditions. We describe here an analytical protocol for extraction of endocannabinoids, anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), endocannabinoid-like lipids such as palmitoyl ethanolamide (PEA) and oleoyl ethanolamide (OEA), as well as arachidonic acid (AA) from biological tissues using liquid-liquid extraction method and simultaneous quantification by liquid chromatography multiple reaction monitoring (LC/MRM).

Key words Endocannabinoids, Endocannabinoid-like lipids, Liquid-chromatography, Multiple reaction monitoring, Lipid extraction

1 Introduction

The endocannabinoids (eCBs), anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), and endocannabinoid-like lipids such as palmitoyl ethanolamide (PEA) and oleoyl ethanolamide (OEA) are bioactive lipids originating from the degradation of phospholipids and serve themselves as substrate to other bioactive lipids, such as the large family of lipid signaling molecules of the prostaglandin class. The endocannabinoid (eCB) system is involved in the regulation of a multitude of physiological and pathophysiological mechanisms in central and peripheral nervous system, immune system and peripheral organs, and is recognized as potential therapeutic target system. Accordingly, the determination of the spatial and temporal dynamics of the eCB levels of various physiological and pathophysiological states in various biological matrices has become a relevant goal in (pre)clinical research.

Mass spectrometry (MS) is nowadays the core technology in molecular characterization of biological matrices, and has been in

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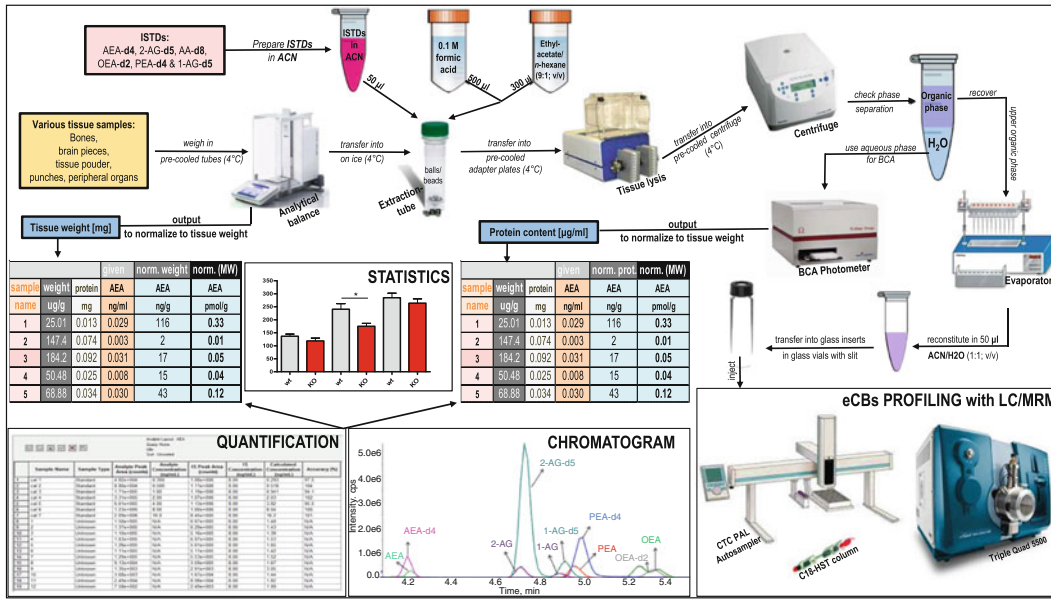
fact the crucial technology in discovery and quantification of endocannabinoids. Advances in the MS technology, particularly in MS-based quantitative assay using liquid chromatography (LC) coupled to multiple reaction monitoring (MRM), have allowed fast and so far the most sensitive quantitative assay for eCBs in various biological matrices, tissues, cells, and biological fluids [1–3]. Main challenges associated with quantification of eCBs stem from their originally low concentration in biological matrices, the diversity of biological matrices where they are present, and their rapidly changing levels during and post sample isolation through ex vivo degradation and synthesis, and chemical modification such as isomerization and oxidation. Such artificial changes in the eCB levels readily occur due to small variations in temperature and time of tissue sampling and handling, materials and equipment for tissue processing, and extraction conditions, making the comparative studies as well as inter- and intra-laboratory reproducibility challenging.

Here we describe an analytical protocol for simultaneous profiling of AEA and 2-AG, 1-AG, PEA, OEA, and AA using liquid-liquid extraction and quantitative assessment by LC/MRM. This protocol is amenable for a variety of biological tissues originating from brain, peripheral organs, bones, fat tissues, and muscles in terms of (a) tissue handling and extraction of eCBs with minimum and controlled ex vivo alteration of their levels, (b) inter- and cross-study reproducibility of the quantitative eCB data, (c) easy adaptability to various type, size, texture, and composition of tissues, and (d) amenability to higher throughput processing [4–7] (Fig. 1).



sample	weight	protein	given AEA	norm. weight AEA	norm. (MW) AEA
name	ug/g	mg	ng/ml	ng/g	pmol/g
1	25.01	0.013	0.029	116	0.33
2	147.4	0.074	0.003	2	0.01
3	184.2	0.092	0.031	17	0.05
4	50.48	0.025	0.008	15	0.04
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Fig. 1 Schematic representation of the analytical workflow for extraction and quantification of AEA, 2-AG, 1-AG, PEA, OEA, and AA

2 Materials

Prepare all solutions for extraction of eCBs and LC/MRM analysis using LC/MS-grade water and solvents. Use only freshly prepared solutions.

2.1 Tissue Extraction Components

1. Homogenization solution: 0.1 M Formic acid.
2. Extraction solution: Ethylacetate/*n*-hexane (9:1; v/v).
3. Extraction tubes: 1.2 ml Strips (*see* **Note 1**).
4. 1.5 ml Tubes for recovery of the lipid extracts (*see* **Note 2**).
5. Steel balls (*see* **Note 3**).
6. Precision balance maintained at 4 °C.
7. Evaporator.
8. Tissue lyser.
9. Centrifuge.
10. Vortex.

2.2 eCB Internal Standards and Calibration Standards

1. Deuterated standards: AEA-d4, 2-AG-d5, AA-d8, OEA-d2, PEA-d4, 1-AG-d5. Aliquot the original samples in acetonitrile and store at –20 °C (*see* **Note 4**).
2. Standard: AEA, 2-AG, AA, OEA, PEA, 1-AG. Aliquot the original samples in acetonitrile and store at –20 °C (*see* **Note 5**).

2.3 LC/MRM

1. LC solvents: Solvent A 0.1 % formic acid in water; solvent B 0.1 % formic acid in acetonitrile.
2. LC glass vials with siliconized inserts (*see* **Note 6**).
3. LC column: 2.5 μm C18(2)-HST column, 100 mm × 2 mm, combined with a pre-column (C18, 4 mm × 2 mm).
4. Autosampler maintained at 4 °C.
5. LC instrument.
6. 5500 QTrap triple-quadrupole linear ion trap mass spectrometer equipped with a Turbo V Ion Source (AB SCIEX) and polarity switching mode or another triple-quadrupole mass spectrometer.
7. LC quality control sample: Mix deuterated eCB standards and standard eCBs to an equimolar mixture in acetonitrile/water (1:1, v/v). Store at –20 °C before use.
8. Acetonitrile/water solution (1:1, v/v), prepare fresh and maintain at 4 °C.
9. Acetonitrile/isopropanol solution (1:1, v/v), prepare fresh and maintain at 4 °C.

2.4 BCA

1. Fluostar instrument.
2. BCA working reagent and calibrants.
3. BCA well plates.
4. 1.5 ml Tubes.

3 Methods

3.1 Tissue Isolation and Pre-processing

1. Isolate the tissue according to its source within the same time frame for all samples to be investigated (*see* **Note 7**).
2. If necessary to dissect tissue or remove specific (sub)areas of tissues, or perform punches, carry out this step on ice or at cold temperature (*see* **Note 8**).
3. Use precooled 1.5 or 2 ml tubes to transfer the tissue following sampling.
4. Snap freeze immediately the tissue and store at −80 °C (*see* **Note 9**).

3.2 Preliminary Steps for Extraction of eCBs from Tissues

1. Equilibrate extraction tubes to 4 °C for 30 min.
2. Equilibrate balance at 4 °C and calibrate.
3. Precool centrifuge at 4 °C.
4. Prepare fresh homogenization and extraction solution, cool, and keep at 4 °C.
5. Place tubes containing the frozen tissue on dry ice.
6. Cool steel balls at 4 °C.

3.3 Tissue Weighing

1. Tare the cooled extraction tubes on balance (*see* **Note 10**).
2. Transfer the frozen tissue from the original tubes to the extraction tubes and weigh (*see* **Notes 11** and **12**).
3. Place the extraction tubes containing the weighed tissue immediately on dry ice.

3.4 Extraction of eCBs

1. Prepare spiking solution of deuterated internal standards: Set the target concentration of each internal standard in the final extracts (50 μl) for LC/MRM analysis. Prepare a mixture of deuterated eCB standards in acetonitrile to a 20 times higher concentration than the target concentration in the final extracts for LC/MRM. The volume of spiking solution needed is 50 μl × no. of samples + no. of calibration solutions × 50 μl. Keep the spiking solution at 4 °C till extraction of samples (*see* **Note 13**).
2. Add cold steel balls in the extraction tubes.
3. Dilute 1:20 in acetonitrile a volume of spiking solution corresponding to 50 μl × no. of samples. Perform this step on ice.

4. Accurately pipette 50 µl of the diluted cold spiking solution to each sample.
5. Add 500 µl of cold homogenization solution, followed by 300 µl cold extraction solution (*see* **Note 14**).
6. Place the extraction tubes in tissue lyser holder and prepare a counterbalance plate.
7. Homogenize without delay in tissue lyser. Typical settings for homogenization: one cycle of 30 s at 30 Hz (*see* **Note 15**).
8. Transfer immediately the extraction tubes from tissue lyser to precooled (4 °C) centrifuge.
9. Centrifuge the extraction tubes for 10 min at 8000 × *g*, at 4 °C.
10. Transfer the extraction tubes in a precooled metal rack and place them at –20 °C for 10–30 min to freeze the lower aqueous phase.
11. Recover the organic phase into 1.5 ml tubes. These tubes will contain the lipid extracts.
12. Place the tubes containing the organic phase into pre-set evaporator at 37 °C and evaporate to dryness. These tubes will contain the extracted lipids, e.g., eCBs (*see* **Note 16**).
13. Store the remaining aqueous phase from **step 10** at –20 °C (*see* **Note 17**).

3.5 LC/MRM

1. Calibration curve solutions: Prepare fresh, on the day of analysis, a mixture of standard eCBs in acetonitrile in an LC glass vial. Pipette increasing volumes of this standard eCB mixture into LC glass vials in order to create a calibration curve. Add 50 µl of spiking solution of internal standards (*see* Subheading 3.4) to each vial and complement with appropriate volume of acetonitrile up to 500 µl and then add 500 µl of water in each vial to make up for 1 ml each calibration solution (*see* **Note 18**).
2. Accurately pipette cold 50 µl acetonitrile/water into the tubes containing the dry lipid extracts.
3. Vortex for 30 s at room temperature.
4. Centrifuge for 3 min at 10,000 × *g* at 4 °C.
5. Transfer 30 µl of the sample solutions from tubes into LC glass inserts, placed into LC glass vials, and close with open lid caps (*see* **Note 19**).
6. Place the sample-containing LC vials and the vials containing calibration standards into autosampler maintained at 4 °C.
7. Set the following conditions for LC/MRM method (*see* **Note 20**):

   LC gradient: Increase acetonitrile containing 0.1 % formic acid over 2 min from 55 to 90 %, and maintain it at 90 % for 5.5 min.

LC flow rate: 300 µl/min.
LC injection volume: 20 µl; use pre- and post-syringe cleaning.
Ion source: +4800 V for positive ion mode, –4500 V for negative ion mode; curtain gas: 40; temperature (TEM): 550 °C; ion source gas 1 and 2: 50; polarity switching time: 50 ms.
MRM transitions in positive ion mode: AEA, *m/z* 348.3 to *m/z* 62.1; AEA-d4, *m/z* 352.3 to *m/z* 62.1; 2-AG, *m/z* 379.1 to *m/z* 287.2; 2-AG-d5, *m/z* 384.2 to *m/z* 287.2; PEA, *m/z* 300.2 to *m/z* 62.1; PEA-d4, *m/z* 304.2 to *m/z* 62.1; OEA, *m/z 326.2 to m/z 62.1*; *OEA-d2*, *m/z 328.2 to m/z 62.1*.
Compound parameters in positive ion mode: EP: 10 V; dwell time: 20 ms; DP: 70 V; CE: for AEA, AEA-d4, 2-AG, 2-AG-d5 22 V, for OEA, PEA, PEA-d4 21 V and for OEA-d2 23 V; CXP: for AEA, AEA-d4 9 V, for 2-AG, 2-AG-d5 30 V, for OEA, OEA-d2, PEA, PEA-d4 10 V.
MRM transitions negative ion mode AA, *m/z* 303.05 to *m/z* 259.1; AA-d8, *m/z* 311.04 to *m/z* 267.0.
Compound parameters in negative ion mode: EP: –10 V; dwell time: 50 ms; DP: –200 V; CE: –19 V; CXP: –12 V.

8. Equilibrate the LC/MRM system with the instrument default method for equilibration, for 2–5 min.
9. Equilibrate LC/MRM system with the method for eCB analysis for 5–10 min.
10. Verify the performance of the LC/MRM using the LC quality control sample.
11. Write a sample batch for LC/MRM analysis. A minimum of three calibration curves are necessary: one at the beginning of the batch, one in the middle of the batch, and one at the end of the batch. Place a wash run before and after every calibration curve. For the wash run inject 20 µl of acetonitrile/isopropanol (1:1, v/v). Run regularly LC quality control samples (it can also be a calibration solution) between samples without pre- or post-quality control wash run.
    Run minimum five washes: by injecting 20 µl of acetonitrile/isopropanol (1:1, v/v) after batch analysis, followed by control sample of the system (*see* **Note 21**).
12. Following analysis remove the LC glass vials containing the samples from the autosampler, replace the open lid caps with closed lid caps, and store the remaining solution at –20 °C.
13. Quantify the data using the Analyst software.
14. Import the Analyst-calculated concentrations of eCBs into an Excel sheet. Input the tissue weight and/or protein amount corresponding to every sample and normalize the values of the Analyst-calculated concentrations of eCBs to tissue weight and/or protein content.

15. Perform statistical analysis when sample groups are to be compared.

3.6 BCA Assay

1. Remove the extraction tubes containing the aqueous phase from −20 °C and let it thaw for 30 min.
2. Dilute 1:10 a volume of 20 μl aqueous phase and run BCA assay according to the provider's instructions. Repeat the measurement with another dilution rate if the protein values are out of range.
3. Retrieve and store the protein concentration data.

4 Notes

1. 2 ml Precellys tubes can alternatively be used when the tissue samples are large and do not fit into the 1.2 ml strips.
2. When processing more samples, in our hands more than 20, we use microtiter plates or deep well plates to increase the speed of pipetting.
3. Even though one steel ball can be used, we find it more effectively to use small steel balls for homogenization with the tissue lyser. Ceramic beads can alternatively be used.
4. We typically dilute the deuterated standards as obtained from provider in acetonitrile and further aliquot. To avoid isomerization or other chemical modifications we do not thaw and freeze these aliquots for multiple use, but use them one time for tissue extraction.
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6. When processing more than 20 samples we use coated (e.g., silic-onized) microtiter or deep well plates for LC/MRM analysis to increase the speed of pipetting. Moreover, the storage of the rest samples post-analysis is more space effective.
7. Because postmortem alteration of eCB levels is readily occurring it is essential that tissue sampling is as fast as possible, irrespective of the tissue origin, to prevent such alterations. Moreover, for reliable comparative studies and minimum variability in the eCB levels due to tissue sampling procedure, the time required for sampling has to be equal for all samples to be compared in one or more biological replicate analysis.
8. When tissue dissection or isolation of specific subregions of tissues is necessary, place the original tissue directly on ice to perform such isolation. Also, any transport of the tissue from

one bench or lab to another has to be carried out on ice. For tissues such as brain, we typically freeze the entire brain at −80 °C prior to dissection, and then for brain region isolation we thaw the brain on ice and perform the isolation at 4 °C. This way the time and temperature conditions necessary to perform region isolation can be controlled to be similar and short for all samples to be compared to prevent variability in the rate of ex vivo synthesis/degradation of eCBs.

9. For tissue samples where multiple types of analysis has to be carried out from the same tissue source, but precluding pitfalls due to tissue heterogeneity, we pulverize the tissue and split for the corresponding analysis. When the tissue samples are hard tissues such as bone, or fibrous tissues such as heart and lungs, we cut the tissue in smaller pieces while frozen using a steel scissors or cutter to enhance the homogenization step.
10. Place the balance in the cold room to carry out the weighing or use a balance with a temperature control, maintaining it at 4 °C to avoid tissue thawing. Also, make sure that you do not hold the tubes in your hands more than necessary to avoid thawing of the tissue. The tissue weighing is time consuming, when analyzing large number of samples. We do weigh the tissue only if the biological question specifically requires normalization of the eCB values to tissue weight, or when reference/comparison to previously published data on eCB levels/tissue weight is necessary (*see* **Note 17**).
11. For larger tissues, we reduce the tissue sample by cutting or by pulverization and splitting the sample. We typically do not process tissue samples larger than 30 mg using this extraction method. This is due to increased difficulty for lipid-protein-phase separation under the limited volume of homogenization and extraction solvent used for these tubes and additionally because the homogenization becomes often ineffective, or requires too many homogenization cycles that lead to temperature increase in the sample, hence ex vivo eCB level alteration.
12. For very small tissues, such as biopsies or punches, we do not weigh the tissue at all, because it leads to fast, uncontrollable thawing during handling, which in turn leads to ex vivo degradation/synthesis of the eCBs and consequently to large variability in the eCB levels. In such cases, protein content (determined by a protein assay of the aqueous phase, see below extraction procedure) will be used to normalize the eCB values.
13. The target concentration of the internal standards in the final extracts is set based on preliminary tests on reference tissues. We typically use a target concentration of deuterated standards corresponding to the middle calibration curve of the LC/MRM, which in turn is tailored using reference tissue to encompass the reference values of eCB levels for the tissue type under investigation.

14. Irrespective of the tissue type, we invariably perform tissue lysis/homogenization with extraction solvent included. In our hands, this gave the highest abundance of the endogenous eCBs in LC/MRM analysis, possibly due to a more effective release of eCBs from their location in the tissue.
15. Visually inspect the homogenized samples after one cycle and assess if pieces of tissue are still visible. If so, repeat for one or two cycles of homogenization. Homogenization and extraction solvent volume can additionally be added, if the phase separation is not appropriate that is usually the case for very large samples.
16. If not possible to analyze immediately the samples by LC/MRM, store the dried extracts at −20 °C.
17. The aqueous phase can be used to determine the protein content of the tissue by a protein assay and normalize the eCB values to this content (*see* also **Note 12**) as an alternative to weight for small tissue samples such as biopsies and punches. We typically use normalization to protein content because of increased throughput possible, as compared to weighing.
18. The calibration curve range is tailored to the tissue type using reference tissue. We use the eCB levels determined in a reference.
19. We typically transfer 30 µl of the extracts into LC vials or well plates for analysis. The leftover 20 µl of extracts are immediately frozen and stored at −20 °C for an additional analytical replicate if necessary, or can be used for additional analysis in another ionization mode, if polarity switching is not available on the mass spectrometer.
20. The parameters and conditions for LC/MRM are valid for the lab equipment mentioned here. For other MS, LC instruments, or other LC column provider type all these settings have to be optimized accordingly. When polarity switching mode is not available, you can use the rest of the eCB extract to analyze the sample in the additional ionization mode.
21. Typically, we run a quality control every 20 biological samples and a calibration curve every 40 biological samples.

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References

1. Zoerner AA, Gutzki FM, 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
2. Buczynski MW, Parsons LH (2010) Quantification of brain endocannabinoid levels: methods, interpretations and pitfalls. Br J Pharmacol 160: 423–442
3. Lerner R, Lutz B, Bindila L (2013) Tricks and tracks in the identification and qualification of endocannabinoids. eLS. Wiley, Chichester. doi:10.1002/9780470015902.a0023407
4. Duerr GD, Heinemann JC, Gestrich C et al (2014) Impaired border zone formation and adverse remodeling after reperfused myocardial infarction in cannabinoid CB2 receptor deficient mice. Life Sci 14:324–325
5. Lomazzo E, Bindila L, Remmers F et al (2015) Therapeutic potential of inhibitors of endocannabinoid degradation for the treatment of stress-related hyperalgesia in an animal model of chronic pain. Neuropsychopharmacology 40:488–501
6. Jergas B, Schulte K, Bindila L et al (2014) O-2050 facilitates noradrenaline release and increases the CB1 receptor inverse agonistic effect of rimonabant in the guinea pig hippocampus. Naunyn Schmiedebergs Arch Pharmacol 387:621–628
7. Wenzel D, Matthey M, Bindila L et al (2013) Endocannabinoid anandamide mediates hypoxic pulmonary vasoconstriction. Proc Natl Acad Sci U S A 110:18710–18715