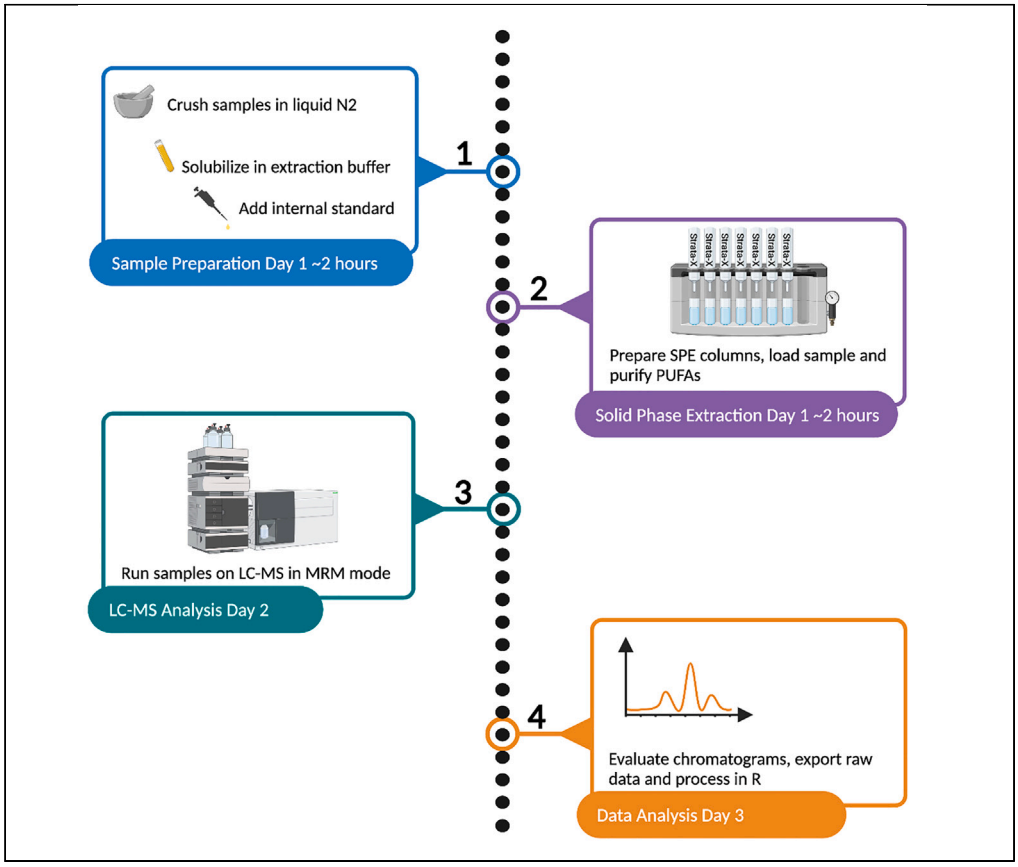
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Multiplexed targeted analysis of polyunsaturated fatty acids and oxylipins using liquid chromatography-tandem mass spectrometry



Polyunsaturated fatty acids (PUFAs) and their oxidized products (oxylipins) are important mediators in intra- and extra-cellular signaling. We describe here the simultaneous quantification of 163 PUFAs and oxylipins using liquid chromatography-mass spectrometry (LC-MS). The protocol details steps for PUFA purification from various biological materials, the conditions for LC-MS analysis, as well as quantitative approaches for data evaluation. We provide an example of PUFA quantification in animal tissue along with the bioinformatic protocol, enabling efficient inter-sample comparison and statistical analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Evgenia Turtoi, Jeremy Jeudy, Gilles Valette, Christine Enjalbal, Isabelle K. Vila, Nadine Laguette, Andrei Turtoi

andrei.turtoi@inserm.fr

Highlights

Isolating and purifying PUFAs from cells, tissue, or blood

Preparing the LC-MS system, MRM transitions, QC evaluation, and injecting the samples

Basic PUFA LC-MS data analysis, peak interpretation, and quantitative evaluation

Bioinformatic data analysis using R, generation of heatmaps, volcano and PLS plots

Turtoi et al., STAR Protocols 4, 102226
September 15, 2023
https://doi.org/10.1016/j.xpro.2023.102226

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Multiplexed targeted analysis of polyunsaturated fatty acids and oxylipins using liquid chromatography-tandem mass spectrometry

Evgenia Turtoi,[1] Jeremy Jeudy,[2] Gilles Valette,[3] Christine Enjalbal,[3] Isabelle K. Vila,[4] Nadine Laguette,[4] and Andrei Turtoi[1,5,6,7,8,*]

[1]Platform for Translational Oncometabolomics, Biocampus, CNRS, INSERM, Université de Montpellier, Montpellier, France

[2]Agilent Technologies, Les-Ulis, France

[3]Laboratoire de Mesures Physiques, Université de Montpellier, Montpellier, France

[4]Institut de Génétique Moléculaire de Montpellier (IGMM), CNRS, Université de Montpellier, Montpellier, France

[5]Tumor Microenvironment and Resistance to Therapy Laboratory, Institut de Recherche en Cancérologie de Montpellier, Université de Montpellier, INSERM U1194, Montpellier, France

[6]Gunma University Initiative for Advanced Research (GIAR), Maebashi, Gunma, Japan

[7]Technical contact

[8]Lead contact

*Correspondence: andrei.turtoi@inserm.fr
https://doi.org/10.1016/j.xpro.2023.102226

SUMMARY

Polyunsaturated fatty acids (PUFAs) and their oxidized products (oxylipins) are important mediators in intra- and extra-cellular signaling. We describe here the simultaneous quantification of 163 PUFAs and oxylipins using liquid chromatography-mass spectrometry (LC-MS). The protocol details steps for PUFA purification from various biological materials, the conditions for LC-MS analysis, as well as quantitative approaches for data evaluation. We provide an example of PUFA quantification in animal tissue along with the bioinformatic protocol, enabling efficient inter-sample comparison and statistical analysis.

For complete details on the use and execution of this protocol, please refer to Vila et al.,[1] Costanza et al.,[2] Blomme et al.,[3] and Blomme et al.[4]

BEFORE YOU BEGIN

Polyunsaturated fatty acids (PUFAs) are oxidized enzymatically and non-enzymatically to produce a plethora of compounds called oxylipins. The protocol outlined below describes a high-throughput assay to profile 163 PUFAs and oxylipins, including prostaglandins, prostacyclins, thromboxanes, dihydroprostaglandins, and isoprostanes, in animal tissues and cells. For the sake of simplicity, herein, we refer to all these compounds as PUFAs. The described method consists of three parts: i) sample preparation, ii) PUFA purification, and iii) mass spectrometry (MS) analysis. It also includes the sample preparation protocol modifications in function of the chosen tissue. Conversely, PUFA purification and MS analysis are the same for all starting materials.

1. Consider how the sample/tissue will be prepared.

Tissue and cell samples will need to be homogenized. This can be achieved by several means. In the present protocol, we describe the use of mortar and pestle (for tissues only) and oscillating/shaking with a tissue homogenizer (Precellys 24 or similar, e.g., FastPrep-24, from MPBio) in the presence of glass beads (for all sample types, with the exception of liquid samples, such as serum). TissueRuptor II (Qiagen) with disposable probes or Ultra-Turrax (Ika) are other examples of tissue homogenization

equipment that can be used with good results. However, tissues should be initially frozen in 15 mL tubes to allow accommodating the homogenizer probe in the tube. Therefore, particularly for soft tissues (e.g., liver), the mortar and pestle step can be omitted if these alternative homogenization methods are used. For more information on tissue homogenization see the method details below.

2. Familiarize yourself with the chemicals used.

General safety instructions: The present protocol requires various hazardous chemicals that must be handled with caution: methanol, isopropanol, acetonitrile, hexane, 6 M HCl, 10 M NaOH, and formic acid. All these chemicals must be handled under a fume hood (fumes are toxic) while wearing protective gloves, goggles, and a laboratory coat. Following are some general and specific Hazard (H) and Precautionary (P) statements related to these chemicals:

Hazard:

H225: Highly flammable liquid and vapor (methanol, acetonitrile, isopropanol, hexane).

H226: Flammable liquid and vapor (formic acid).

H228: Flammable solid (SDS).

H290: May be corrosive to metals (formic acid, HCl, NaOH).

H301 + H311 + H331: Toxic if swallowed, in contact with skin or if inhaled (methanol, formic acid).

H302 + H332: Harmful if swallowed or if inhaled (all).

H304: May be fatal if swallowed and enters airways (hexane).

H314: Causes severe skin burns and eye damage (formic acid, HCl, NaOH).

H315: Causes skin irritation (SDS, hexane).

H318: Causes serious eye damage (SDS).

H319: Causes serious eye irritation (acetonitrile, isopropanol).

H335: May cause respiratory irritation (SDS, HCl).

H336: May cause drowsiness or dizziness (isopropanol, hexane).

H361f: Suspected of damaging fertility.

H370: Causes damage to organs (Eyes, Central nervous system) (methanol).

H373: May cause damage to organs (nervous system) through prolonged/repeated exposure (if inhaled) (hexane).

H412: Harmful to aquatic life with long lasting effects (SDS, methanol).

Precaution:

P210: Keep away from heat, sparks, open flames, hot surfaces. No smoking (all).

P223: Keep container tightly closed (all).

P261: Avoid breathing dust/fume/gas/mist/vapours/spray (all).

P271: Use only outdoors or in a well-ventilated area (HCl).

P273: Avoid release to the environment (all).

P280: Wear protective clothing/eye protection (all).

P301 + P312 + P330: IF SWALLOWED: Call a POISON CENTER/ doctor if you feel unwell. Rinse mouth.

P302 + P352: IF ON SKIN: Wash with plenty of water (all).

P304 + P340 + P311: IF INHALED: Remove person to fresh air and keep comfortable for breathing. Call a POISON CENTER/ doctor (all).

P305 + P351 + P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing (all).

P308 + P313: IF exposed or concerned: Get medical advice/ attention (all).

P403: Store in a well-ventilated place (all).

For detailed information on the safety precautions to be applied and on the steps to be taken in case of exposure, please refer to the "Safety Datasheet" that is provided with each chemical by the supplier. Consult your local regulations and your safety officer on the rules that must be followed for manipulating and disposing these chemicals.

3. Wash & dry glass beads and tubes.
   In the present protocol, glass beads are used for sample homogenization. To avoid sample contamination, they must be washed, dried, and stocked ready for use as described below.
   a. Weigh 20 g of glass beads in a 50 mL Falcon tube.
   b. Add into the tube 20 mL of the solution mix made of 50% methanol, 49% water and 1% formic acid.
   c. Shake vigorously for 1 min, decant the wash solution.
   d. Add 20 mL of 100% methanol and shake vigorously for 1 min.
   e. Decant the wash solution and place the beads on a clean sheet of aluminum foil.
   f. Heat the beads in an oven at 300°C for at least 2 h.
   g. Cool the beads to room temperature and place them in a clean 50 mL Falcon tube for storage.
   Concerning the glass tubes used at the elution step, they need to be washed using a brush in this sequential manner:
   h. Wash with 2% sodium dodecylsulfate (SDS) in water.
   i. Rinse abundantly with deionized water (sink them in a beaker filled with water).
   j. Wash in 0.5 mL of methanol.
   k. Place in a metal rack (upside down), cover with aluminum foil.
   l. Bake in an oven at 300°C for 16 h.

Institutional permissions

The present protocol uses mouse liver and adipose tissue samples. Animal experimentation was performed in accordance with the French and European Animal Care Facility guidelines. All experiments were approved by the Languedoc-Roussillon Animal Welfare and Ethical Review Body, France. Housing and experimental procedures were approved by the French Agriculture and Forestry Ministry (A34-172-13 & 15040-2018050214043878). Note that replicating the present protocol with animal samples requires the permission of the Animal Welfare and Ethical Review Body of the relevant institution.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Water, LC/MS grade	Biosolve	0023214102BS
Acetonitrile, LC/MS grade	Biosolve	0001204102BS
Methanol, LC/MS grade	Biosolve	0010002884
Isopropanol	Sigma-Aldrich	34863
Formic acid	Sigma-Aldrich	33015
2-Morpholinoethansulphonic acid	Sigma-Aldrich	M8250
MASS SPECTOMETRY STANDARDS		
Arachidonic acid oxylipin LC-MS mixture	Cayman	20666
Linoleic acid oxylipins LC-MS mixture	Cayman	20794
Polyunsaturated fatty acid LC-MS mixture	Cayman	17941
8-iso prostaglandin E_2	Cayman	14350
Leukotriene E_4	Cayman	20410
Prostaglandin A_2	Cayman	10210
Thromboxane B_2	Cayman	19030
Thromboxane B_3	Cayman	19990
MASS SPECTROMETRY INTERNAL STANDARDS		
6-keto prostaglandin $F_{1\alpha}$-d_4	Cayman	315210
12(S)-HETE-d_8	Cayman	334570
Leukotriene B_4-d_4	Cayman	320110
Oleoyl ethanolamide-d_4	Cayman	9000552
PAF C-16-d_4	Cayman	360900
Prostaglandin E_2-d_4	Cayman	314010
Thromboxane B_2-d_4	Cayman	319030
CELL CULTURE		
DMEM	Thermo Fisher	10566016
0.25% Trypsin EDTA	Thermo Fisher	15050065
Fetal bovine serum (FBS)	Thermo Fisher	A3160501
PBS	Thermo Fisher	14190144
Experimental models: Organism/strains		
Mouse liver from 6 weeks old female mice	Charles River	C57BL/6 Mice
Software and algorithms		
MassHunter LC-MS Data Acquisition	Agilent	10.1.67
MassHunter Workstation Quantitative Analysis	Agilent	10.1.733.0
R studio	RStudio, PBC	Version 1.3.1056
Metaboanalyst	Jeff Xia Lab, McGill University, Canada	3.0.3 https://www.metaboanalyst.ca/home.xhtml
Other		
1.5 mL tubes	Sarstedt	72.692.405
15 mL tubes	Corning	430766
Glass beads	Retsch	22.222.0002
Tissue Homogenizer Precellys 24	Brevet Bertin Technologies	03119.200.RD000
Freeze dryer	Labconco	710201030

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Vacuum pump	Millipore	WP6122050
Centrifuge	Eppendorf	5430R
15 mL rotor	Eppendorf	F-35-6-30
SpeedVac	Thermo Fisher	SPD2030
Culture tubes, glass	BOREX	N/A
Solvent A, needle wash, seal wash bottles	Agilent	9301-6528
Solvent B bottle	Agilent	9301-6526
Strata-X 33 µm SPE Column; 10 mg	Phenomenex	8B-S100-AAK
Vacuum Manifold	Supelco	
Screw Vial, Fixed Insert, Clear	Agilent	5188-6591
Screw Blue Cap, 9 mm, PTFE/S	Agilent	5182-0720
Biocompatible HPLC System	Agilent	1290 Infinity II
LC-MS/MS	Agilent	6495C
Kinetex® 2.6 µm C8 100 Å, LC Column 150 × 2.1 mm	Phenomenex	00F-4497-AN

MATERIALS AND EQUIPMENT

LC-MS instrument

The present method presumes the availability of a high-performance liquid chromatography-MS/MS (HPLC-MS/MS) system. This may include a binary HPLC pump that can reproducibly deliver a mix of two solvents at 250 µL/min (e.g., the combination of two single-channel pumps or a quaternary pump). The LC system should include an automatic, online solvent degasser, a chilled autosampler, and a temperature-controlled column oven. The mass spectrometer must be a triple-quadrupole system that includes a nitrogen collision cell and is equipped with an electrospray source operated with nitrogen gas (other collision and source gases are possible, depending on the manufacturers' recommendations).

Here, we used the Agilent 6495C LC-MS system. TSQ Altis (ThermoFisher), Xevo TQ-XS (Waters), QTrap 6500+ (Sciex), and LCMS-8060 (Shimadzu) are examples of alternative LC-MS systems from other vendors. Some vendors have application notes or method packages that can be used to quickly set up the MS system for PUFA analysis.

Analytical standards for the Quality control (QC) mix

The method requires for QC purposes an analytical standard mix that contains unlabeled PUFAs (see list in the Mass Spectrometry Standards part of the key resources table).

⚠ CRITICAL: Methanol, acetonitrile, hexane and formic acid are flammable liquids; gases can be explosive. Hexane, methanol and formic acid vapors are particularly harmful/toxic. HCl, NaOH and formic acid are corrosive, the vapors also are corrosive. Methanol, hexane and acetonitrile should never be discarded directly into sewers, and require dedicated recycling/waste management procedures.

Note: PUFAs have different names and this can lead to confusion. For example, the compound defined as 5-oxo-ETE in the "Arachidonic Acid Oxylipin LC-MS Mixture" datasheet is included in the present method under its alternative name of 5-KETE.

STEP-BY-STEP METHOD DETAILS

Preparing the internal standard (ISTD) stock solution

Timing: 30 min

Table 1. ISTD pipetting scheme and their grouping in the respective Compound Groups

PUFAs (heavy labeled)	Final concentration	Amount	Compound group
6-keto Prostaglandin $F_{1\alpha}$-d_4	0.5 μg/mL	2 μL	1
12(S)-HETE-d_8	0.5 μg/mL	2 μL	2
Oleoyl Ethanolamide-d_4	0.5 μg/mL	2 μL	3
Leukotriene B_4-d_4	0.5 μg/mL	2 μL	4
PAF C-16-d_4	0.5 μg/mL	2 μL	5
Prostaglandin E_2-d_4	0.5 μg/mL	2 μL	6
Thromboxane B_2-d_4	0.5 μg/mL	2 μL	7
Acetonitrile (MS grade)		986 μL	

⚠ CRITICAL: The ISTD stock solution can be stored at −80°C stably for years. It is recommended to store small aliquots in glass vials and to overlay these with N_2. The inexpensive N_2 gas can be replaced by Ar gas that is more inert and is considered a better choice.

Note: The PUFAs included in the ISTD are all soluble in acetonitrile, but may be shipped in other solvents. Depending on the starting amount, intermediate dilutions may be needed when preparing the ISTD mix. These can be prepared using acetonitrile. The ISTD and each PUFA are grouped in Compound Groups that are indicated in Table 1 above and in Table S1. The ISTD of a specific compound group is used to normalize the results of all PUFAs belonging to that group (the list of PUFAs measured by the present method is provided in Table 2). The number of compound groups may be expanded and more internal standards (heavy labeled PUFAs) may be included. In our experience this does not increase the protocol accuracy. However, multiple reaction monitoring (MRM) transitions for additional heavy-labeled internal standards are included in the compound list provided in Table S1.

Preparing control samples

Timing: 1 h (does not include time needed for liver tissue harvest)

To perform the LC-MS system QC, blank, positive control, and QC samples are required. Their preparation is described below.

1. *Blank sample*, transfer 980 μL of methanol and 20 μL of ISTD to a 1.5 mL polypropylene tube with screw cap (e.g., Sarstedt cat. # 72.687 or similar). Add 0.5 g of glass beads (Sigma Aldrich) and vortex vigorously for 1 min. Homogenize the blank sample using the Tissue Homogenizer Precellys 24 and the following program: speed 4 m/s, 2 cycles of 15 s. Proceed to the PUFA Purification section below.

   Note: Optionally, a matrix-adjusted blank can be prepared by mixing 100 μL of DMEM (without serum) with 880 μL of methanol and 20 μL of ISTD in a 1.5 mL polypropylene tube. The advantages are outlined below.

2. *Positive control*, prepare a sample consisting of 50 mg of mouse liver tissue (we recommend C57BL/6 mice), prepared as described in the sample preparation section below. If specific PUFAs must be analyzed, these can be included by spiking (10 μM final concentration) the sample when the ISTD is added. An alternative source of free PUFAs is animal serum, such as fetal bovine serum (FBS) (see the key resources table). A 200 μL aliquot of FBS can be prepared as positive control, as indicated in the sample preparation section below.
3. QC, prepare a 100 μL mix of all Mass Spectrometry Standards (indicated in the key resources table), at 10 μM final concentration, in methanol.

Table 2. List of PUFAs measured using the present method (163 unique molecules and 16 heavy-labeled PUFA ISTDs)

Nr.	Name	Nr.	Name	Nr.	Name
1	10,17-DiHDoHE	61	18-HETE	121	Adrenic acid
2	10-HDoHE	62	19-HETE	122	AEA
3	11,12-DHET	63	1a1b-dihomo-PGF2a	123	Azelaoyl PAF
4	11,12-EET	64	2,3-dinor-8-iso-PGF2a	124	DHA-d5
5	11,12-EET-EA	65	20-carboxy-AA	125	Dihomo-gamma-linoleic acid
6	11-beta-13,14-dihydro-15-keto-PGF2a	66	20-carboxy-LTB4	126	Docosahexaenoic acid
7	11-beta-PGF2a	67	20-HDoHE	127	Docosapentaenoic acid
8	11-dehydro-TXB2	68	20-HETE	128	Eicosapentaenoic acid
9	11-HDoHE	69	20-hydroxy-LTB4	129	EPA-d5
10	11-HETE	70	20-hydroxy-PGE2	130	iPF2a-IV
11	11-trans-LTC4	71	20-hydroxy-PGF2a	131	Linoleic acid
12	11-trans-LTE4	72	4-HDoHE	132	Linolenic acid
13	12,13-DiHOME	73	5,15-DiHETE	133	LTB4
14	12,13-EpOME	74	5,6-DHET	134	LTB4 d4
15	12-HEPE	75	5,6-DHET-lactone	135	LTB4-EA
16	12-HETE	76	5,6-DiHETE	136	LTC4
17	12-HETE-d8	77	5,6-EET	137	LTC4-d5
18	12-HHT	78	5,6-EET-EA	138	LTD4
19	12-HpEPE	79	5-HEPE	139	LTE4
20	12-KETE	80	5-HETE	140	LTF4
21	12-keto-LTB4	81	5-HETE-d8	141	LXA5
22	13,14-dihydro-15-keto PGJ2	82	5-HpEPE	142	Lyso-PAF
23	13,14-dihydro-15-keto-PGD2	83	5-HpETE	143	Maresin1
24	13,14-dihydro-15-keto-PGE2	84	5-iPF2a-VI	144	N-acetyl-LTE4
25	13,14-dihydro-15-keto-PGF2a	85	5-KETE	145	OEA
26	13,14-dihydro-15-keto-tetranor-PGD2	86	5S,14R-LXB4	146	OEA-d4
27	13,14-dihydro-15-keto-tetranor-PGE2	87	5S,6R-LXA4	147	PAF
28	13,14-dihydro-15-keto-tetranor-PGF1a	88	5S,6S-LXA4	148	PAF-d4
29	13,14-dihydro-15-keto-tetranor-PGF1b	89	6,15-diketo-13,14-dihydro-PGF1a	149	PGA1
30	13-HDoHE	90	6-keto-PGF1a	150	PGA2
31	13-HODE	91	6-keto-PGF1a-d4	151	PGB2
32	13-HOTrE	92	6-trans-LTB4	152	PGD1
33	13-HpODE	93	7,17-hydroxy-DPA	153	PGD2
34	13-KODE	94	7-HDoHE	154	PGD2-d4
35	14,15-DHET	95	8,12-iso-iPF2a-VI-1,5-lactone	155	PGD2-EA
36	14,15-DiHETE	96	8,15-DiHETE	156	PGD3
37	14,15-EET	97	8,9-DHET	157	PGE1
38	14,15-EET-EA	98	8,9-EET	158	PGE1-EA
39	14,15-LTC4	99	8,9-EET-EA	159	PGE2
40	14,15-LTE4	100	8-HDoHE	160	PGE2-d4
41	14-HDoHE	101	8-HETE	161	PGE2-EA
42	15-deoxy-delta-12,14-PGJ2	102	8-iso-13,14-dihydro-15-keto-PGF2a	162	PGE3
43	15-HEDE	103	8-iso-15(R)-PGF2a	163	PGF2a
44	15-HEPE	104	8-iso-15-keto-PGF2a	164	PGF2a-d4
45	15-HETE	105	8-iso-PGA1	165	PGF2a-EA
46	15-HETE-d8	106	8-iso-PGA2	166	PGF3a
47	15-HETrE	107	8-iso-PGE1	167	PGJ2
48	15-HpEPE	108	8-iso-PGE2	168	PGK2
49	15-HpETE	109	8-iso-PGF1a	169	Resolvin D1
50	15-KEDE	110	8-iso-PGF2a	170	Resolvin D2
51	15-keto-PGE2	111	8-iso-PGF3a	171	Stearidonic acid
52	15-keto-PGF2a	112	9,10-DiHOME	172	Tetranor-PGDM
53	16-HDoHE	113	9,10-EpOME	173	Tetranor-PGEM
54	16-HETE	114	9-HETE	174	Tetranor-PGEM-d6

(*Continued on next page*)

Table 2. *Continued*

Nr.	Name	Nr.	Name	Nr.	Name
55	17,18-DiHETE	115	9-HODE	175	Tetranor-PGFM
56	17,18-EpETE	116	9-HOTrE	176	TXB1
57	17-HDoHE	117	9-HpODE	177	TXB2
58	17-HETE	118	9-KODE	178	TXB2-d4
59	18-carboxy-dinor-LTB4	119	AA	179	TXB3
60	18-HEPE	120	AA-d8		

Note: It is good practice to prepare a batch of positive control samples (10 or more aliquots of tissue samples) and keep them ready to use (at −80°C) when a new sample preparation is started.

Sample preparation

General considerations

A schematic overview of the sample preparation process is outlined in the Figure 1. Before starting the sample preparation protocol it is importnat to determine the number of samples needed per condition as well as the sample homogenisation process.

In metabolomic analyses, we recurrently observe strong inter-sample variation. This means that 30%–40% of relative standard deviation for a given metabolite (comparison of replicates) is not uncommon. This is mainly caused by the biological variations of metabolite changes due to the protocol steps/experimental conditions. For example, animal diet (feeding habits) directly influences the free PUFA concentration in serum. To detect significant differences below the 2-fold change, we recommend using five replicates.

For frozen sample homogenization, particularly tissues, we recommend using mortar and pestle to crush the tissue into small pieces and then to complete the homogenization using glass beads (0.25–0.5 mm diameter; glass or ceramic beads with larger diameters gave less good results) and the tissue homogenizer Precellys 24 (or similar). However, for smaller tissue pieces (up to 30 mg), we found that the mortar and pestle step can be skipped and that the glass bead-based homogenization alone works equally well. This may need to be preliminarily tested in the laboratory to decide how to proceed for the specific tissue under study, especially when processing large sample series.

At the end of sample preparation (see the custom-made protocols for four specific sample types) one can proceed rapidly to the PUFA Extraction step or store samples overlaid with N_2 (or Ar) for up to 1 year at −80°C (longer storage times have not been tested).

Except for adipose tissue samples, the present protocol also offers the possibility to keep general water-soluble metabolites for targeted or untargeted metabolomics. If these compounds are of interest, 2-morpholinoethansulfonic acid may be spiked as additional internal standard in the samples below (5 µL/sample of a 100 µM solution); further details on the method for their analysis are in Turtoi et al.[5].

⚠ CRITICAL: The sample quantities indicated in the subsequent steps are the recommended quantities that have been tested and have provided robust results. We recommend to use these amounts with minimal variation (± 10%) among the different samples to be compared in the same batch. If this is not possible and samples in one batch contain different amounts of starting material, consider first crushing/lysing them using the same volume of methanol for all of them. After the homogenization step, the volume of sample transferred to a new tube should be adjusted in function of its initial weight (larger volumes for smaller samples and smaller volumes for larger ones). Subsequently, adjust

with fresh methanol to reach the same volume in all samples, spike with the ISTD, and proceed to the next protocol steps. It should be noted that there is no ideal procedure to handle samples with different sizes in the same batch. Any correction introduces a source of error that reduces the data robustness and therefore, necessitates additional biological replicates to verify potential modulations.

Note: During the homogenization steps (described below), samples can easily leak or be entirely lost if the specific tubes (screw-caps with o-rings) referenced in the present protocol are not used.

Cell extracts

Timing: 1 h (excluding the time for growing cell cultures, step (a) below)

4. Start from adherent cells in 15 cm Petri dishes.
   a. Grow 5–8 million cells/15 cm dish to 80% of confluence.
   b. Wash the cell monolayer twice with ice-cold PBS (10 mL/wash).
   c. Detach cells using 1 mL trypsin (0.25% trypsin-EDTA, phenol red) at 37°C for 5 min. Stop the reaction with 10 mL of culture medium (containing 10% FBS; e.g., DMEM with 10% FBS). Dissociate cells by resuspension and collect them in a 15 mL tube (polypropylene screw cap, e.g., Corning cat. # 352096, or similar). Count cells to determine the number of cells/mL (e.g., Countess™ Automated Cell Counter).
   d. Transfer a volume containing 3 million cells in a fresh 15 mL tube. Centrifuge the tube at 200g (swing-bucket rotor), at 4°C, for 5 min. Remove as much as possible of the liquid and wash the cell pellet in 10 mL of ice-cold PBS. Centrifuge the tube again (same conditions as above) and remove the maximum of PBS, keep the pellet.
5. Crush the cell pellet in 980 µL of ice-cold methanol, add 20 µL of ISTD and transfer to a 1.5 mL polypropylene tube with a screw cap (e.g., Sarstedt cat. # 72.687, or similar). Add 0.5 g of glass beads and vortex vigorously for 1 min. Thoroughly homogenize the sample using the tissue homogenizer Precellys 24 and the following program: speed 4 m/s, 2 cycles of 15 s.
   a. Agitate the sample at 4°C for 1 h.
   b. Remove the debris by centrifugation at 20,000 × *g* (4°C) for 10 min.
   c. Transfer the supernatant to a 15 mL tube.
   d. Proceed to the PUFA Extraction step or store samples at −80°C.

⚠ CRITICAL: Timing is very important in step 4. Once the cells leave the controlled atmosphere of the cell incubator, metabolism starts changing. This rather uncontrollable event contributes to metabolite inter-replicate variability (described also above). As enzymatic reactions stop when cells are crushed in methanol (step 5), it is better to limit the number of samples concomitantly handled in order to rapidly proceed through step 4 and add methanol as quickly as possible. Lastly, all PBS should be aspirated (removed) in step 4d because this will dilute the methanol and introduce salts in the sample. Only LC-MS grade methanol should be used.

Note: After step 5a, it is possible first to store the homogenized sample at −20°C for 16 h, and then recover proteins from the sample by centrifugation. The resulting pellet can be lightly air-dried under the hood (2–3 min) to remove the residual methanol and then lysed in 500 µL of lysis buffer (50 mM Tris-HCl, 1% SDS, pH 8.0). Add lysis buffer to the pellet, vortex vigorously for 1 min, and leave the lysate on ice for 20 min. Then, vortex again vigorously and centrifuge at 15,000 × *g* (4°C) for 10 min. Recover the supernatant and proceed to protein quantification using a colorimetric assay, such as the bicinchoninic acid (BCA) assay. The protein quantity can be used to readjust the sample volumes transferred at step 5c above. If this adjustment is performed, the final volume in step 5d must be re-adjusted to 980 µL using methanol.

Importantly, if proteins are recovered, the ISTD cannot be added at the start of step 5, but only at step 5d after all samples have (again) the same volume.

Liver and other solid tissues

Timing: 2 h

6. Start from fresh mouse liver tissue that was immediately snap-frozen in liquid nitrogen after collection.
   a. Resect liver from euthanized mice, wash briefly in ice-cold PBS (remove excess PBS using filter paper), and weigh 50 mg.
   b. Transfer the 50 mg of tissue to liquid nitrogen for snap freezing and then convert the frozen tissue into powder by crushing it in liquid nitrogen with mortar and pestle. Transfer the tissue powder to a 1.5 mL polypropylene tube with screw cap (e.g., Sarstedt cat. # 72.687 or similar), add 980 μL of ice-cold methanol and 20 μL of ISTD. Add 0.5 g of glass beads and vortex vigorously for 1 min. Thoroughly homogenize the sample using the Precellys 24 tissue homogenizer and the following program: speed 4 m/s, 2 cycles of 15 s.
   c. Agitate the sample at 4°C for 1 h.
   d. Remove debris by centrifugation at 20,000 × *g* (4°C) for 10 min. If the protein pellet is recovered, refer to the **NOTE** above.
   e. Transfer the supernatant to a 15 mL tube.
   f. Proceed to the PUFA Extraction step or store samples at −80°C.

⚠ CRITICAL: When crushing with mortar and pestle, we suggest to wrap the tissue in aluminum foil and crush it in the foil to avoid sample contamination and sample loss (all equipment coming in contact with the tissue must be pre-chilled in liquid nitrogen). All equipment items that come into contact with the tissue powder must be rinsed with de-ionized water and wiped dry before re-use.

Note: Tissue samples can originate also from humans or other animal species. Human, mouse and snail samples have been tested. Other species have not been tested, but are expected to behave similarly.

Serum/plasma/cell culture medium

Timing: 2 h (except the lyophilization step that takes 10–12 h)

7. Start from a serum/plasma sample (refer to step **a** below) or cell-free culture supernatants (refer to step **b** below).
   a. Transfer 100 μL of serum or plasma sample to a 1.5 mL polypropylene tube with screw cap.
   b. Starting from a 15 cm Petri dish (5–8 million cells at 80% of confluence), collect 10 mL of cell-free culture supernatant and lyophilize it by freeze drying. Dissolve the sample in 100 μL of water and transfer to a 1.5 mL polypropylene tube with screw cap.
   c. To the samples prepared in **1a** or **1b** add 880 μL of ice-cold methanol and 20 μL of ISTD.
   d. Agitate the sample at 4°C for 1 h.
   e. Remove precipitates by centrifugation at 20,000 × *g* (4°C) for 10 min.
   f. Transfer the supernatant to a 15 mL tube.
   g. Proceed to the PUFA Extraction step or store samples at −80°C.

Adipose tissue

Timing: 2 h 30 min

Unlike other tissues, adipose tissue entraps PUFAs and their derivatives in a very hydrophobic environment, preventing their efficient release when using only methanol. Therefore, to obtain a high yield, first fat must be hydrolyzed into free fatty acids and then PUFAs can be extracted. However, it should be noted that this will result in the hydrolysis of all ester bonds, and PUFAs bound to the cell membranes will become free. Thus, unlike the other methods described above, this adipose tissue sample preparation method will allow obtaining data on both bound and free PUFAs. If only free PUFAs are required, proceed as described above for liver and other solid tissues.

8. Start from fresh mouse visceral adipose tissue that was immediately snap-frozen in liquid nitrogen after collection.
   a. Resect adipose tissue from euthanized mice, wash briefly in ice-cold PBS, and weight 50 mg.
   b. Transfer the tissue to liquid nitrogen for snap freezing and then convert frozen tissue into powder by crushing it in liquid nitrogen with mortar and pestle.
   c. Transfer the tissue powder to a 1.5 mL tube. Add 1 mL of methanol and spike with 20 µL of ISTD. Add 0.5 g of glass beads.
   d. Homogenize the sample using the Precellys 24 tissue homogenizer and the following program: speed 4 m/s, 2 cycles of 15 s.
   e. Transfer the homogenate to a 15 mL tube and hydrolyze the tissue by adding 1 mL of water, 2 mL of methanol and 1 mL of 10 M NaOH. Overlay with a stream of N_2 and vortex the tube at maximum speed for 1 min. Incubate at 90°C for 1 h. Cool the sample on ice for 10 min.
   f. Acidify the sample with 2 mL of 6 M HCl and add 3 mL of *n*-hexane. Vortex the tube at maximum speed for 1 min and centrifuge at 7,000 × *g* at room temperature (fixed angle rotor) for 5 min.
   g. Recover the organic phase (visible top layer) in a fresh 15 mL tube and dry the sample under a gentle stream of N_2. Solubilize the sample in 1 mL of ice-cold methanol.
   h. Proceed to the PUFA Extraction step or store samples at −80°C.

   Note: Avoid prolonged exposure to hexane/high temperature of plastic labware (polypropylene). Plastic will eventually leach and may increase the noise in the MS analysis. Proceed with sample preparation rapidly.

   ⚠ CRITICAL: Step 8e should be performed in a fume hood with protective eyewear and gloves. 10 M NaOH and 6 M HCl solutions are corrosive. Consult the safety datasheet provided by the suppliers before starting the experiments.

PUFA purification using solid phase extraction (SPE) columns

Timing: 2 h (except for the evaporation step [16] that is usually performed for 16 h overnight)

Before proceeding with the next steps prepare the following solutions (volumes sufficient for 15 samples):

Loading solution: 100 mL of water with 0.1% formic acid.

Wash solution: 20 mL of 15% ethanol solution in water.

⚠ CRITICAL: Prepare the Loading and Wash solutions with LC-MS grade chemicals just before use.

Note: Acetonitrile is flammable, irritant and toxic by skin adsorption. Working under the fume hood, wearing laboratory coat, googles and gloves are mandatory when using this solvent.

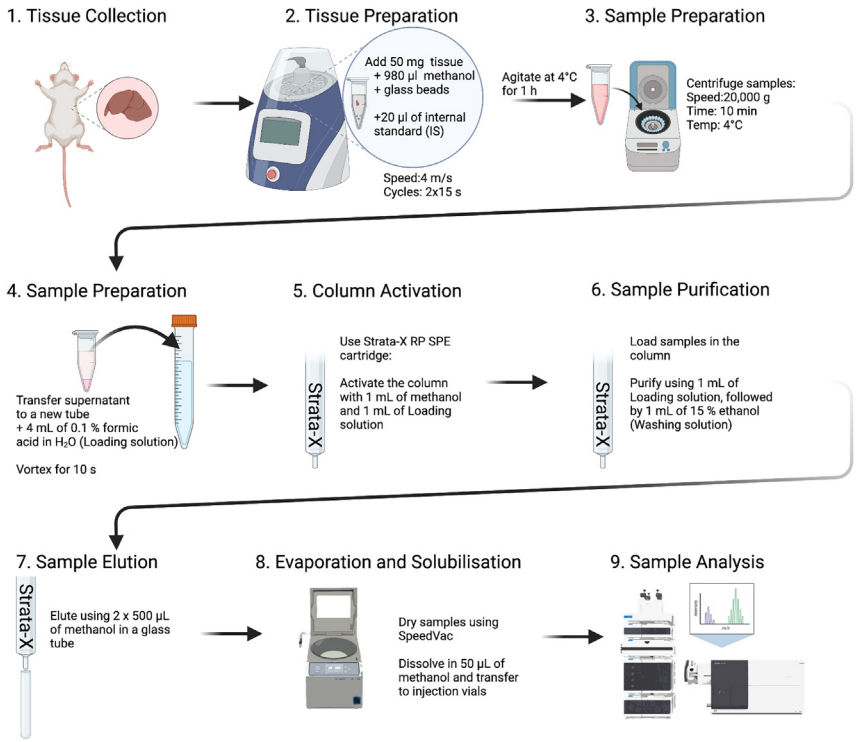


Figure 1. Workflow of the PUFA sample preparation and analysis

9. Vortex the sample vigorously for 1 min. If starting from frozen samples, allow them first to thaw at 4°C.
10. Add 4 mL of Loading solution to the supernatant to obtain a total sample volume of 5 mL.
11. Leave samples on ice for 15 min.

⚠ CRITICAL: Proceed rapidly and without any stop throughout all steps.

Mount Strata-X 30 mg SPE cartridges (Phenomenex) on a SPE manifold, attach the vacuum pump to the manifold (for alternatives see also the **OPTIONAL** section below) and perform the washing, loading, rinsing and elution steps described below. Volumes are always indicated for 1 column.

⚠ CRITICAL: Ideally, all steps on the SPE manifold should be completed under gravity flow. PUFA recovery will be higher with slower flow. However, viscous samples may need the application of additional (low) vacuum (-10 to -20 kPa) for better throughput. Vacuum can be controlled directly on the specified pump model thanks to the presence of a dedicated manometer and control valve. In any case, do not exceed a flow of 1 drop per second.

12. Washing and conditioning.
    a. Add 1 mL of methanol.
    b. Add 1 mL of Loading solution.
    c. Discard the wash solutions and proceed rapidly to step 13.
13. Sample loading.
    a. Gently agitate the sample before loading onto the SPE column.
    b. Add the sample stepwise by loading 1 mL onto the SPE column.
    c. Discard the filtrate.

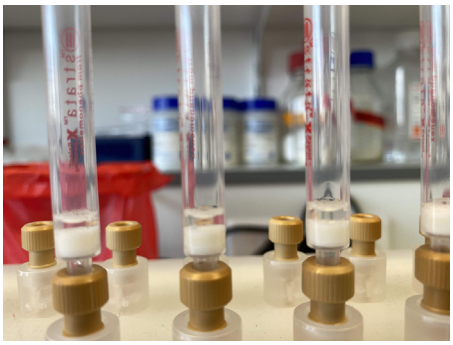


Figure 2. SPE purification of PUFA
Example of residual liquid level left above the SPE column bed to prevent sample oxidation.

Note: At step 13c, the filtrate can be stored to analyze water-soluble metabolites. To this aim, lyophilize the sample (or evaporate in a SpeedVac) to dryness and refer to the "Before you begin" section of the "Analysis of Polar Primary Metabolites in Animal Samples Using Targeted Metabolomics and LC-MS" protocol in Turtoi et al.[5]

14. Rinsing.
    a. Add 1 mL of Loading solution.
    b. Add 1 mL of Wash solution, and let it run completely through the column (proceed quickly to step 15 below).
    c. Discard the filtrate.
15. Elution.
    a. Add 500 µL of methanol.
    b. Collect the eluate in a glass tube.
    c. Repeat steps 15a and 15b for a total of 1 mL.
16. Dry samples in a SpeedVac.

Optional: Alternatively, vacuum can be controlled by using a specific positive pressure manifold, such as Pressure+ 48 (Biotage). Other vendors propose similar equipment, such as HyperSep (ThermoFisher) and PPM48 (Agilent). Several vendors provide alternative SPE products with similar specifications, for instance Oasis HLB by Strata-X Waters. Other SPE columns with different chemistry also may be suitable, such as Bond Elut Lipid Extraction (Agilent).

Note: At steps 14 and 15, ideally do not allow the SPE columns to run dry because this may increase sample oxidation. It is good practice to always leave 1 mm of liquid on top of the SPE resin bed (see Figure 2).

Pause point: At step 16, dried samples can be overlaid with N_2 (or Ar) and stored at −80°C for a short time (less than one month). Longer storage periods have not been tested.

LC-MS/MS analysis

Preparation of solvents and LC-MS/MS set-up

Timing: 30 min

17. Prepare 4 solvent mixtures for the Agilent Infinity II UHPLC (used in this protocol): A and B to make the analytical gradient, Needle Wash to rinse the needle used to inject the sample, and Seal Wash to rinse the pump seals in the system. An identical set-up is available on all standard HPLC systems from major commercial vendors.

Solvent A: water/0.1% formic acid	
Chemical	Amount
Water	1,000 mL
Formic Acid	1 mL

Solvent B: acetonitrile/0.1% formic acid	
Chemical	Amount
Acetonitrile	1,000 mL
Formic Acid	1 mL

Needle Wash: methanol/water 50/50 and 0.1% formic acid	
Chemical	Amount
Methanol	500 mL
Water	500 mL
Formic Acid	1 mL

Seal Wash: methanol/water 10/90 and 0.1% formic acid	
Chemical	Amount
Isopropanol	100 mL
Water	900 mL
Formic Acid	1 mL

Note: All recipes for the liquids used during the analysis are given as 1 L volumes (sufficient for at least 200 injections). However, always prepare fresh solvents by calculating the required amount (plus 20% as a safety margin). An easy manner to calculate the required amounts of solvents A and B is to multiply the number of injections to be performed (including blanks and QCs) by 0.25 mL (flow rate per minute) and by 30 min (duration of one analysis). The resulting volume is then increased by 20% and divided by 2 to obtain the volume of solvents A and B.

⚠ CRITICAL: Keeping liquids in bottles for extended periods of time (longer than 10 days) will result in their deterioration; algae and fungi may also start to grow, particularly in solvent A. Atmospheric CO_2 will change their pH. Lastly, solvent B is light sensitive because acetonitrile in acidic conditions tends to polymerize with time. We strongly recommend the use of brown color bottles at least for solvent B (see key resources table). If large volumes need to be stored for extended periods of time, we recommend to remove them from the HPLC station and store them at 4°C.

MS parameter optimization

Timing: 4–16 h (includes possible automatic steps performed overnight)

Prior to starting the actual measurement, a set of compound-dependent and compound-independent parameters must be optimized. Compound-dependent parameters are those that depend on the physical features of each measured molecule. Compound-independent parameters concern all compounds in the analysis because they are mainly influenced by the solvent composition and flow rate. The single most critical compound-dependent parameter that requires optimization is the collision energy (CE) for the individual MRM transitions. The most relevant compound-independent parameters concern the source.

18. Adjust the CE for the individual MRM transitions. Users who operate any Agilent 64xy triple-quad instruments (e.g., 6495C) will be able to use the MRM CE, as described in the present protocol (Table S1). Instruments from other vendors will require CE adjustments. PUFA have very similar CE behavior in function of their subclass (referred to here as Compound Group). The Compound Group numbers are given in the compound list (included in Table S1). If using a different MS system, carry out CE optimization based on, at least, a set of PUFAs (Table S1). The same CE can be applied to other PUFAs in the same Compound Group. This is a good start that can be refined later by performing further CE optimizations for the compounds of interest.
19. Adjust the source parameters.Unless using the same source as presented here (Agilent Jet Stream), individual optimization on the used instrument will be required, considering the solvent composition and flow rate. For this, refer to the User Manual of the used instrument. After optimizing gas temperature, sheath gas temperature and flow, iFunnel values on Agilent 6495 instruments also must be optimized to improve the performance. However, one compromise setting can be applied for all compounds (cannot be varied for each compound during the run, hence compound-independent). To optimize the iFunnel refer to the 6495 series user manual. Note that iFunnel parameters only apply to the 6495 series, while other (e.g., 6475) may not have this feature and therefore users operating these instruments can skip the iFunnel parameter optimization.

Note: Users of the Shimadzu 8040, 8050 and 8060 series of LC-MS devices can access a commercially available MS method file from the instrument vendor (Lipid Mediators, Shimadzu) that contains a similar set of analytes. Users of the Waters TQ-S and TQ-XS instruments should refer to the Waters application note 720004664EN from 2015 that provides transitions and CE for 123 PUFAs and oxylipins. Users of the Sciex QTRAP instruments can refer to the Lipid Mediator technical note.

Source Parameters	
Gas temperature	250°C
Gas Flow	12 L/min
Nebulizer	35 psi
Sheath Gas Temperature	400°C
Sheath Gas Flow	12 L/min
Capillary Voltage (neg. mode)	4000 V
Capillary Voltage (pos. mode)	4000 V
Nozzle Voltage	500 V
iFunnel	
High Pressure RF	Pos: 150 V Neg: 90 V
Low Pressure RF	Pos: 60 V Neg: 60 V

20. Program the LC gradient in the analytical method.

LC Gradient	
Time, min	Solution B, %
0	10
5	25
10	35
20	75
20.1	95
28	95

(*Continued on next page*)

Continued	
Time, min	Solution B, %
28.1	10
30	10
Flow Rate	0.250 µL/min
Oven Temperature	40°C
Autosampler Temperature	8°C

Note: If the MS instrument is equipped with a divert valve, use the valve to divert the flow from the MS to waste after 25 min. This will ensure that the mass spectrometer remains operational longer, minimizing the need of instrument cleaning.

For further details concerning the LC and MS parameters, please refer to the Table S1.

Systems performance check

Timing: 3 h

Before starting the analysis of experimental samples, the LC-MS system performance needs to be verified. At this step, all retention times (RT) and CEs should have been properly included in the instrument method file.

For the system performance check, the following samples need to be prepared in LC-MS vials:

21. 100 µL of 10 µM QC mix in methanol prepared from the MS Standards described in the materials and equipment section.
22. Blank sample (add 50 µL of methanol to the prepared blank).
23. Positive control (add 50 µL of methanol to the prepared positive control).
24. Vortex samples vigorously for 1 min.
25. Inject 1 µL of each sample into the LC-MS system.
26. Verify first the performance check results by confirming the absence of signal for the blank sample (if significant traces of PUFAs are present, refer to the troubleshooting section below); signals close to background are considered normal for some compounds because they are generally abundant and may be present as contaminants. Then, examine the QC mix result and check that the tested compounds elute at the expected RT with the expected intensities and signal-to-noise (S/N) ratio (for RT shift problems, refer to the troubleshooting section). For more information on the S/N value, calculation and interpretation, see the article by Dolan.[6]
27. Verify the results for the positive control sample. If using liver, as in the present protocol, verify that most of the metabolites listed in Table 3 are present; otherwise, refer to the troubleshooting section. In case of other LC-MS system issues, refer to the troubleshooting section for the most common ones and how to solve them.

Note: Each MS system has its own sensitivity and performance features. Therefore, we cannot provide universal values (i.e., absolute intensities) for the system performance check. In this context, it is more important to establish the "normal" performance of the available instrument (ideally just after preventive maintenance, when performance and function have been verified), document the obtained values, and each time compare the values obtained in the new analysis with the recorded values. In this manner, each laboratory can define its own QC parameters, quickly spot problems and system deteriorations, thus avoiding the loss of precious samples in case of problems. As guidance for users of the 6495C system, we provide

Table 3. Quantification of the major PUFAs found in mouse liver extracts

	Blank	Liver Rep. 1	Liver Rep. 2	Liver Rep. 3				
Compound name	Average area	Area Corr.	Area Corr.	Area Corr.	Average (area)	SD	RSD	c (μM)
11,12-DHET	3.4	205.9	248.4	287.8	247.4	40.9	16.6	N/A
11-HETE	42.0	169.0	170.8	296.1	212.0	72.9	34.4	N/A
12,13-DiHOME	68.6	718.2	689.5	617.8	675.2	51.7	7.7	N/A
12-HETE	24.4	290.6	209.0	109.4	203.0	90.8	44.7	N/A
12-HETE-d8	**8.2**	**5057.4**	**4267.2**	**4641.8**	**4655.5**	**395.3**	**8.5**	**N/A**
12-HpETE	3.4	133.8	103.9	133.6	123.8	17.2	13.9	N/A
12-KETE	33.0	94.8	149.6	47.6	97.3	51.0	52.4	N/A
13-HDoHE	4.0	95.3	82.3	165.8	114.5	45.0	39.3	N/A
13-HODE	79.3	1355.1	1576.8	1370.9	1434.3	123.7	8.6	0.012
13-HOTrE	7.0	105.4	200.9	173.1	159.8	49.1	30.7	N/A
13-HpODE	13.6	417.3	662.1	583.4	554.3	124.9	22.5	N/A
13-KODE	30.1	712.4	844.2	813.2	789.9	68.9	8.7	0.009
14,15-DHET	10.1	666.2	635.2	696.9	666.1	30.8	4.6	N/A
14,15-DiHETE	11.6	135.3	96.2	96.1	109.2	22.6	20.7	N/A
15-HETE	30.7	196.3	75.2	191.4	154.3	68.6	44.4	N/A
16-HDoHE	3.2	173.1	166.1	143.0	160.7	15.7	9.8	N/A
17,18-DiHETE	13.1	305.8	172.4	304.9	261.0	76.8	29.4	N/A
19-HETE	11.4	150.6	242.2	230.2	207.7	49.8	24.0	N/A
1a1b-dihomo-PGF2a	33.2	691.8	729.2	620.4	680.5	55.3	8.1	N/A
20-carboxy-AA	44.2	8957.2	8597.6	7400.1	8318.3	815.3	9.8	N/A
20-hydroxy-PGE2	1.1	225.6	221.8	220.4	222.6	2.7	1.2	N/A
4-HDoHE	3.3	90.8	85.2	130.1	102.1	24.5	24.0	N/A
5-HEPE	1.5	96.7	135.6	105.2	112.5	20.5	18.2	N/A
5-HETE	37.8	464.5	457.0	524.5	482.0	37.0	7.7	N/A
6-keto-PGF1a-d4	**2.6**	**5517.1**	**5408.5**	**5730.0**	**5551.9**	**163.6**	**2.9**	**N/A**
13,14-dih-15-keto-PGD2	3.0	14744.2	14237.0	15411.5	14797.6	589.1	4.0	N/A
13,14-dih-15-keto-tetra-PGF1a	2.6	122.1	131.0	189.7	147.6	36.7	24.9	N/A
8-iso-PGA2	19.8	1578.8	1388.6	1447.7	1471.7	97.4	6.6	N/A
8-iso-PGE2	5.0	6054.2	5541.2	5961.6	5852.3	273.4	4.7	N/A
9,10-DiHOME	88.2	339.1	423.9	324.5	362.5	53.7	14.8	N/A
9-HODE	15.9	496.0	557.6	569.9	541.2	39.6	7.3	N/A
9-HpODE	5.6	674.3	1149.4	803.8	875.8	245.6	28.0	N/A
9-KODE	12.2	172.0	215.6	213.5	200.4	24.6	12.3	N/A
AA	191.6	869563.8	933805.8	873615.3	892328.3	35977.6	4.0	5.09
AEA	1.3	151.8	198.8	162.6	171.1	24.6	14.4	N/A
DHA	289.6	298687.9	318227.5	306465.7	307793.7	9837.3	3.2	5.33
Eicosapentaenoic Acid	18.6	49289.8	44842.2	52169.3	48767.1	3691.4	7.6	1.87
Linoleic Acid	22.0	455.6	398.9	640.2	498.2	126.2	25.3	N/A
Linolenic Acid	6.7	144.1	120.5	142.8	135.8	13.2	9.8	N/A
LTB4 d4	**5.1**	**4879.3**	**4567.9**	**4885.5**	**4777.6**	**181.6**	**3.8**	**N/A**
LTE4	3.9	766.4	568.1	803.1	712.5	126.4	17.7	N/A
OEA	178.0	12104.7	11958.7	12885.6	12316.3	498.4	4.0	N/A
OEA-d4	**15.7**	**794000.4**	**759037.1**	**793822.0**	**782286.5**	**20134.8**	**2.6**	**N/A**
Lyso-PAF	25.6	799623.7	733163.4	757393.1	763393.4	33634.0	4.4	N/A
PAF	1.3	232.9	165.6	138.0	178.8	48.8	27.3	N/A
PAF-d4	**11.3**	**831214.8**	**796151.0**	**870049.9**	**832471.9**	**36965.5**	**4.4**	**N/A**
PGA1	4.1	1.5	20.2	7.4	9.7	9.6	98.6	N/A
PGA2	65.2	5107.0	5273.6	5770.2	5383.6	345.0	6.4	N/A
PGB2	8.4	8.4	42.7	47.2	32.8	21.2	64.8	N/A
PGD2	2.7	6147.0	5393.2	5602.9	5714.4	389.1	6.8	N/A
PGE2	1.6	5710.2	5331.9	5732.7	5591.6	225.2	4.0	N/A
PGE2-d4	**9.5**	**17709.5**	**17523.2**	**18065.2**	**17765.9**	**275.4**	**1.6**	**N/A**

(*Continued on next page*)

Table 3. *Continued*

Compound name	Blank Average area	Liver Rep. 1 Area Corr.	Liver Rep. 2 Area Corr.	Liver Rep. 3 Area Corr.	Average (area)	SD	RSD	c (μM)
PGF2a-EA	0.7	180.2	170.4	225.3	192.0	29.3	15.3	N/A
PGJ2	14.7	6585.5	6158.1	6526.0	6423.2	231.5	3.6	N/A
Stearidonic Acid	19.4	1321.1	1158.4	1694.5	1391.3	274.8	19.8	N/A
TXB2-d4	**1.4**	**7698.8**	**6731.1**	**7715.0**	**7381.6**	**563.5**	**7.6**	**N/A**
TXB2	8.5	360.8	432.9	491.0	428.2	65.2	15.2	N/A

Shown are three technical replicates (including sample preparation and measurements). Note that the area of each PUFA is normalized with the internal standard (ISTD) of the corresponding Compound Group, as outlined in the compound list provided in Table S1, MS Parameters section. Absolute quantities were calculated for selected PUFAs using the calibration curves and the PUFA non-normalized areas. The standard deviation of means (SD) and the relative standard deviation (RSD) is calculated for each individual compound from three replicates. The Blank value is the average area of three replicates. For non-corrected areas of individual samples see supplemental information, "Example" section.

the absolute intensity values for a set of QC molecules after injection of 1 μL of 10 μM QC mix (see Table S2).

LC-MS/MS sample analysis

Timing: 30 min/sample

If the performance check results are satisfactory, proceed to the actual sample analysis.

28. Resuspend all dry samples in 50 μL of methanol.
29. Vortex samples vigorously for 1 min.
30. Transfer samples in glass vials for LC-MS (Agilent, cat. # 5188-6591).
31. Inject 2 μL of sample onto the LC-MS system.

Note: Ideally, the sample injection sequence should be randomized. After the last sample, inject again blank, QC mix, and positive control to verify the performance. Injection of pure methanol between samples to check for carryover should be avoided because this may cause an RT shift for some molecules in the next sample. If a carryover check is needed (i.e., injections between experimental samples), it is recommended to use the matrix-adjusted blank because this will not affect the RT of the next sample in the sequence. The sample injection volume can be increased safely up to 5 μL without significant changes in RT. The injection volume should be increased if the quantities of starting materials are below the amounts indicated in the Sample preparation section. We recommend a test injection using one random sample to determine the quantity that does not saturate the MS system. If using the 6495C MS system, avoid signal intensities >10^7 counts per second.

EXPECTED OUTCOMES

Free PUFAs are abundant in serum where they are readily secreted. Among the measured compounds, ~100 are detectable in serum. In tissues, PUFA concentration is much lower, and typically 50–60 compounds are observed. In cell culture medium and cell samples (without any stimulation) ~30 PUFAs can be readily detected.

PUFA metabolites are rich in structural isomers (i.e., molecules with the same molecular weight and very similar MRM fragmentation patterns). Thus, as indicated in the Table S1, the present method detects isomers in the same MRM event; however, depending on the sample, the chromatogram may display multiple peaks that are separated by minor RT shifts (often <10 s). Peak integrations need to be performed very carefully for these metabolites, and if necessary, adequate pure

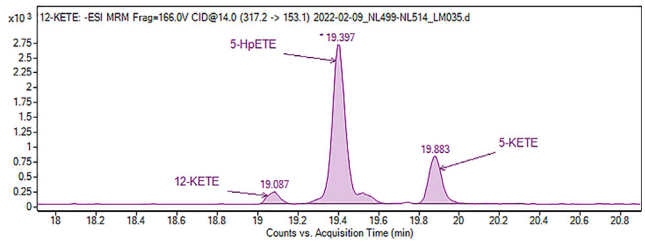


Figure 3. Elution profiles of 12-KETE, 5-HpETE and 5-KETE from liver extracts

standards should be used to establish their exact nature. The following PUFA groups are affected (the order of elution is indicated by ">", from first to last eluting; as an example, see Figure 3):

8-iso-15(R)-PGF2a > 8-iso-PGF2a > 11-beta-PGF2a > PGF2a (11–12.5 min).

8-iso-PGE2 > PGE2 > PGD2 (12–13.5 min).

8-iso-PGE1 > PGE1 > PGD1 (12.5–13.5 min).

8-iso-PGA2 > PGA2 > PGJ2 (14.5–15.5 min).

6-trans-LTB4 > LTB4 (15.5–16.5 min).

19-HETE > 20-HETE > 18-HETE > 17-HETE > 16-HETE (17.5–19 min).

13-KODE > 9-KODE (18.5–19.5 min).

12-KETE > 5-HpETE > 5-KETE (19–20 min).

> ***Note:*** The thromboxanes TXB1, TXB2, TXB2-d4 and TXB3 display peak tailing caused by tautomerism (Figure 4).

Following sample measurement, raw data are processed using the MassHunter analysis software (or similar) that integrates the respective peaks and provides areas for each detected compound. Figure 5 shows examples of peak integration (area calculation) for four compounds (13-HODE, 13-KODE, AA, and eicosapentaenoic acid).

In the frame of the present protocol we have analyzed fresh livers from mice. Table 3 outlines the compounds that were identified and quantified in those samples (see also Table S3).

Absolute quantification of selected molecules is also possible provided a calibration curve has been constructed. Figure 6 shows some examples of calibration curves. Additional examples are given in the Figure S1.

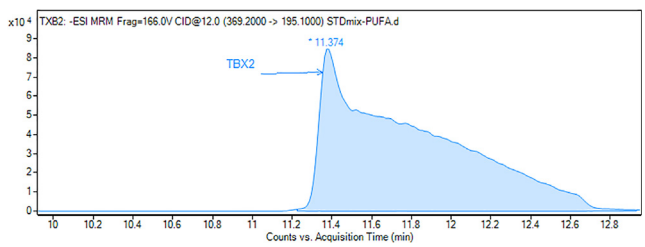


Figure 4. LC-MS chromatogram obtained with the pure standard mixture of PUFAs containing TXB2

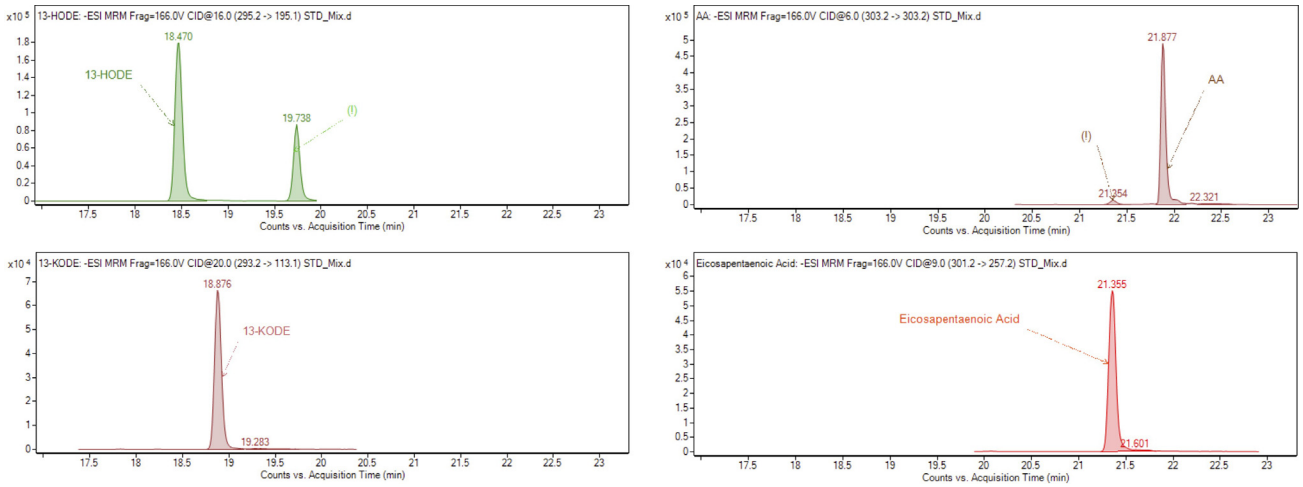


Figure 5. Integration of quantifier transitions for the selected compounds

The area under the curve of the peak is proportional to the concentration and is used to generate the calibration curves. Note the existence of unspecific, smaller peaks (marked with "!").

Using the calibration points, a linear or quadratic equation can be established in a given concentration range. This curve outlines the relationship between area of the signal and its quantity for a specific metabolite.

Example: The 13-HODE calibration curve linear equation is y = 89629x + 363.7 (Figure 6), where y is the peak area of 13-HODE and x is the concentration in μM. In Table 3, for 13-HODE, we measured an average peak area of 1434.267. When this value is inserted in the equation, it gives: x= (1434.267–363.7)/89629 and thus, a value of 0.012 μM.

To illustrate the analysis of different conditions in a typical metabolomics experiment, we have re-analyzed the previously published[4] PUFA data using MetaboAnalystR package and PUFAMetaboR script. Our test dataset contains the results of the PUFA analysis in breast cancer cell xenograft samples in which the *MYOF* gene was silenced (shMYOF) or not (negative control; shNT). This dataset includes five biological replicates per condition.

For details on the script and to download the test dataset see: https://github.com/DirtyHarry80/PUFAMetaboR.git. Briefly, ISTD normalized data were loaded in PUFAMetaboR. "`Normalization`" parameters were set to: "QuantileNorm", "NULL", "AutoNorm". Following data normalization, the Grubbs' test showed that the shNT4 replicate behaved as an outlier. Therefore, it was removed from the analysis. The remainder of the script contained standard parameters as described in MetaboAnalystR tutorials.

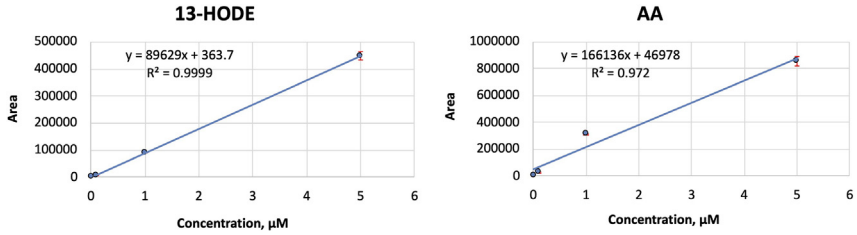


Figure 6. Examples of calibration curves for selected PUFAs in the low μM range

Raw data and calibration curves for 13-KODE, EPA and DHA are provided in the Figure S1.

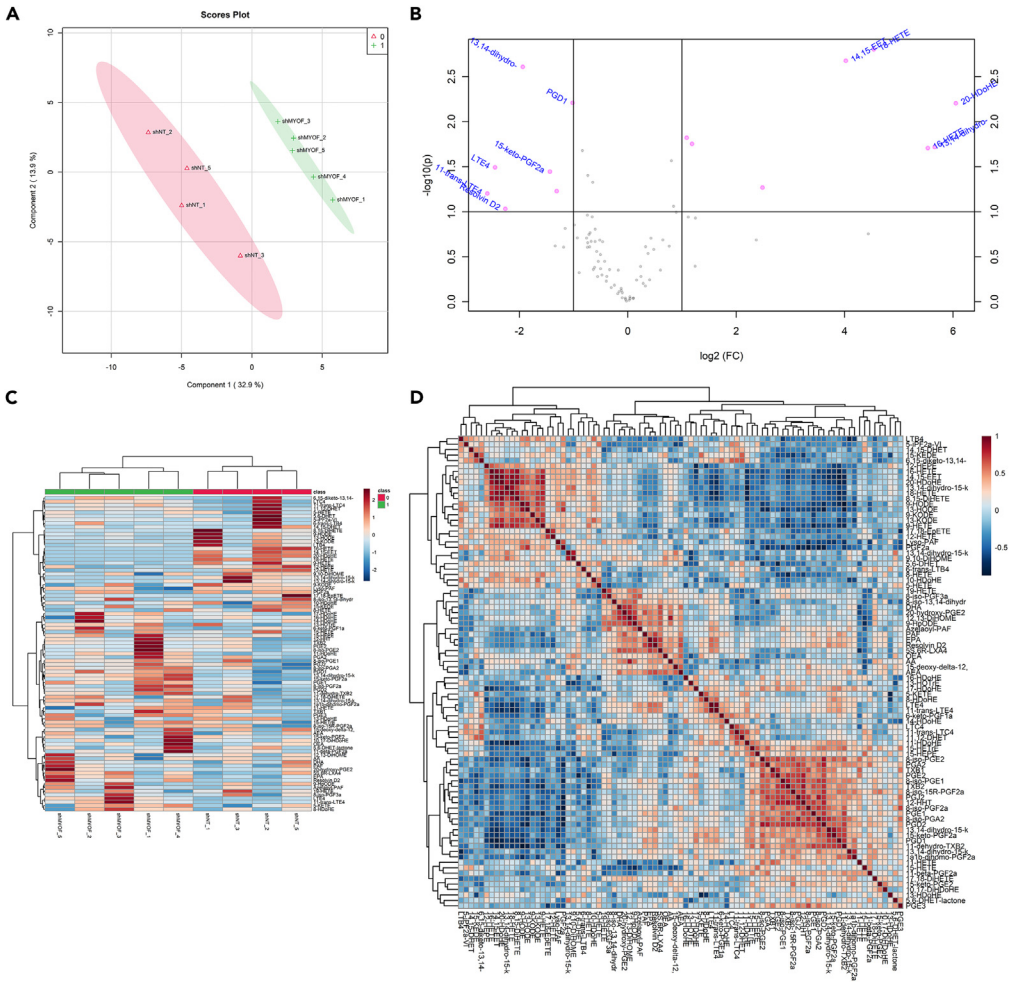


Figure 7. Example of data analysis using the MetaboAnalystR package and the PUFAMetaboR script

Displayed are PUFA quantification data for MDA-MB231 cancer cell-derived xenografts, following myoferlin (*MYOF*) gene silencing using short hairpin RNAs (shMYOF). For details see Blomme et al.[4]

(A) PLS plot outlining the control (shNT) and shMYOF tumors.

(B) Volcano plot showing PUFAs with at least 2-fold changes in concentration between conditions (shNT and shMYOF).

(C and D) (C) Heatmap and (D) Correlation matrix of all compounds in the dataset.

MetaboAnalystR can offer a wide variety of graphical representations. In our opinion, the partial least squares regression (PLS) plot, volcano plot, heatmap and correlation matrix are the most useful. Figure 7 below outlines the plots generated using the PUFAMetaboR script and the test dataset.

The PLS plot shows a clear distinction between the individual experimental conditions, indicating the presence of differences between sample groups (shMYOF and shNT). Following *MYOF* silencing, the concentration of several PUFAs showed variations above the 2-fold change. The heatmap and correlation matrix are particularly interesting. The heatmap revealed clear clustering of samples in two groups. The metabolites also were divided in two major clusters (one group of up- and another group of down-regulated PUFAs in shMYOF samples). The correlation matrix showed structures (clusters) of highly correlated (red) and anti-correlated PUFAs (blue). The PUFAs in these clusters behaved similarly in all shMYOF samples, suggesting a common functional relevance.

QUANTIFICATION AND STATISTICAL ANALYSIS

Basic data analysis

For relative quantification, the peak areas obtained for each PUFA are first subtracted from the blank measurement and then divided by the area of the ISTD that corresponds to the Compound Group to which that PUFA had been assigned. This is the normalized area value that can then be averaged among replicates and used to compare individual samples in a relative fashion. If reporting small numbers with decimals is an issue, these values can be scaled across all samples by multiplying them by a specific factor (e.g., 1000).

If absolute quantification is required, separate measurements of standards and their dilutions can be performed with the same method. For selected compounds, calibration curves need to be made.

Nevertheless, some aspects need to be clarified beforehand to choose the type of absolute quantification modality. The following two modalities are the most frequently used: semiquantitative or absolute method (described below).

Semiquantitative modality (used here): The external calibration curve is made using non-isotopically labeled standards that are diluted in methanol to provide different concentrations. They are not subjected to SPE sample preparation and are separately measured by LC-MS. Curves are fitted to the calibration points that describe the relationship between analyte area and concentration. Equations describing the curves are then used for calculating the quantities. Examples are provided in Figure 6 and in Figure S1, where the calibration curves for selected PUFAs in methanol are shown. The absolute quantities are reported in Table 3.

> *Note:* In this setting the ISTD can be used to correct the areas between samples. By choosing one sample as reference, the ISTD ratios of all other samples relative to this reference sample will give correction coefficients that can be used to correct the areas between samples. Spiking ISTD in the external calibration curve is not useful because due to the lack of sample complexity and matrix, the ISTD signal intensity will be different compared to the actual matrix loaded samples.

Absolute modality: The calibration curve is made using isotopically labeled standards that are spiked at different concentrations directly in the calibration samples. The calibration samples are ideally created from a pool of individual samples used in the study. For each sample (e.g., after sample crushing using methanol), a small aliquot is transferred in a separate tube and then all tubes are pooled. Then, this pool is divided into individual tubes, the number depending on the calibration points. After spiking of the isotopically labeled standards, samples also are spiked with ISTD (described above) and then processed for PUFA extraction using SPE columns. After the LC-MS analysis, the analyte area to ISTD area ratio is used to build the calibration curve (rather than just the analyte area as done in the semiquantitative approach).

Comparison across multiple conditions

Typical metabolomics experiments contain different conditions (e.g., treated/untreated) that are to be compared. The sample groups also include different biological replicates. Therefore, a single normalization using only ISTD is not sufficient, because additional (e.g., biological and physical) effects come into play, modifying the actual metabolite intensities. An extensive overview of different normalization methods for metabolomics can be found elsewhere.[7] A simple yet effective manner to normalize samples in such biological experiments is to blank-subtract and ISTD-correct the peak areas of each detected PUFA (as described in basic data analysis section), followed by the final normalization (division) to the median, upper quantile or total signal intensity of all metabolites detected in the respective sample. These types of analyses are most efficiently done using dedicated R

Table 4. Example of data table format (here containing only two PUFAs) that is usable with the Metaboanalyst R script, as outlined in this manuscript

	CTRL1	CTRL2	CTRL3	CTRL4	Treat1	Treat2	Treat3	Treat4
Label	0	0	0	0	1	1	1	1
TXB3	6.75E-06	2.18E-06	6.28E-06	6.70E-07	1.49E-06	0.0003668	0.00030319	0.0001274
PGE2	4.01E-06	6.54E-06	4.63E-06	1.50E-06	8.52E-07	6.2404E-06	5.46E-06	2.43E-06

Especially important are the first two rows of the table and the first column that need to be kept as indicated. "Label" indicates the grouping of samples into conditions (for more than two conditions, use label value 2, 3, etc.). Other table formats are possible; however, they will require script adjustments of the "`Read.TextData`" function (for details see https://www.metaboanalyst.ca/home.xhtml).

language packages, for example MetaboAnalystR.[8] To provide an example on how to use MetaboAnalystR, we deposited an R script and a test dataset in the public repository GitHub, accessible at the following address: https://github.com/DirtyHarry80/PUFAMetaboR.git.

> *Note:* To be usable in MetaboAnalystR, data need to be saved as tab-delimited text files (decimals separated by point) and must follow a number of criteria. The R code lines as outlined in the PUFAMetaboR will only work with the following data table format (Table 4; measurements are in columns, "Label" indicates the condition group):

Depending on the type of data pre-normalization performed, the function "`Normalization`" needs different parameter adjustments (see MetaboAnalystR User's Manual). Finally, PUFAMetaboR has an integrated outlier test based on Grubbs. This permits to quickly identify if any of the replicates are outliers and hence to remove them from further analysis.

LIMITATIONS

This protocol requires relatively high amounts of starting material, which for some experiments (particularly small organs or precious cell lines) is a limiting factor. To improve this, the column diameter can be reduced from 2.1 to 1 mm. However, the column used here is not available in diameters smaller than 2.1 mm. The modified flow rates using other columns will require re-adjusting the RT and source parameters. Lastly, the present protocol is adapted for the analysis of PUFAs and its applicability to mono-unsaturated fatty acids (MUFAs) or saturated fatty acids (SFAs) has not been determined yet. A starting point for this evaluation could be the extraction and the elution step on the SPE columns. Methanol (used here) may not be sufficiently hydrophobic to extract/elute MUFAs and SFAs. Thus, it could be replaced with acetonitrile for extraction/elution. These modifications should be tested in future works.

TROUBLESHOOTING

Problem 1

It is difficult to filter samples through the SPE cartridge.

Potential solution

Verify the presence of particulate matter in the sample and if necessary, perform a centrifugation step before loading the sample into the SPE cartridge.

Problem 2

PUFA results are very variable among replicates.

Potential solution

High PUFA variability can have technical and/or biological causes. If the ISTD spiked in the sample do not show any variability, then the reason for the variation is probably of biological nature. Check that the time between sample collection and crushing in methanol was as short as possible and that

the sample has been stored properly (at −80°C) before use. Finally, consider increasing the number of biological replicates (5 are considered as the minimum requirement).

Problem 3

No flow from the HPLC (backpressure is < 10 bar, no liquid coming out of the capillary that connects the HPLC to the MS).

Potential solution

Check for the presence of air bubbles in the tubing from the bottle to the selection valves. Try purging the pumps. If the problem persists, check the solvent filters, remove them and test without them (regular cleaning in a sonication bath is necessary). Check for leaks between the bottle compartment and the pump.

Problem 4

High pressure in the system (e.g., 10% or more than 270–280 bar, which is usual for this column at 0.25 mL/min, 90% solvent A and the used HPLC system).

Potential solution

Column or/and capillaries could be clogged. Start checking on the MS side by removing gradually the capillaries that connect to the source and controlling the pressure. At each step one may usually see a drop of few bars if the capillary is not clogged. Proceed to remove the column. If the pressure is still high, clogging might be present until the pump. If the pressure drops significantly (pressure with no column attached should be around 30 bar) the column might be clogged. Proceed to wash the column as described in the Problem 6 below for 16 h (overnight). If an overnight wash does not give satisfactory results, a backflush might be the last attempt to save the column. To backflush the column: i) disconnect the column and reverse the flow direction, ii) connect the column-end to the waste directly, iii) inject a 1:1:1 mix of isopropanol : acetonitrile : water at 45°C for 30 min (0.15 mL/min), and iv) reconnect the column in the correct flow direction and use the initial method settings (90% A).

Note that if using a column pre-heater, this also is a likely place where deposits can block the liquid flow and cause high pressure. Unmount the pre-heater and column and sonicate them separately in methanol/water : 50/50 mix under a fume hood for 20 min.

Problem 5

No signal or poor signal in the MS.

Potential solution

Narrow down the possible source of the problem by checking the tune solution on the MS. Check that the tune criteria set by the instrument vendor are met. This will exclude any issue with the MS instrument. If the tune criteria are not satisfied, consider cleaning the instrument, particularly the spray unit in the source, cones and transfer line. Refer to the instrument User Manual for the exact procedure.

Problem 6

The blank sample shows significant signal.

Potential solution

Run the analysis using a methanol sample. If still positive, the cause is a needle contamination, or most probably the analytical column needs cleaning. Prepare a mix of 1:1:1 of isopropanol : acetonitrile : water, purge pump A, and inject the mix in the column at 0.1 mL/min, 40°C oven temperature, for 16 h. Change the autosampler needle wash solution, perform a wash, and purge. Re-inject at least 2–3 methanol samples, run the complete gradient (make sure that solvents A and B are

properly set). Repeat the measurement of the blank sample. If the methanol sample is negative and blank is still positive, check the sample preparation procedure: the used glassware and solvents may need cleaning/change. If the methanol sample continues to be positive, proceed to column back-flush (invert the column flow and inject isopropanol : acetonitrile : water 1:1:1 mix at 40°C oven temperature for 30 min, 0.2 mL/min) because some contaminants may have accumulated in the column head (if this occurs frequently, use an appropriate pre-column mounted on the analytical column).

Problem 7

QC shows RT shift.

Potential solution

Consider changing the HPLC solvents (they should not be older than 1 week). If the problem persists, check the pump volumetry and whether the indicated flow rates correspond to the actual ones. Dead volumes in the connections after the column also can be a source of problems with the chromatogram. Particularly, the dead volume can lead to peak broadening and loss of separation when many compounds are eluted in a short time frame (e.g., Figure 3). Check for dead volume presence (at the junctions of two capillaries, of capillary and valve, of capillary and column) by verifying the connections of the capillaries after the column. Check the pressure curve (during the run) compared with previous injections (e.g., instability, pressure increase). If the pressure is unstable, purge the pumps. If the problem persists, consider changing the column because it may have deteriorated (a column of this type should usually last for 800 injections).

Problem 8

Few PUFAs are identified in the sample.

Potential solution

- (internal standards are present); Consider increasing the amount of starting tissue material. When dealing with cell lines, at least 1×10^6 cells are required. Concentrating the sample after the PUFA Elution step also is an option. Do not inject more than 5 μL in the column because the sample contains 100% methanol that will lead to RT shift of PUFAs or tailing.
- (internal standards are not present); Consider checking carefully the PUFA purification steps. Verify that the protocol was followed correctly and ensure that PUFAs are not exposed to air by, for example, letting the SPE column dry while purifying the sample (Figure 2). Check that the flow rate of the sample or of the washing/eluting solutions through the SPE column is as low as indicated in the CRITICAL section of PUFA purification protocol (1 drop/sec).

Problem 9

Fewer or additional peaks are visible for a given compound.

Potential solution

For some PUFAs in the protocol, multiple peaks are normal and indicate different compounds, as suggested in the "expected outcomes" section. However, several unexpected peaks may be visible for some compounds (as shown for the peak marked with "!" in the 13-HODE panel of Figure 5). In this event, if no RT shift is observed for the QC sample, the peak at the expected RT should be considered as valid. If no peak is present at the expected RT, but additional peaks are present close by, proceed to check the RT. This is done by spiking the expected compound as pure standard in the sample and by re-injecting the sample (use a small aliquot of the actual sample). The peak the intensity of which significantly increases is the correct compound.

Problem 10

Peak tailing/shoulder is observed for some compounds.

Potential solution

In this protocol, some highly polar PUFAs will tend to peak-tailing (e.g., tetranor-PGEM, tetranor-PGDM, tetranor-PGFM, 20-hydroxy-PGE2, 20-hydroxy-PGF2α). If these peaks become difficult to integrate, a specific injection sequence can be performed in which water is co-injected with the methanol sample. Try co-injecting different volumes of water (5–15 μL) with the sample to improve the results. In the co-injection mode, the sample loop is first filled with the sample followed by water from a dedicated vial. Then, the whole liquid in the loop is injected into the column.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources (e.g., MS method file) and reagents should be directed to and will be fulfilled by the lead contact, Andrei Turtoi (andrei.turtoi@inserm.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The dataset supporting the current study has been included in Tables 3 and S3. The code used to create Figure 7 and the test dataset were deposited in the public repository GitHub, accessible at the following address: https://github.com/DirtyHarry80/PUFAMetaboR.git. The instrument specific raw data are available from the corresponding author on request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102226.

ACKNOWLEDGMENTS

The authors wish to acknowledge French Ministry of Higher Education and Research for supporting the Platform for Translational Oncometabolomics through a CPER grant (IBDLR).

AUTHOR CONTRIBUTIONS

Conceptualization, A.T.; Methodology, E.T., J.J., G.V., I.K.V.; Investigation, E.T., J.J., G.V., I.K.V., N.L., C.E., A.T.; Formal Analysis, E.T., A.T.; Writing – Original Draft, E.T., A.T.; Writing – Review & Editing, J.J., G.V., I.K.V., N.L., C.E., A.T.; Funding Acquisition, A.T., C.E.; Resources, N.L., A.T.; Supervision, N.L, C.E., A.T.

DECLARATION OF INTERESTS

J.J. is employed by Agilent France, the vendor of the LC-MS system used in the present protocol.

REFERENCES

1. Vila, I.K., Chamma, H., Steer, A., Saccas, M., Taffoni, C., Turtoi, E., Reinert, L.S., Hussain, S., Marines, J., Jin, L., et al. (2022). STING orchestrates the crosstalk between polyunsaturated fatty acid metabolism and inflammatory responses. Cell Metab. *34*, 125–139.e8.

2. Costanza, B., Turtoi, A., Bellahcène, A., Hirano, T., Peulen, O., Blomme, A., Hennequière, V., Mutijima, E., Boniver, J., Meuwis, M.A., et al. (2018). Innovative methodology for the identification of soluble biomarkers in fresh tissues. Oncotarget *9*, 10665–10680.

3. Blomme, A., Van Simaeys, G., Doumont, G., Costanza, B., Bellier, J., Otaka, Y., Sherer, F., Lovinfosse, P., Boutry, S., Palacios, A.P., et al. (2018). Murine stroma adopts a human-like metabolic phenotype in the PDX model of colorectal cancer and liver metastases. Oncogene *37*, 1237–1250.

4. Blomme, A., Costanza, B., de Tullio, P., Thiry, M., Van Simaeys, G., Boutry, S., Doumont, G., Di Valentin, E., Hirano, T., Yokobori, T., et al. (2017). Myoferlin regulates cellular lipid metabolism and promotes metastases in triple-negative breast cancer. Oncogene *36*, 2116–2130.

5. Turtoi, E., Jeudy, J., Henry, S., Dadi, I., Valette, G., Enjalbal, C., and Turtoi, A. (2023). Analysis of Polar Primary Metabolites in Biological Samples Using Targeted Metabolomics and LC-MS. STAR Protocols. https://doi.org/10.1016/j.xpro.2023.102400.

6. Dolan, J.W. (2006). The role of the signal-to-noise ratio in precision and accuracy. LC GC Eur. *19*, 12–16.

7. De Livera, A.M., Sysi-Aho, M., Jacob, L., Gagnon-Bartsch, J.A., Castillo, S., Simpson, J.A., and Speed, T.P. (2015). Statistical methods for handling unwanted variation in metabolomics data. Anal. Chem. *87*, 3606–3615.

8. Pang, Z., Chong, J., Zhou, G., de Lima Morais, D.A., Chang, L., Barrette, M., Gauthier, C., Jacques, P.É., Li, S., and Xia, J. (2021). MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights Nucl. Nucleic Acids Res. *49*, W388–W396.