Université catholique de Louvain

Louvain Drug Research Institute

Bioanalysis and Pharmacology of Bioactive Lipids Research Group

SETTING UP, VALIDATION AND APPLICATION OF LIPID QUANTIFICATION METHODS: A POWERFUL TOOL TO STUDY INFLAMMATORY DISEASES

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THE EXAMPLES OF PROSTAGLANDINS AND SHORT CHAIN FATTY ACIDS

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JURY COMPOSITION

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LIST OF ABBREVIATION

abbreviation	meaning
2-AG	2-arachidonoylglcyerol
2-MeBA	2-methylbutyrate
2-MePA	2-methylpentanoate
3-MePA	3-methylpentanoate
3-NPH	3-nitrophenylhydrazine
4-MeVA	4-methylvalerate
AA	arachidonic acid
ACN	acetonitrile
AEA	anandamide
AUC	area under the curve
BA	butyrate
BALF	bronchoalveolar lavages fluid
BCFA	branched chain fatty acids
BHT	butylated hydroxytoluene
CA	citrate
CCM	citrate cycle metabolites
CE	collision energy
CH_2Cl_2	dichloromethane
$CHCl_3$	chloroform
CNS	central nervous system
COX2	cyclooxygenase 2
$cPLA_2$	cytosolic phospholipase A_2
CV	cone voltage
DOE	design of experiment
DSS	dextran sodium sulfate
EDC	N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide
EDTA	ethylenediaminetetraacetic acid
FID	flame ionization detector
GC	gas chromatography
GPCR	G protein-coupled receptors
HA	hexanoate

HETE	hydroxyeicosatetraenoic acids
*i*BA	isobutyrate
IBD	inflammatory bowel diseases
iPOH	isopropanol
*i*VA	isovalerate
KB	ketone bodies
LA	lactate
LC-MS	liquid chomatography coupled to mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
LPS	lipopolysaccharides
MA	malate
MeOH	methanol
MRM	multiple reaction monitoring
MS	mass spectrometry
NMR	nuclear magnetic resonance
PA	propionate
PG	prostaglandin
PG-EA	prostaglandin ethanolamide
PG-G	prostaglandin glycerol ester
PyA	pyruvate
Q-TOF	quadrupole time of flight
S/N	signal to noise
SA	succinate
SCFA	short chain fatty acids
SPE	solid phase extraction
TLC	thin layer chromatography
UHPLC	ultra-high-performance liquid chromatography
VA	valerate
αKA	α-ketoglutarate
βHA	β-hydroxybutyrate

Table des matières

INTRODUCTION

Part I. Lipids – diversity of classes

I.1 – Lipid definition and classification

The definition of a "lipid" differs depending on the domain of activity. When in physics we will talk about "any substance insoluble in water", in chemistry we will describe them as "any molecule presenting a hydrocarbon backbone" and in biology we might refer to "a membrane constituent able to serve as energy source for the cells". Since the first attempts at defining lipids [1], there has been an important evolution in their study, notably due to the realization of the large molecular diversity of this class of molecules [2-4].

The study of lipids gained further interest with the emergence of the concept of "bioactive lipid" [5]. Indeed, besides being an energy source and an energy storage form [6], it is now well established that many lipid levels respond to pathophysiological stimuli and can activate downstream pathways [5, 7, 8]. This translates in an increasing number of reports showing a link between lipid species and biological activities. Moreover, new lipid species continue to be described in the literature, even in recent years [9-13]. This large and increasing number of lipid species support the need of a complete lipid classification.

In 2005, Eoin Fahy et al. within the "international lipid classification committee" established a consensus classification system (updated in 2009 and 2020) based on the general structure of the lipid as well as the functional moieties that could be present on the lipid backbone [14-16]. The evolution in lipid classification led to the creation of classes and subclasses of lipids [17-21]. Moreover, synthetic lipids or bacteria-guided synthetized lipids appear in the classification. For instance, several water-soluble molecules (thus in opposition with the common definition of a lipid) possessing a hydrocarbon backbone and presenting different physiochemical properties have been synthesized (e.g. premonensin analogs) [22-24]. The last

update applied to LipidMaps® aimed to fine tune the classification subclasses by adding more information on the functional groups' diversity, going from the type of atom (e.g. iodide, bromide, …) to the type of carbohydrate structures [16]. This "international lipid classification committee" determined eight principal lipid classes described as (1) fatty acyls, (2) glycerolipids, (3) glycerophospholipids, (4) sphingolipids, (5) sterol lipids, (6) prenol lipids, (7) saccharolipids and (8) polyketides [14]. I will briefly summarize below their main characteristics.

I.1.1 – Fatty acyls

The term fatty acyl gathers all the compounds presenting an acyl chain consisting in the repetition of methylene groups (going from 1 to more than 27 carbons) as well as a functional final group **(figure 1)**. The diversity inside the family is due to the presence of modifications on the acyl chain. These modifications can be unsaturations, chemical substitutions (such as a hydroxyl or a methyl group), cis/trans conformation, as well as the presence of a linker inside the acyl chain [25-27].

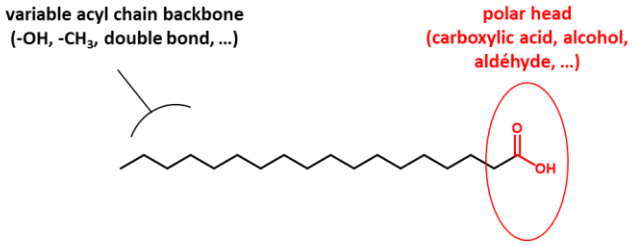


Figure 1: general structure of the fatty acyls

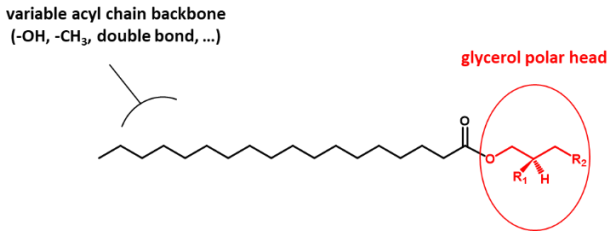
The biological source of these lipids is mainly the dietary fat assimilation (as such or following the degradation of more complex acylated lipids). Firstly, fatty acyls coming from the diet are mainly middle chain fatty acids such as linoleic acid or palmitic acid, often present in alimentary oils or in nuts [28-30]. They can be quickly assimilated during the digestion process

and can then play several roles in the body [31, 32]. Fatty acyls can also be obtained through several synthetic pathways inside the body and mobilised from more complex lipids [33, 34]. This mobilisation can occur to maintain the energy balance of the body, but also when signalling lipids are produced [34-36].

For instance, several fatty acyls (e.g. arachidonic acid) can enter in synthetic pathways leading to the formation of whole fatty acyl families such as the hydroxyeicosatetraenoic acids (HETE), the hydroxyoctadecadienoic acids (HODE) or the well-known prostaglandins (PG) [37, 38]. These newly synthetized lipids have numerous roles, typically during inflammation [39-41]. Moreover, reports emerged to link the fatty acyl lipids to cardiovascular diseases [42], inflammatory bowel diseases [43], cancer [44], neurological disorders [45, 46], …

I.1.2 – Glycerolipids

The glycerolipid lipid class consists in all the glycerol-containing lipids, with the exception of the glycerophospholipids that are regrouped in a distinct class **(figure 2)**. Triglycerides, for which the three hydroxyl of the glycerol are linked to an acyl chain, are typical glycerolipids.



compound type	R_1	R_2
monoacylglycerols (MAG)	-OH	-OH
diacylglycerols (DAG)	-acyl chain	-OH
alkylacylglycerols	-alkyl chain	-OH
alkenylacylglycerol	-alkenyl chain	-OH
diacylglycerol glycans	-acyl chain	-glycan
triglycerides (TG)	-acyl chain	-acyl chain

Figure 2: general structure of the glycerolipids

However, there is a wide diversity of mono-, bi- and trisubstituted glycerolipids depending of the substituent present at the hydroxyl positions. For example, glyceroglycans contain a sugar moiety, while the caldarchaeol are cyclic lipids made of two disubstituted glycerols joined by two acyl chains.

Glycerolipids are found in the adipose tissue where triglycerides are abundant [47]. Moreover, monoacylglycerols and diacylglycerols are often implicated in endogenous metabolic pathways and lead to the formation of a wide diversity of lipids [48, 49].

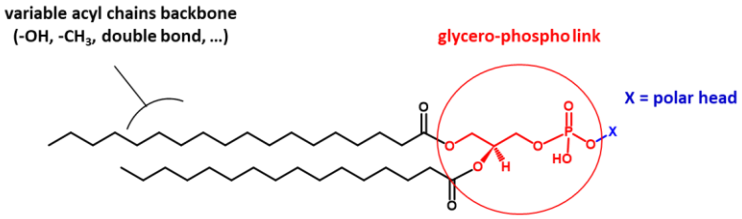
Besides their role as energy storage (i.e. triglycerides), glycerolipids can also plays several roles in health and disease thus their metabolic enzymes are often targeted as potential therapeutic approach. Their roles range from the control of glycemia [50] to inflammation [51], or cancer [52]. Triglycerides have also been involved in the initiation of cardiovascular diseases [53]. Moreover, a part of the effects observed with glycerolipids are linked to their ability to serve as precursors of several other lipids such as the acyl lipids described above.

I.1.3 – Glycerophospholipids

This class of lipids is similar to the glycerolipids but presents a phosphatidic moiety serving as a link between the acyl chain(s) and a polar head. The polar head can be for instance an ethanolamine, an inositol or a choline in the case of the phosphatidylcholines **(figure 3)**. This class of lipids is important in term of abundance (part of the most abundant lipids) but also in term of diversity (important number of acyl chains combinations). Some differences also exist between mammalian and bacteria or yeast species.

Glycerophospholipids are the main cell membrane lipid component. The nature and proportion of the acyl chains can differ between cells and species [54-56]. While 16:0, 18:0, 18:1 and 20:4 acyl chains are the most abundant in mammalian, we can find in other species some acyl chains (such as 15:0 or 17:0) usually not present in mammalians. Those differences are linked to the diversity of enzymes between species [57].

Similarly to the acyl chain diversity, polar head nature and membrane abundance can also differ between cells. Moreover, the mono acylated glycerophospholipids are called lysophospholipids, a subclass of glycerophospholipids representing a wide variety of lipids.



compound type	polar head
phosphatidic acids (PA)	/
pyrophosphates	phosphate
phosphatidylcholines (PC)	choline
phosphatidylethanolamines (PE)	ethanolamine
phosphatidylserines (PS)	serine
phosphoglycerophosphates	glycerophosphate
phosphatidylglycerol (PG)	glycerol
phosphatidylinositol (PI)	inositol
cardiolipines	other glycerophospholipid
phosphoinositolglycans	inositol(poly)glycan(s)

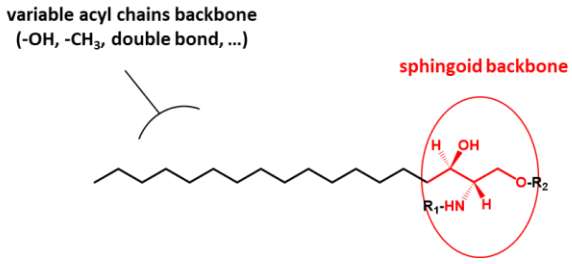
Figure 3: general structure of glycerophospholipids

In recent years, lysophospholipids and glycerophospholipids gained in interest due to their ability to bind several receptors, leading to several downstream effects. For example, lysophosphatidylinositols are described to bind to G protein-coupled receptors such as the GPR55 [58, 59]. The understanding of the role of this family of lipids progressively evolved due to an increasing number of studies that showed their ability to bind to several receptors [59] or to exert biological functions [60-63]. Besides acting on receptors, glycerophospholipids are also involved in lipid raft formation within cell membranes. Studies have also shown that the composition of the lipid raft (phospholipid nature, presence of others lipids such as the ceramides or cholesterol) could influence its function [64].

I.1.4 – Sphingolipids

The lipids constituting the sphingolipid class are characterized by a sphingoid backbone linked to one (i.e. sphingosines) or two (i.e. ceramides) acyl chains **(figure 4)**. Even if in mammals the 18:1 acyl chain is predominant on one of the two chains, variations exist between species. Moreover, depending on the presence of unsaturations on the acyl backbone, other derivatives exists (i.e. dihydroceramides). Finally, several sphingolipids have one or several

sugar moieties linked to the sphingoid moiety. This is the case for the galactosyl (or glucosyl) cerebrosides as well as for the gangliosides (having two or more sugar moieties).



compound type	R_1	R_2
sphinganines/ sphingosines	-H	-H
sphingosine-1-phosphate	-H	-phosphate
ceramides	-acyl chain	-H
sphingomyelins	-acyl chain	-phosphocholine
phosphonosphingolipids	-acyl chain	-2-aminoethylphosphonate
glycosphingolipids	-acyl chain	-glycan
gangliosides	-acyl chain	-(poly)glycans
sulfosphingolipids	-acyl chain	-sulfoglycans

Figure 4: general structure of the sphingolipids

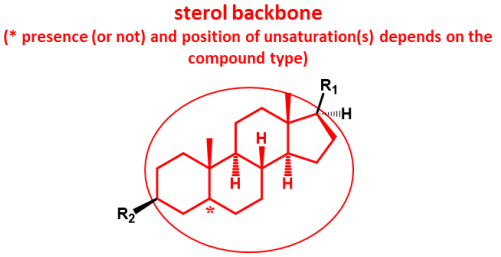
Sphingolipids in general (sphingosines, ceramides, sphingomyelins, gangliosides,…) were first isolated from the brain [65]. They are major constituents of the central nervous system where they are highly present in the myelin sheet. In the cell membranes, they are important for cell-to-cell interaction and in vesiculation processes [5].

Due to their high content in the central nervous system, they play numerous physiological roles in the brain such as the myelin sheet maintenance [66] or the synaptic communication integrity [67]. Ceramides are also linked to neuroinflammation or even peripheral inflammation [68-70]. Sphingolipids are also involved in several processes related to cancer such as cell invasion and migration [71, 72]. Ceramides are also important in the skin where they contribute to the impermeabilization and have antioxidant functions [73, 74].

I.1.5 – Sterol lipids

The sterol class mainly derives from cholesterol metabolism in mammalians. It is characterized by a sterol nucleus, consisting in three cyclohexane units and one cyclopentane unit, and a side chain **(figure 5)**. Moreover, besides cholesterol derivatives, several sterols derivatives with unique structural characteristics can be found in plant, fungal or even algae sources. As

several positions of the sterol backbone can bear a substituent, there is an important diversity of molecules in this class of lipids. Although most of the compounds are derived from cholesterol, the diversity in metabolic enzymes results in several families of sterol such as oxysterols, steroid hormones, bile acids, vitamins, glucuronides, …



compound type	R_1	R_2
cholesterol	C_8H_{19}	-OH
cholesteryl esters	C_8H_{19}	-acyl chain
estrogens (e.g. estradiol)	-OH/=O	-OH
androgens (e.g. testosterone)	=O/-OH	-OH/=O
bile acids	-variable chain	-H
glucuronides	=O	-glycan
sulfosteroides	=O/-OH	-sulfonyl

Figure 5: General structure of the sterol lipids

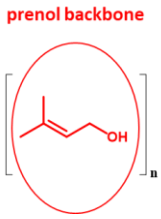
As a membrane constituent, cholesterol (or phytosterols such as the β-sitosterol) is found in a variety of dietary sources from meat to plants and algae. Sterols are easily assimilated during digestion and can be mobilized in several parts of the body. De novo synthesis of sterols is provided from HMG-CoA by the mevalonate pathway.

One of the main roles of cholesterol is to rigidify the cellular membranes [75, 76]. More recently, the ability of cholesterol to form lipid domains at the surface of the cells was described [64]. Sterol lipids are implicated in a broad range of effects, physiologically and pathologically. For instance, oxysterols, the direct metabolites of cholesterol, are linked to inflammation [77-81]. Cholesterol is also a precursor of various key fat-soluble hormones (e.g. sexual hormones) and vitamins such as D vitamin. Bile acids, besides acting as dietary emulsifiers through bile secretion [82], also act as lipid mediators (e.g. by binding to FXR and PXR receptors). Indeed, primary bile acids can bind FXR receptor to lead to several effects in

the digestive system [83, 84]. Besides, it now appears that secondary bile acids are in part responsible for some of the beneficial effects of the microbiota [84].

I.1.6 – Prenol lipids

Originated in mammalians from the mevalonate pathway, this family of lipids consists in the repetition of C_5 prenol units. The differences between prenol lipids come from the number of repetitions, the orientation of the prenol units as well as the terminal substituent of the molecules **(figure 6)**. This lipid class is well represented in plants and bacteria (e.g. carotenoids), while the most known mammalian derivative is retinol (i.e. vitamin A).



compound type	-OH substitution
isoprenoids	n prenols
prenylphosphates	phosphate
ubiquinones/ K vitamin	quinone
bactoprenols	diphospho peptydoglycans

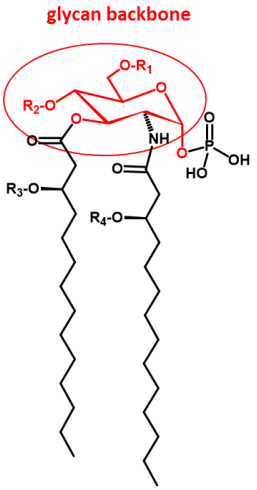
Figure 6: General structure of the prenol lipids

As the mevalonate pathway is a key pathway for cholesterol synthesis (as mentioned above), endogenous synthesis of prenol lipids is possible via a deviation of the cholesterol synthetic pathway. Besides, several vegetal dietary sources of prenol lipids exist where they serve as precursors of triterpene synthesis or as distinct compounds such as β-caroten. Retinol is also obtained from dietary sources in plants.

Even if this class of lipid contains several biologically important molecules, such as vitamin A, our knowledge regarding their roles is more limited compared to other lipid classes. For instance, dolichols are suggested to play a role in the *N*-glycosylation process [85]. Most of the knowledge on prenols is related to their properties in bacteria physiology [86, 87].

I.1.7 – Saccharolipids

This category of lipids refers to glycan derivatives directly bound to acyl chains **(figure 7)**. This class contains all the lipopolysaccharides (LPS)-derived lipids, such as lipid A, that can be present at the surface of bacteria. The large diversity of saccharolipids is due to the fact that besides the diversity of glycans (e.g. glucose, galactose, mannose, …) the different hydroxyl groups present at the surface of glycans can be mono- or polysubstituted by a broad diversity of acyl chains. This large number of isomers in the family leads to the complexity that characterizes LPS derivatives.



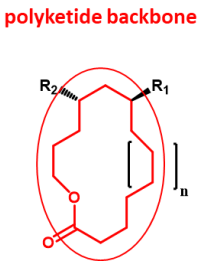
lateral chain	composition
R_1	-H or others glycans
R_2	-H or -phosphate
R_3	-H or acyl chain
R_4	-H or acyl chain

Figure 7: structure of the saccharolipids

These high molecular weight lipids are usually not detected (or present at low levels) in the circulation or tissues in physiological conditions. However, conditions altering the intestinal barrier function (e.g. inflammation, obesity, stress) result in increased LPS levels in the circulation [88, 89].

I.1.8 – Polyketides

Polyketide refers to molecules containing a macrocyclic lactone backbone on which several substituents (e.g. hydroxyl, glycosyl, methyl, …) can be present **(figure 8)**. Typically, these lipids are present in plants, bacteria or fungi where classical fatty acid synthases together with specific polyketides synthases generate a broad range of natural products.



lateral chain	composition
R_1	-OH, -H, -methyl, =O, …
R_2	-OH, -H, -methyl, =O, …

Figure 8: structure of the polyketides

Polyketides are not produced in humans, but they originate from bacteria or plants even if they have low *per os* bioavailability.

I.1.9 - Lipidome

With the important diversity of lipid species, the term “lipidome” emerged in the 21st century [90] and refers to the complete lipid content present in a given biological sample. With untargeted lipidomic analysis (i.e. the quantitative analysis of the lipidome without focusing on a particular lipid family), researchers are aiming at characterizing the entire lipidome of a sample, or rather its variations between experimental conditions. However, despite methodological improvements, given the large variety of structures and the increasingly large

number of lipid species (47718 lipids listed on the LipidMaps® database, figure 9) it is still complex to have the complete lipidic fingerprint of a sample.

Lipid Category	Curated	Computationally-generated	All
Fatty Acyls [FA]	8634	1875	10509
Glycerolipids [GL]	352	7379	7731
Glycerophospholipids [GP]	1737	8327	10064
Sphingolipids [SP]	1793	3168	4961
Sterol Lipids [ST]	3581	0	3581
Prenol Lipids [PR]	2381	0	2381
Saccharolipids [SL]	51	1294	1345
Polyketides [PK]	7146	0	7146
TOTAL	**25675**	**22043**	**47718**

Figure 9: Number of lipid species (per class) listed on LipidMaps® database, 2022 October. Curated: reported in the literature excluding computationally generated structures; computationally-generated: generated using a database covering +30.000 bulk species.

I.2 – Lipid analytical methods: description and limitations

Over the years several analytical strategies have been developed to analyse as comprehensively as possible the lipid content. Linked to the diversity of lipid classes and the number of molecules inside each families of lipids, the analysis of the complete endogenous lipidome - in a limited number of analysis - remains a challenge. This remains true even if novel lipidomic strategies are regularly described. Around eight hundred new articles are published each year discussing of “lipid quantification” (based on PubMed). This tends to confirm the increasing interest for lipid analysis in the recent years.

As I will describe in the following sections, there are several analytical techniques and instruments allowing either the characterization of a whole lipid class or the quantification of specific lipid species. For most approaches, particularly when analysing complex matrices, several steps are needed for sample preparation before analysing the lipid content. An

extraction and/or a purification step will allow the clean-up of complex samples, while maintaining lipid content integrity, as well as concentrate the lipids of interest which can be particularly relevant for the analysis of less abundant species. Among the strategies used, liquid-liquid extraction (e.g. Folch's technique [91] or Bligh and Dyer method [92, 93]) or protein precipitation are common, as are prepurification steps using solid phase extraction (SPE). As it is important to have a good confidence regarding the identified specie (especially given the diversity existing in the structures as well as the number of isomeric structures), the development of new strategies often requires a validation step. Among the different instrumentation techniques available, the sensitivity and the selectivity/specificity vary resulting in different limitations depending on the type of technique applied [94]. Altogether, the set up and the control of those different parameters makes the development of new strategies to quantify lipids challenging.

In the sections below, I will summarize important elements in the different steps of lipid analysis (**figure 10**). As many strategies have been described, in this section I will not be exhaustive, but I will rather focus on key points based on the literature and my own experience.

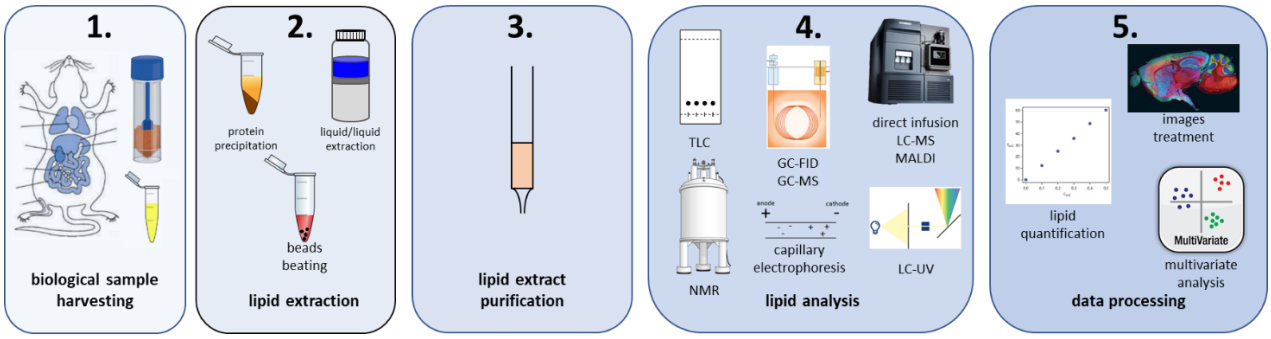


Figure 10: general workflow in lipid analysis

I.2.1 Biological matrices recovery and storage

The first step in lipid analysis is obtaining and storing until its use the sample of interest. Depending of the analysed lipid, it is important to consider several potential issues that can arise upon collection and/or storage.

Firstly, alteration of the lipid content can be fast as in most tissues there are numerous enzymes able to metabolize lipids. Thus, if matrices are not quickly stored under proper conditions, the lipid pool can be affected by fast metabolization. Snap freezing the samples or quenching enzymatic activities using organic solvents (e.g. methanol) before storage should then be recommended.

Secondly, it is important to keep in mind the tissue heterogeneity that can affect the sampling. It is, for example, the case for liver for which vascularisation is different depending on the liver part or for faecal mater in which heterogeneity appears depending on the diet composition and the digestion.

Finally, some researchers reported lipid degradation during process or long-term storage condition, even in extreme cold condition such as -80°C [95, 96]. Thus, ideally, storage before processing should be minimized as much as possible to avoid potential changes in lipid content.

I.2.2 Lipid extraction techniques

Once the sample obtained, most of the time the next important step consists in the extraction of the lipids. This will decomplexify the sample by removing proteins and ideally molecules that are not the target analytes. Moreover, the extraction step often provides a first concentration step crucial for some lipid analysis (e.g. for low level lipids).

Prior to extracting the lipids from a solid matrix, a homogenization is required. Once again, the choice of solvent for the homogenization and the selection of appropriate work conditions are important to minimize early degradation and maximize recovery.

Through degradation processes, some lipid species (i.e. unsaturated fatty acids or cholesterol) are sensitive to oxidation and can therefore be chemically altered [97, 98] in the presence of oxygen or even some solvents. It is also described that several lipids can undergo interconversion into isomeric forms in the presence of specific solvents (e.g. 2-arachidonoylglycerol in the presence of methanol) [99].

Another potential issue is the use of plastic tubes to recover or store the samples. In fact, it is described that many lipids can adsorb on surfaces such as plastic [100]. This can lead to loss of analytes for example when plasma is recovered or transferred. In this context the use of specially treated plastic (e.g. silanized) can help decrease this issue.

Finally, some lipids (e.g. erucamide acetate) can be introduced in the sample during the analytical process because of their presence in the materials (e.g. glass vials, pipettes) and solvents used. Other lipids such as short chain fatty acids (SCFA) are volatile compounds and can easily contaminate samples. For those reasons, it is important to assess the potential contamination level of the studied lipid (for instance by using “blank” conditions, see result section II). All these points should be kept in mind when selecting and optimizing the analytical process.

The easiest way to homogenize biological matrices is the use of cold water as this allows for a homogeneous tissue suspension. For the mechanical disruption of the tissues, a dispersing

element such as the Ultra-Turrax® digital disperser or beads-related techniques can be used [101].

In some cases, other approaches can be used. Firstly, some researchers replace the water by an organic solvent to minimize early enzymatic conversion possible with the use of water. Other modifications, such as acidification of the extraction mixture can be employed to promote the non-ionized form of lipid and improve the extraction yield [102]. Finally, it is also possible to add compounds aiming at protecting the lipid (e.g. from oxidation). This is typically the case for ethylenediaminetetraacetic acid (EDTA), an iron chelator limiting pro-oxidant conditions or the addition of butylated hydroxytoluene (BHT), an antioxidant substance limiting once again oxidation processes [98].

Several lipid extraction and purification steps are listed in **table 1**. The most used extraction technique, but probably the one resulting in less clean samples, is the so-called “protein precipitation” [103-105]. One advantage of this technique is the speed as the homogenized matrix only needs to be supplemented by internal standard (in the majority of cases), centrifuged and can then be analysed as such. This is for example the case in shotgun lipidomic applications. The use of organic solvent will disrupt quaternary and tertiary protein structure to allow their precipitation without affecting the other matrix constituents. The sample will thus contain a wide diversity of lipids. The issue linked to that is the complexity of lipid content and the risk of matrix effect that some abundant lipid such as triglycerides or phospholipids can induce on other less abundant species. Nonetheless, protein precipitation is often used in lipid quantification as first analytical step before applying other preparative techniques.

technique	description	advantages	inconvenients
protein precipitation	sample homogenate + appropriate solvent mixture and physicochemical properties.	- fast technique - recovery of large amount of lipid	- risk of matrix effect - recovery of others non-lipidic substances
Folch's method	homogenize in $CHCl_3$-MeOH (2:1; v/v). Recover the lower organic layer.	- applicable to large amount of tissue - good lipid recovery yield for many lipid species - adjustable to the lipid of interest	- use of important volumes of toxic solvents - need to recover the lower organic layer
Bligh and Dyer's method	homogenize in $CHCl_3$-MeOH (1:2; v/v) and then add $CHCl_3$ and water. Recover the lower organic layer.	- good lipid recovery yield for many lipid species - adjustable to the lipid of interest	- use of important volumes of toxic solvents - need to recover the lower organic layer
Matyash's method	Add MTBE on the sample and then incubate 1h at room temperature. Add water in MTBE-water (4:1;v/v) proportion. Recover the upper organic layer.	- good lipid recovery yield - recovery of some polar lipid species - recovery of the upper organic layer	- only effective for low amount of sample - unadjustable conditions - risk of degradation during incubation
solid phase microextraction (SPME) method	insertion of the SPME canalicule inside the tissue of interest. Use of the coating device to recover sample inside an appropriate injection solvent. Analyse lipids.	- usefull for monitoring studies - animal sacrifice not necessarly required - small volume of solvent used	- only available for low amount of sample - extract less puriefied
solid phase extraction (SPE) method	Load sample on pre-conditionned SPE columns. Wash with appropriate solvent mixture. Recover lipids of interest.	- allow specific recovery of certain species - reduce the matrix charge - allow concentration of sample	- difficult to apply without pre-extraction step - time consuming technique

Table 1: description of the main extraction and purification techniques

In the mid of the twentieth century, two other widely used lipid extraction methodologies emerged. They are known as the Folch's method [91] and Bligh and Dyer's method [92]. Both methods rely on the use of a mixture of chloroform ($CHCl_3$), methanol (MeOH) and water (H_2O). This allows to create three different phases in the mixture with the lower organic phase containing the lipids and other lipophilic molecules (main part of the lipid content of the sample) and the upper aqueous phase containing all the water-soluble compounds such as amino acids or small polar compounds. The interphase is constituted of insoluble cellular debris (e.g. precipitated proteins). These two liquid-liquid extraction methods are among the most used methods in lipid analysis. Some modifications, such as pH variation or solvent proportions changes, can be used to fine tune the conditions in order to focus on specific lipid species [106, 107]. Another possibility is the replacement of $CHCl_3$ by dichloromethane (CH_2Cl_2) to decrease toxicity [108]. One issue with these methods is that less lipophilic lipids can partially or completely partition in the aqueous phase. This is for instance the case with the 25-hydroxycholesterol-3-sulfate we analysed recently at our laboratory [78] showing a preference for the aqueous layer. Nonetheless, both methods are applicable to a broad diversity of matrices and are easy to implement in several lipid analysis methods.

Another liquid-liquid extraction technique developed for lipid analysis is the use of methyl-tert-butyl-ether (MTBE) [109]. In this technique, methanol is first added to the sample and then MTBE is added before mixing. After the addition of water, the upper organic phase containing the lipid fraction is recovered. This method is a good substitution to Folch's and Bligh and Dyer's methods as similar recoveries are obtained for lipid species such as phospholipids and the lipids can be recovered from the upper phase and is therefore more amenable to automatisation. Interestingly, this method uses fewer toxic solvents than the Folch (and Bligh and Dyer) method.

While the above-mentioned methods still represent the main strategies used in the development of new lipid quantification methods [110], other methodologies emerged to allow on-line extraction methods. Methodologies such as the solid phase microextraction (SPME) allow to decrease the issues related to degradation during storage as well as fast enzymatic metabolization related to several lipid species [111-113]. The use of this methodology can also spare time as it allows direct lipid analysis and there is lower need of an additional purification step as small size biological samples are used and lipid species concentrated before analysis. Moreover, this technique requires lower amount of biological sample and facilitates monitoring applications [114]. Nonetheless, as small amount is used and even if matrix effects can be reduced, detection difficulties for low abundant lipids are possible.

I.2.3 Lipid purification techniques

Once the lipid extract is obtained, it is sometimes necessary to add a purification step. In fact, as lipid-containing extracts are often highly concentrated in many compounds, the addition of purification steps provides a reduction in matrix effect or, for less abundant lipids, allows to

concentrate the final extract to achieve a detection. This is also useful to increase instrument life time as routine analysis of concentrated samples is detrimental for the chromatographic columns and mass spectrometers.

The main, and almost only, purification method widely used in lipid analytical methods is solid phase extraction (SPE) [115, 116]. During SPE, the lipid extract is added on a (pre-conditioned) solid phase column consisting in modified (or unmodified) silica or resins particles. The extract is washed and then the purified fraction of interest (i.e. containing the targeted lipids) is recovered. The selection of appropriate SPE stationary phase among the large number of phases available will depend on the lipid species of interest, the extraction method previously used, as well as the type of analytical instrument used to measure the lipid content [116, 117]. Some researchers use commercially available SPE columns when others design and generate specific SPE columns more adapted to the lipid species analysed. It is also possible to use SPE columns directly as extraction and purification step, however the amount of biological sample added to the SPE is a critical element to avoid saturating the columns. For this reason, if SPE is used alone without a pre-extraction step, lower amount of sample is analysed but it remains of high interest in the case of biofluids such as plasma and urine. This strategy fits to clinical applications whereas the use of Folch's technique or MTBE liquid-liquid extraction strategy in clinical routine is not possible due to the longer processing time.

Altogether, those elements make the SPE technique very interesting in the case of the concentrated lipid matrices. Nonetheless, several optimizations are needed to develop methods using this type of technique.

I.2.4 Lipid analysis

Several analytical instruments allow the separation and detection of lipids. Nonetheless, it is very important to keep in mind the limitations of each instrument as well as the purpose of the study to be sure that the selected technique will give useful information. All the technologies differ on several points: (1) specificity; (2) sensitivity; (3) type of information. Moreover, depending on the level of method development, some techniques will give only qualitative information when a full validation will provide quantitative information. However, some instruments are better than others to provide powerful information concerning identity of the lipid species, one of the main challenges being the number and diversity of lipids as mentioned above.

I.2.4.1 Analysis by thin layer chromatography

Thin layer chromatography (TLC) can be used to separate lipid classes. Although it is relatively inexpensive and fast, the interest for this technique decreased over the years. Indeed, one major drawback is the poor resolutive power of this method, even when 2D separations are used. Nonetheless, TLC provides interesting qualitative information about complex samples [118]. In fact, with the emergence of the use of high-performance silica (grafted or no) together with the commercial availability of a diversity of pre-coated TLC plates it is now possible to characterize the nature of the main lipid classes present in a sample. Moreover, coupled with different detectors, with or without the use of visualization reagents, it is also possible to obtain some quantitative information (still only about the lipid classes) of a sample.

I.2.4.2 Analysis by nuclear magnetic resonance

Nuclear magnetic resonance (NMR) is a widely used analytical technique providing qualitative as well as quantitative data after appropriate method validation. With the use of complementary NMR techniques (e.g. ^{1}H and ^{13}C NMR or 2D NMR) isomeric information can be accessible, event in complex samples. Moreover, depending of the type of NMR technique used, it is possible to obtain imaging information in a non-invasive manner [119, 120]. It allows to monitor for example the evolution of tumour environment or the biodistribution of isotopically labelled lipids [121]. Altogether, the different possibilities available in NMR techniques give us an interesting tool to study lipids. The main limitation could be the fact that NMR is less sensitive than other techniques (such as mass spectrometry) which limits its interest for low abundant lipids. Nonetheless, an increasing number of studies describe lipidomic analysis using NMR as analyser [122].

I.2.4.3 Analysis by gas chromatography coupled to specific detectors

Gas chromatography (GC) was considered as a gold standard for a long time for the analysis of fatty acyls derivatives, often after their derivatization into fatty acyls methyl esters (FAMEs) [123]. GC offers an excellent resolutive power, although long runs can be necessary. Besides FAMEs other derivatization methods were also efficiently applied to lipids, for example using trimethyl silane (TMS) or pentafluorobenzyl reagents. Those reagents allow to increase the volatility of compounds exhibiting higher evaporation temperatures, such as lipids, necessary for their analysis by GC. Nonetheless, even with the use of those type of techniques, GC is complex to apply in the majority of cases and the need of using a derivatization method can impact structural information elucidation.

Following GC-based separation, several detectors providing a signal proportional to the amount of lipids and allowing their absolute quantification are available. The two most popular remain the flame ionization detector (FID) and the mass spectrometer detector (MS).

I.2.4.4 Analysis by capillary electrophoresis

Capillary electrophoresis (CE) is an interesting technique in the case of lipids as it brings another separation criterion with the intrinsic charge present on many lipids. This can provide separation between some isomers as the position of the ionisable function can differ on the molecule surface exhibiting differential separation on the system. CE gains in interest at the beginning of the twenty-first century with the development of CE-MS systems combining the high separation capacity of the capillary electrophoresis and the sensitivity and structure information provided by mass spectrometry [124, 125]. However, even if CE is well adapted to proteomic analysis, the switch to lipidomic analysis is not straightforward and some improvements are still necessary to develop global methods in the case of lipids.

I.2.4.5 Analysis by liquid chromatography coupled to mass spectrometry

Nowadays mass spectrometry (MS) coupled or not to liquid chromatography (LC) is considered the most convenient analytical strategy for lipid analysis. The existing technologies allow, for example, the spatial characterization and repartition of the lipids. It is the case with the MALDI interface coupled to mass spectrometry which allows the obtention of high-resolution pictures. Other instruments such as the Orbitrap® or the time of flight (TOF) instruments allow structure elucidation after high fragmentation or lipidome characterization in high resolution mass spectrometers [126, 127]. Finally, tandem and triple quadrupole instruments have high sensitivity capacities allowing the detection and quantification of low

abundant lipids. Tandem (and triple) quadrupole technologies are mostly used for targeted analysis (targeting specific lipid species), while Orbitrap® and TOF instrument are more used for untargeted lipidomic analysis (characterizing several lipid classes in the sample). Even if all those technologies are complementary, the main limitation existing is the high cost linked to this type of instrument. Nonetheless, mass spectrometry allowed to develop efficient lipidomic methods allowing isomeric separation, efficient quantification of numerous lipid species or even high fragmentation procedures to allow structural elucidation.

I.2.5 Data processing and result interpretation

Once raw data are collected, there is a need to normalize, adjust or convert those data to answer to the biological question. Actually, before comparing experimental groups, it is important to pay attention to the potential analytical variabilities. These variabilities can be linked to the operator, the environmental conditions during the experiment (e.g. temperature, moisture, …) or the instrument used. To take into consideration these variabilities, normalization standards are often used. These internal standards will face the same variability-inducing events than the lipids of interest during the analysis. Thus, the analytical signal for the lipid of interest can be normalized to the signal of the internal standard before comparing the conditions. Consequently, it is important to select standards having the same properties than the compounds of interest (molecular form, lipophilicities (or hydrophilicities), physiochemical properties, …) but that are not present in the biological sample. Isotopic forms of the compound of interest (e.g. deuterated forms) are often used but represent additional costs. Once variabilities are smoothed with the normalization step, the groups of interest can be compared.

The first way to use lipid raw data is to compare the signal obtained for both control (or physiological) and treatment (or pathological) conditions. This rather simple data treatment requires to normalize the signal obtained for both conditions to the amount of sample used for the experiment. This will allow to get a direct interpretation of the biological question. Another approach consists in transforming the raw signal into an amount of lipid. However, this will require the complete validation of the technique used to ensure a reliable information. Indeed, it is sometimes interesting to be able to compare the abundance of some lipid species in a selected matrix. Moreover, the relation between instrument signal change and biological amount change are not always strictly proportional leading to some misinterpretation of the changes. The validation of the analytical method is therefore important to bypass those potential issues as small changes in lipid content can sometimes have relevant biological or physiological consequences.

Another potential issue when quantifying lipids could be the absence of commercially available standards resulting in increased complexity during method validation. Moreover, in some cases, there is a need to consider isomer or similar structure than the lipid of interest to determine validation criteria.

As mentioned in the foreword, my work aimed at developing an LC-MS/MS method for quantifying prostaglandins and an LC-MS method to quantify SCFA. In the next pages, I will discuss what is already known about those two families of lipids in terms of biological roles as well as quantification methods already described.

Part II. Prostaglandin derivatives analysis

II.1 Prostaglandin derivatives as bioactive lipids

Prostaglandins are a family of lipids **(Figure 11)** described for the first time in 1936 as lipidic compounds extracted from human prostate gland (leading to their name) and able to lower blood pressure [128]. We know now that this observation was associated with the action of some prostaglandins on receptors at the surface of platelets or smooth muscle (one important constituent of the arterial wall) [129]. Since this first observation, many other studies followed describing the actions of prostaglandins. The effects mediated by prostaglandins and the description of specific receptors led to consider these lipids as bioactive lipids [130-132].

It is well-established that prostaglandins bind and activate several receptors (known as DP, EP, FP and IP receptors) leading to downstream signalling [129, 133, 134]. Those receptors are G protein-coupled receptors (GPCR) and their activation results in different effects depending on the physiological environment around them as well as the levels of prostaglandin [135-139]. Moreover, the expression of these receptors varies depending on the tissues and cells [135, 137, 139]. The expression of the enzymes controlling prostaglandin levels (from the precursors, cytosolic phospholipase A_2 ($cPLA_2$), to downstream metabolites, cyclooxygenase 2 (COX-2) and prostaglandin synthases) are often overexpressed in inflammatory conditions [140]. Altogether, those characteristics lead to an important diversity of action of prostaglandins in pathophysiological conditions [129, 136, 137, 140].

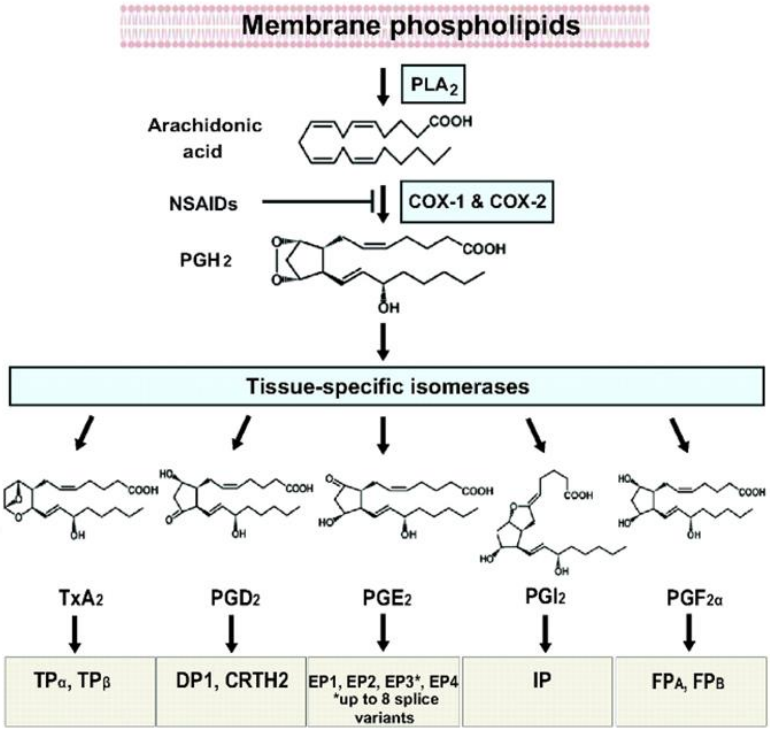


Figure 11: Simplified representation of the production and molecular targets of the prostaglandins (adapted from [141])

One of the historical effects linked to prostaglandins is their capacity to act on smooth muscle cells to induce either their contraction or their relaxation [142] **(figure 12)**. Those effects were documented in the context of airway inflammation (e.g. in asthma) [143-145] or in several reproductive events (e.g. sperm delivery, oocyte maturation or blastocyst migration) [146-148]. Prostaglandin analogues (e.g. dinoprostone or misoprostol) are even clinically used during the delivery process [149]. Other effects described for prostaglandins is their implication in platelet physiology and blood fluidity [150-152]. This led to the pharmacological use of acetylsalicylic acid (an irreversible COX inhibitor [153-155]) for blood fluidification and cardiovascular events prevention.

Prostaglandins are largely involved in inflammation (from its initiation to its resolution) [131, 133, 140, 156]. Firstly, as mentioned above, COX-2, one of the rates limiting enzyme of the pathway, expression is increased in inflammatory settings due to increased NF-κB signalling

[157, 158]. This is leading to the generation of prostaglandin I_2 and E_2 inducing vasodilation and allowing immune cells recruitment [159-161]. Prostaglandins are also able to induce cytokines release [161] or modulation of macrophages activation [156, 160, 162]. Altogether, those effects contribute to the set-up of an inflammatory state at the inflammation site. Thus, targeting COX-2 using acetylsalicylic acid or non-steroidal inflammatory drugs (NSAID) such as ibuprofen **(Figure 11 & 12)** remains one of the most important pharmacological approaches worldwide allowing to tackle a wide diversity of inflammatory processes [163]. Besides, COX-2 is overexpressed in a broad range of cancers [164] and prostaglandins participate to treatment resistance and metastasis [165].

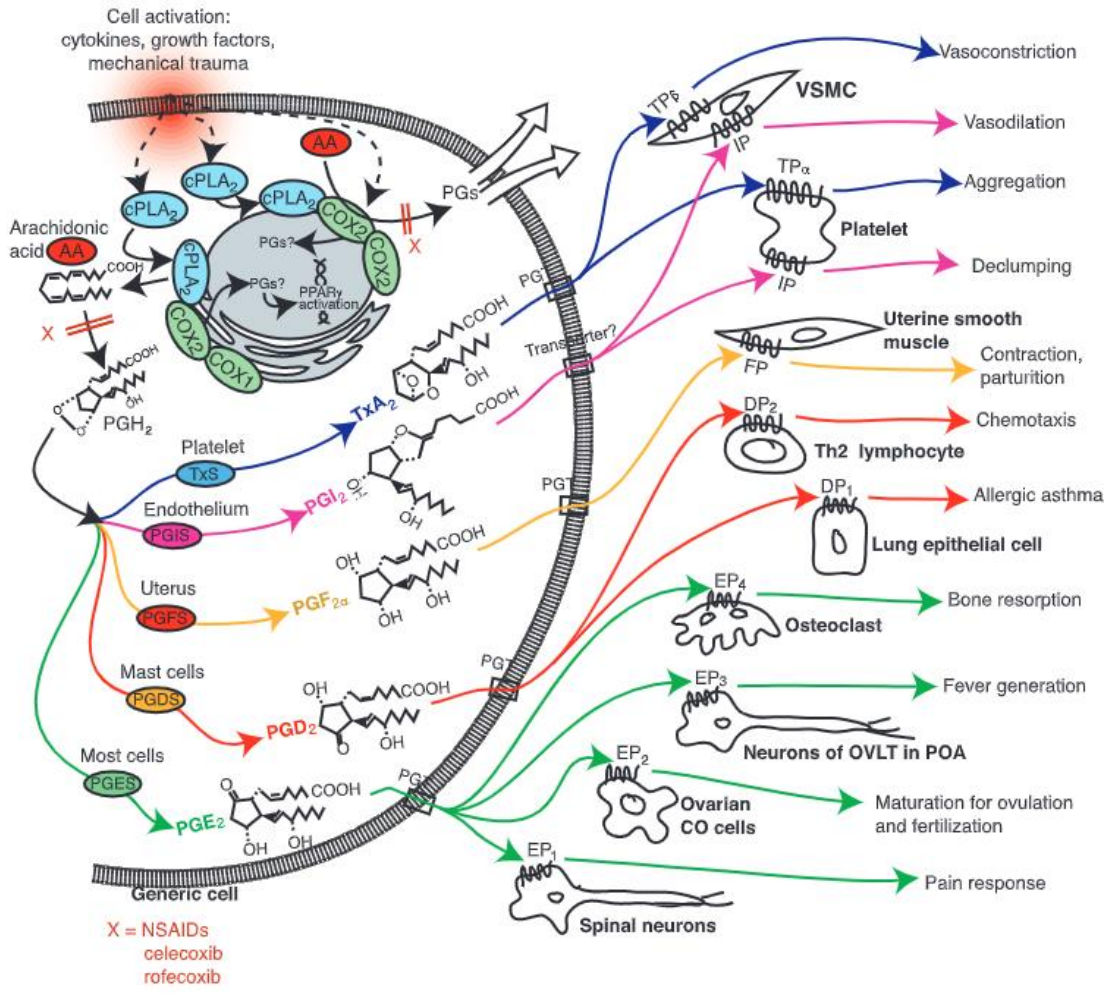


Figure 12: Proposed prostaglandin synthesis activation and downstream effects [129]

Further increasing the complexity of this system's pharmacology, Marnett's group discovered in the early 21th century that COX-2 is able to oxidize two arachidonic acid derivatives, namely 2-arachidonoylglcyerol (2-AG) and *N*-arachidonoylethanolamine (anandamide, AEA) to generate new prostaglandin analogues known as prostaglandin glycerol esters (PG-G) and prostaglandin ethanolamides (prostamides, PG-EA), respectively [166, 167] **(Figure 13)**.

As the same enzyme has several substrates, a competition between substrates for the catalytic pocket exist and can be altered when the proportions between substrate tissue levels are changed [168]. Of note, while the initial reports suggested that COX-1 catalytic pocket cannot accommodate 2-AG and AEA, later reports did not exclude the possibility of COX-1 metabolism of 2-AG and AEA [133]. Nonetheless, it is well described that COX-2 can metabolize 2-AG and AEA and, as the enzyme activity can be modulated by the biological conditions, it is difficult to predict the substrate utilization. Finally, while it appears that the same enzymes are shared by the three substrates (AA, 2-AG and AEA), thus leading to glycerol esters and ethanolamide derivatives of the conventional prostaglandins (**Figure 13**), it is less clear to what extent these lipid mediators share similar receptors [133, 169].

Most likely, a preferential formation of prostaglandins, PG-G or PG-EA can occur depending on the conditions [168]. Moreover, a lot of different compounds (prostaglandins as well as other lipid mediators such as HETE, lipoxins, …) can be generated in a specific physiological or pathological condition [170-172]. These newly generated metabolites can also interact with each other [173, 174]. It increases the number of targets potentially activated by these lipid families at the same time.

As shown in **Figure 13**, PG can be synthesized from their respective glycerol ester derivatives through the action of several enzymes. However, it has not been demonstrated whether

ethanolamide derivatives possess a similar capability to produce PG. Likewise, even if Di Marzo's and Marnett's groups worked on these metabolic pathways, it remains unexplored whether prostaglandins can undergo condensation with either glycerol or ethanolamine moieties to yield their glycerol ester and ethanolamide derivatives [48, 175]. Further research is necessary to elucidate these aspects of the metabolic pathways.

We previously showed that PGD_2-G is able to bind DP1 receptor with similar affinity than PGD_2 [176] (**Figure 13**). It is also described that PGE_2-EA is able to bind the all four EP receptors [177]. 15d-PGJ_2-G was also shown to interact with PPARγ receptor [178]. Part of the effect of $PGF_{2\alpha}$-EA appears to be mediated through a heterodimer between the FP receptor and a splicing variant subunit [179]. Finally, PGE_2-G acts as an $P2Y_6$ receptor endogenous agonist [180].

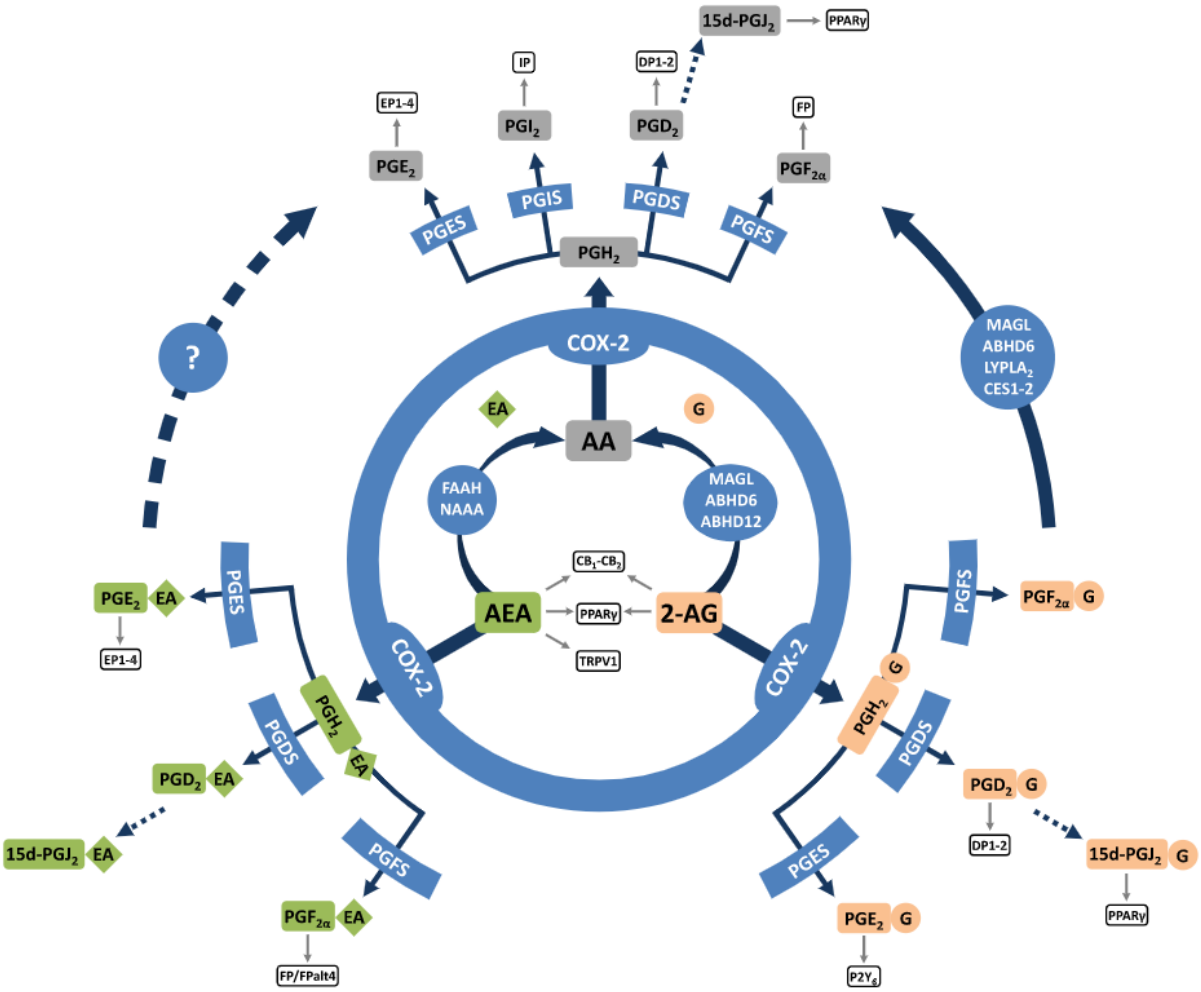


Figure 13: Synthetic pathways, main enzymes and described receptors of prostaglandins, prostamides and prostaglandin glycerol esters as well as their precursors. [133] While it has been shown that PG-G can undergo hydrolysis to yield the corresponding PG, this has not been clearly established for PG-EA.

As their biochemical characterization is quite recent and their tissue detection complex, only a limited number of studies describe the biological properties of the PG-G and PG-EA derivatives. Walker's group reported that PGE_2-G induced hyperalgesia in a model of pain induced by hind paw incision (HPI) [181]. Bimatoprost, a $PGF_{2\alpha}$-EA analogue, is used as antiglaucoma drug [182]. PGD_2-EA was reported to have protective effects against skin cancer through his metabolite, the 15deoxy$\delta^{12,14}$ PGJ_2-EA [183]. Our group initially described the anti-inflammatory properties of PGD_2-G using LPS-induced inflammation in mice [162]. Later, we reported its beneficial effects in DSS-induced colitis in mice [176]. We have also showed that

PGD_2-G reduces inflammation and pain in a carrageenan-induced hyperalgesia model [184]. More recently, we have explored its potential in the context of CNS inflammation, notably using the experimental autoimmune encephalomyelitis model of multiple sclerosis [185]. The group of Van Dross explored the interest of PGJ_2-EA as antitumor drug [186]. These different observations put forth the interest to study PG-G and PG-EA as bioactive molecules.

To decipher the complex roles of PG-G and PG-EA, pharmacological tools (e.g. enzyme inhibitors [187, 188] and receptor ligands [187, 189, 190]) are essential. However, these approaches will not help understand how a given physiopathological condition affects the endogenous levels of these lipid mediators and to what extent they are interconnected in vivo. Thus, as I will discuss in the next section, several groups reported analytical methods to measure the levels of these compounds. It is important to note that, besides COX-2, lipoxygenases and cytochrome P450 are also described as 2-AG and AEA metabolizing enzymes [169] generating HETE-G and EET-G, respectively, in the case of 2-AG and HETE-EA and EET-EA, respectively, in the case of AEA. This adds further biochemical pathways depicted above.

II.2 Challenges and current methods for PG-G and PG-EA quantification

Even if a large number of reports describe methods for the accurate and sensitive quantification of the prostaglandins, to date only a few reports describe the quantification of some PG-G or PG-EA species. In fact, quantifying with a single method prostaglandin, PG-G and PG-EA is a complex task with several challenges to overcome as I will discuss below. As their quantification is probably less challenging, I will not discuss here the methods described for the quantification of 2-AG and AEA. The interested reader can refer to several reviews on the topic [191-194].

II.2.1 Challenges

II.2.1.1 Low biological abundance

One of the main issues in the quantification of these mediators is their endogenous levels at least in physiological conditions where they are often low in abundance **(Appendix table 1 and related references)**. Even in the case of prostaglandins, the described levels are around 0,5-100 fmol/mg (or mL in the case of plasma) [195]. This leads to reduced possibilities in the choice of the analytical technique to use. Moreover, the challenge is bigger in the case of PG-G where the few described endogenous amounts are in the order of the fmol/mg of tissue **(Appendix table 1 and related references)**. In the case of prostamides, the picture is probably even more complex as there are not so many reports of endogenous levels for this type of compounds. On the contrary, the precursors of these three families of compounds (arachidonic acid, 2-AG and AEA) are present at several orders of magnitude higher levels compared to their respective metabolites. This brings an additional challenge if we aim at developing a single method for the prostaglandin derivatives and their precursors.

II.2.1.2 Similarity of structures

The other main challenge consists in the fact that prostaglandins, prostaglandin glycerol esters and prostamides share similar chemical backbones. Moreover, some mediators are position isomers (e.g. PGD_2-EA and PGE_2-EA) but also some structural isomers (e.g. PGD_2-1-G and PGD_2-2-G) as shown on **figure 14**. Adding complexity to the complexity, a large number of endogenous lipids sharing the same molecular masses or similar structure patterns than prostaglandins are present in biological matrices. For instance, when looking on LipidMaps® for compounds having a similar mass on charge ratio (± 0.1) than PGD_2/PGE_2 238 matches are

found. It is possible that the same diversity of compounds could exist in the case of PG-G and PG-EA [196]. In fact, as already reported in the case of prostaglandins, reports emerged in the past years showing that other oxidases than COX-2 can also metabolize 2-AG and AEA, the two precursors of PG-G and PG-EA respectively [169, 197, 198]. This high number of compounds can then logically interfere with PG-G and PG-EA analysis in the extraction and purification procedure, or in the detection step (e.g. increase matrix effect) or lead to mis-identification of the compounds of interest depending of the analytical method used.

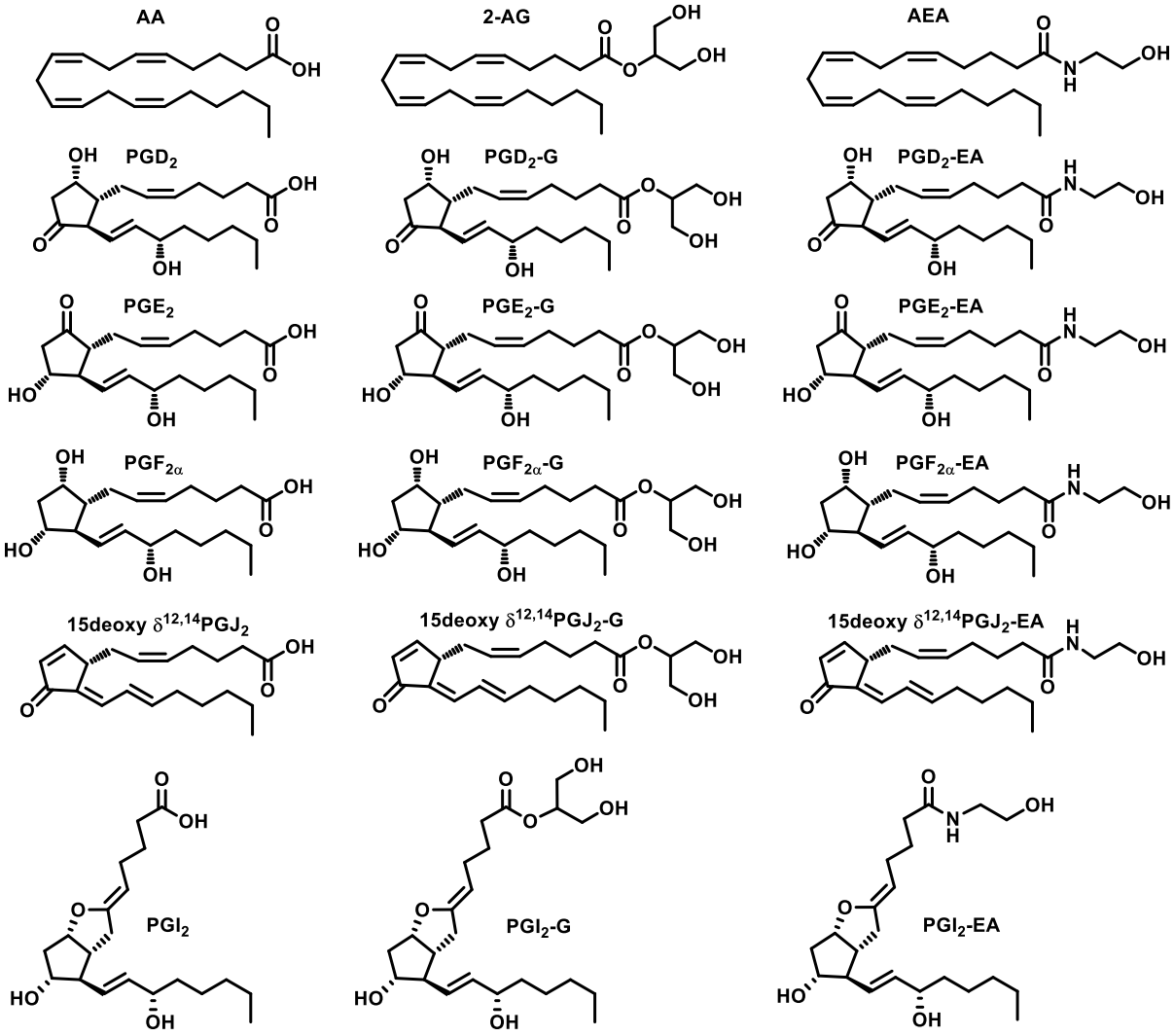


Figure 14: Structures of three pathways of selected prostaglandin derivatives. The glycerol derivatives are shown as the 2-isomer but 1(3)-isomers also exist.

II.2.1.3 Availability of compounds

As PG-G and PG-EA pathways are newly described, another limitation in the development of analytical methods is the poor commercial availability of compounds. Indeed, having access to pure analytical standards is a crucial step for species identification in analytical method development. Even if the number of derivatives commercially available is increasing (e.g. thanks to Cayman Chemical™), several molecules such as 15d-PGJ_2-EA are not yet available via commercial sources. This forced Ladin et al. to synthesize 15d-PGJ_2-EA to assess its antitumoral activities [186]. Concerning PGD_2-G, two position isomers known as PGD_2-1-G and PGD_2-2-G exist. Unfortunately, only one of the two isomers (i.e. PGD_2-1-G) is commercially available. It is known, on the basis of 2-AG and 1-AG, that they can undergo interconversion between both isomers without enzymatic action [99]. Nonetheless, it is not known if the two isomers have similar properties (e.g. affinity for receptors).

II.2.1.4 Stability

As it is crucial in all the bioanalytical methods, compounds stability is a key point to take into consideration when developing methods [199-201]. Of course, it is important to avoid any modification of the analyte content during tissue harvesting or storage. This point is even more important in the case of prostaglandin derivatives as they exhibit short half-life time. For example, Kozak et al. determined for PG-G and PG-EA a half-life time of only 5 min in rat plasma [166]. During long term storage, degradation can occur for some of the member family even when stored at -80°C [202-204]. It is therefore important to consider this fast degradation of the metabolites (within some minutes) in the method development (as well as when molecules are administered).

II.2.2 Current methods

As detailed above, it is not easy to develop methods able to detect and determine endogenous levels in the case of prostaglandins. The above-mentioned challenges result in difficulties to find approaches sensitive and specific enough for prostaglandin quantification. The two main techniques applied to the quantification of those metabolites are the use of ELISA strategy as well as the use of mass spectrometry (associated with various separation techniques) [205-209].

II.2.2.1 ELISA approaches

In the case of ELISA **(Figure 15)**, several limitations exist when quantifying prostaglandins. The first one could be the lack of specificity for metabolite families exhibiting similar structures such as prostaglandins. Even if the binding can be selective to certain structures, it is complicated to know if misidentification of lipids occurs [205] for instance due to shared affinities of the monoclonal antibody used for several compounds, leading also sometimes to increased variability [206]. The other limitation inherent to the use of ELISA is the number of lipids that can be detected at the same time with one ELISA kit. Even if the emergence of multiplex increases the number of analytes quantifiable in the same sample, it remains one major limitation of the technique as an important number of compounds needs to be considered in the case of prostaglandin families. Finally, the third limitation, shared with other techniques, is the sensitivity of ELISA that could not be sufficient in the case of PG-G and PG-EA.

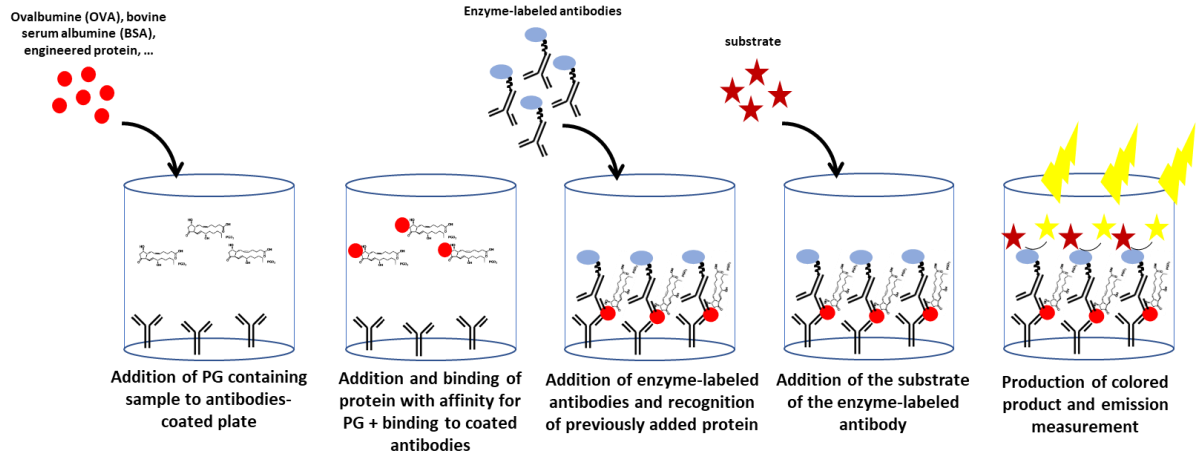


Figure 15: ELISA technique applied to the detection of prostaglandin derivatives (inspired from [210]).

From a technical point of view, ELISA (used as multiplex strategy) could allow the quantification of maximum a dozen prostaglandin derivatives at the same time on one sample. The sensitivities of the commercially available ELISA kits are around 10-20 pg/mL of sample in the case of prostaglandins. On the other hand, ELISA remains a fast technique as results can be available within 3 hours. Nonetheless, as explained above, the lack of specificity could be a major issue in the case of prostaglandins. The question remains as to know whether an ELISA kit designed for a prostaglandin could also detect a PG-G or a PG-EA. Finally, it does not seem that ELISA kits dedicated to the quantification of PG-G and PG-EA derivatives are currently available. Taken together, these elements support the use of other techniques in the case of PG, PG-G and PG-EA.

II.2.2.2 Separation approaches prior to mass spectrometry

Even if mass spectrometry allows sensitivities similar than the ones obtained with ELISA, this technique brings the advantage of allowing the quantification of a broad range of molecules at the same time and often in a single analytical process. But more than the number of molecules, the main advantage of mass spectrometry is, depending to the setup and the type

of instrument selected, the possibility to achieve separated isomers analysis, a key challenge of prostaglandin derivatives analysis.

Several separation techniques were coupled to mass spectrometry and efficiently applied to prostaglandin quantification [211-214]. The main are GC, LC, capillary electrophoresis (CE), supercritical fluid chromatography (SFC) and shotgun lipidomic strategies without any prior separation.

II.2.2.2.a Gas chromatography

As prostaglandins are low molecular weight and poorly volatile molecules, their analysis by GC requires a derivatization step [213, 215, 216]. Moreover, relatively longer analytical runs compared with other techniques (e.g. UPLC) are required. Nonetheless, GC, besides being sensitive when coupled to mass spectrometry, provides reliable results [213, 216]. One classical way to analyse prostaglandins using GC is to add a fluorobenzyl moiety or trimethyl silane moiety during the derivatisation step. However, the application of a derivatization method in addition to the temperature gradient required for GC separation could favour the degradation of these lipid derivatives.

II.2.2.2.b Liquid chromatography

Nowadays LC is largely used in lipid analysis, including for prostaglandins. The progressive switch from high performance liquid chromatography (HPLC) to ultra-high-performance liquid chromatography (UHPLC) allows to increase sensitivity by improving peak shape as well as reduce analysis times. This was a crucial step to facilitate the analysis of prostaglandin derivatives by LC-MS. Moreover, the diversity of available stationary phases continues to increase with the development of fused core columns or traditional columns with better

engraftment properties, … [217] Finally, the development of nanoflow strategies emerged facilitating the analysis of biological sample of reduced size as well as decreasing the use of toxic organic solvents such as methanol. Applied to prostaglandins, the increase in sensitivity and specificity allows to detect new interesting metabolites or detect them in challenging matrices [217, 218].

II.2.2.2.c Capillary electrophoresis

The advantage of the capillary electrophoresis for the quantification of ionizable molecules such as prostaglandins is the addition of a new parameter, i.e. the charge, in the interactions between analytes and the analytical environment. In fact, CE allows to separate compounds due to their affinities with mobile phase (e.g. electrolyte solution), the stationary phase of the capillary selected (e.g. silica, grafted silica or polymers)[219] as well as their charges **(figure 16)**. The coupling to a mass spectrometer as detector makes CE-MS a high sensitive, specific and selective method similarly to UPLC-MS [220-222].

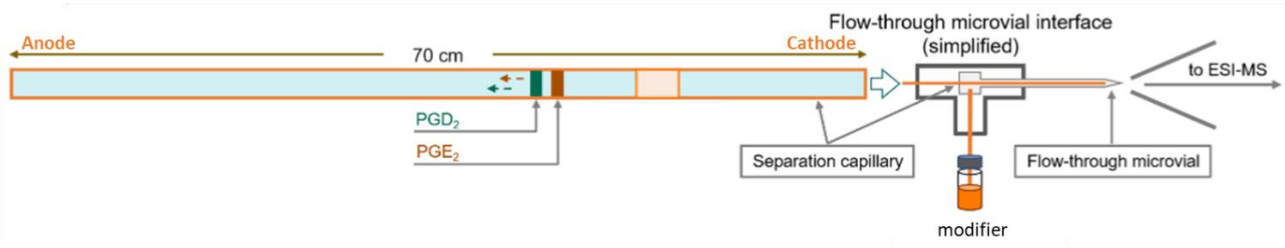


Figure 16: Example of a capillary electrophoresis assembly applied to PG quantification [220]

For example, Huang Zi-Ao et al. described a sensitive method to determine urinary PGE_2 (able to detect around 0.17 ng/mL of PGE_2 in one urine sample) [220]. They show satisfactory separation between PGD_2 and PGE_2 with complete resolution of peaks. However, capillary electrophoresis, to achieve good separation of the compounds, required longer run times than the ones provided with the use of LC. On the other hand, this technique requires small volume

of biological sample, requiring the pre-concentration of compounds from complex matrices and the limitation in the use of organic solvents. Nonetheless, CE has not been applied yet to the quantification of PG-G and PG-EA.

II.2.2.2.d Supercritical fluid chromatography

Another separation technique efficiently applied to prostaglandins is the use of supercritical fluids (mixed gas-liquid chromatography) [214]. CO_2 is the main mobile phase for supercritical fluid chromatography offering unique advantages such as non-toxicity, environmental compatibility, and applicability to a wide range of compounds. The work at selected temperature and pressure, above the thermodynamic critical point, allows to reduce the viscosity of the mobile phase and increases the diffusivity of analytes through the solvent. Both characteristics, applied to prostaglandins, lead to increased flow rates and reduction of retention time [223-225]. The improved selectivity conferred by supercritical fluid chromatography brings additional interests for prostaglandin analysis. It is mainly for those reasons and the development of bioanalytical methods for quantification of some of the prostaglandin species (as biomarkers) that SFC methods were developed in the case of prostaglandins. Kumari et al. reported the separation of several prostaglandin isomers within 3 min of run [224]. As they reported, it is possible to achieve good separation of prostaglandins on this type of chromatography by selecting appropriate stationary phases and using very short analytical runs.

II.2.2.2.e Shotgun lipidomic

Shotgun lipidomic is another approach to analyse the lipidome. In shotgun lipidomic, the idea is not to obtain absolute quantification of some specific species but more to characterize the

global variation of the family (using their “fingerprinting” moiety) before going more in detail with more quantitative analysis [226, 227]. The advantage of this technique is to use small tissue amount (as there is reduced loss of analytes due to the classical extraction, purification and separation steps) and then use mass spectrometry fingerprinting of the compounds to characterize large lipidome from the sample [227]. As prostaglandin derivatives are leading to important variations in pathological conditions, the use of shotgun lipidomic could be interesting to quickly point out the activation of these pathways. Moreover, this technique would allow for the concomitant quantification of prostaglandins, as well as lysophospholipids and phospholipids, their biological precursors, to have a more global view of the pathways activated [228, 229]. However, as mentioned above, the high similarity among the prostaglandins and their low levels compared to other lipids classes represent true challenges for the quantification of prostaglandins by shotgun lipidomics. In fact, probably the most important limitation of the approach is the ion suppression that could easily occur inside the ionization source leading to misevaluation of the compound abundance or variation [230]. These elements could explain the absence of reports describing prostaglandin quantification by shotgun approaches.

II.2.2.3 Quantification by mass spectrometry

Once separation is properly achieved using the above-mentioned techniques, several possibilities exist to characterize prostaglandin derivatives levels. The choice of the analyser will provide differential information concerning the sample. However, given the relatively low levels of prostaglandin, the choice of mass spectrometers that can be used is relatively reduced.

First of all, new generation of high-resolution mass spectrometers (HRMS) (e. g. Obritrap from ThermoFisher Scientific or quadrupole time of flight (Q-TOF) mass spectrometer from various suppliers) allow the characterization of complex samples. The advantage of these mass spectrometers in the case of prostaglandin derivatives is the ability, in a single run, to analyse and detect a broad range of compounds as well as identify potential new interesting derivatives, a key point in prostaglandin derivatives as the family counts hundreds of metabolites. Moreover, the use of these analysers allows, in the majority of cases, to conduct fragmentations of the compounds (MS^n fragmentation) to characterize, ensure the identity and identify potential isomeric forms of the metabolites. For these reasons HRMS has an important place in untargeted lipidomics applied to prostaglandins [225, 231-233]. However, even if the new generation of HRMS has good sensitivity capacities, there are still a large number of compounds undetectable in endogenous conditions using high resolution mass spectrometers.

Of note, new devices based, for instance, on ion mobility or on high fragmentation patterns techniques, allow together with the high-resolution signatures to obtain additional analytical information [234-236]. Unfortunately, to date due to the limitations linked to sensitivity, these techniques were applied only to standard solutions or to cell culture samples.

Since the first mention of the use of a multiple reaction monitoring (MRM) strategy to quantify prostaglandins [237] (applied to GC), a large number of publications have described sensitive and validated methods allowing the quantification of prostaglandin derivatives inside complex biological matrices [211, 238-248]. In fact, most of the methods described for prostaglandin analysis are essentially based on the use of triple quadrupole (or tandem quadrupole (TQ)) to quantify those derivatives as the use of MRM strategies allows to reach better sensitivities

when compared to HRMS. Indeed, the use of MRM approaches drastically reduce the background noise allowing to increase the signal to noise ratio, a critical criterion for compound quantification. Even if the mass precision is lower as compared with HRMS, the use of two fragments from a same molecule allows to increase the confidence in compound identification. By selecting specific mass transitions, it is, in theory, possible to analyse separately mass isomers avoiding selectivity issues. Moreover, MRM strategies allow to follow several mass transitions from the compound of interest, supporting the confidence in the identification.

Thus, tandem quadrupoles are probably the best compromise between whole lipid families quantification and quantification of low abundant compounds. This makes tandem quadrupole mass spectrometry a method of choice to quantify prostaglandins (and eicosanoids in general) [249]. Moreover, the acquisition frequencies provided by the new instruments allow to analyse at the same time a larger number of analytes without affecting signal integrity.

II.4 Abundance and tissue distribution

Since the first extraction of prostaglandins from the prostate gland [128], a large number of publications reported endogenous levels of prostaglandins and sometimes prostaglandin-glycerol ester and prostamides levels. However, probably linked to the different methodologies used over the years, and the variable quality of the reports, a large variability in the levels of these molecules is apparent when surveying the literature. In the context of this introduction chapter, I conducted a rather extensive literature search based on the criteria described in **Figure 17**. The tables in the Appendix of this manuscript report the amounts of prostaglandin, PG-G, PG-EA as well as the amount of their precursors (namely arachidonic

acid, 2-AG and AEA) in several human (**Appendix Table 1**) and animal (**Appendix Table 2**) tissues.

While numerous data are available regarding the prostaglandins and the “precursors” much less reports describe endogenous levels of PG-G and PG-EA. Regarding the clinical data, most of them report data obtained in human biofluids (e.g. plasma, urine, …).

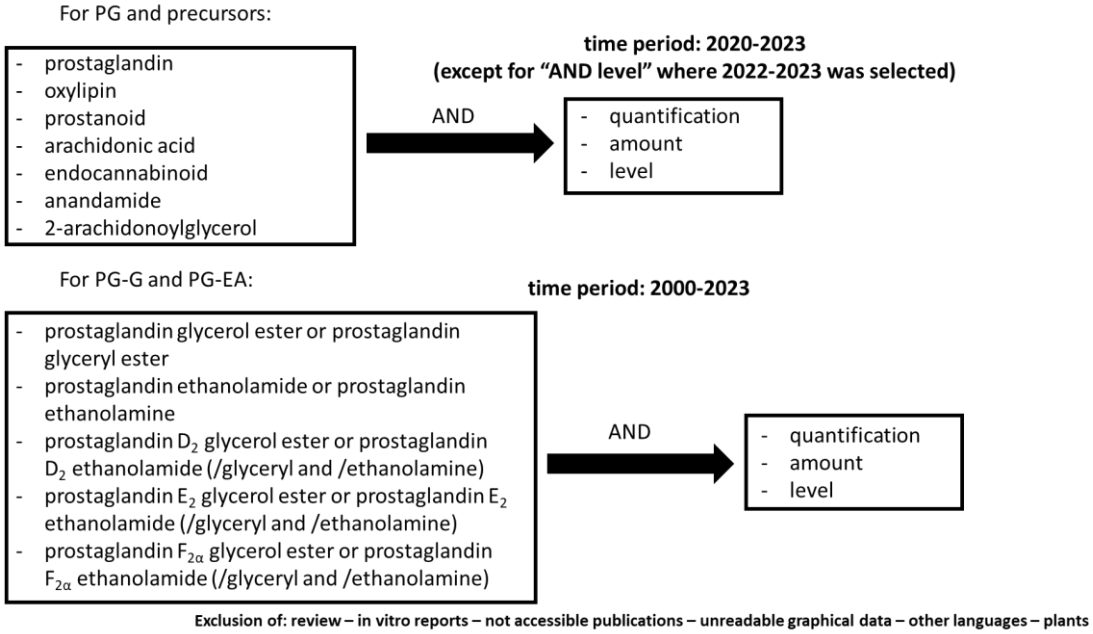


Figure 17: Methodology used to identify the papers reporting endogenous amounts of prostaglandin derivatives.

When looking at the reported levels for a given metabolite in the same tissue and for the same animal species the diversity of reported levels is quite evident. This could be due to the animal strain (e.g. C57BL/6, SWISS, Balb/c, etc), the analytical technique used (lack of specificity, issue in sensibility, misuse, …), or potentially some calculation errors, ... Nevertheless, these values can give an indication on the tissue where a particular analyte is more or less abundant. While abundance does not necessarily indicate an important role, it is indicative of potential “hot spots” in terms of metabolic activity of the pathway.

To have a better grasp on the differences, **figure 18** (based on the Appendix tables) shows for arachidonic acid, 2-AG and AEA, the reported amounts in human and animal plasma.

Given the rather large variability in the reported levels it is complex to determine what are the actual circulating levels of arachidonic acid, 2-AG and anandamide (**Figure 18).** When looking at human plasma, it is considered that 2-AG is somewhat more abundant than anandamide, this is especially the case when looking at papers reporting the levels of both endocannabinoids.

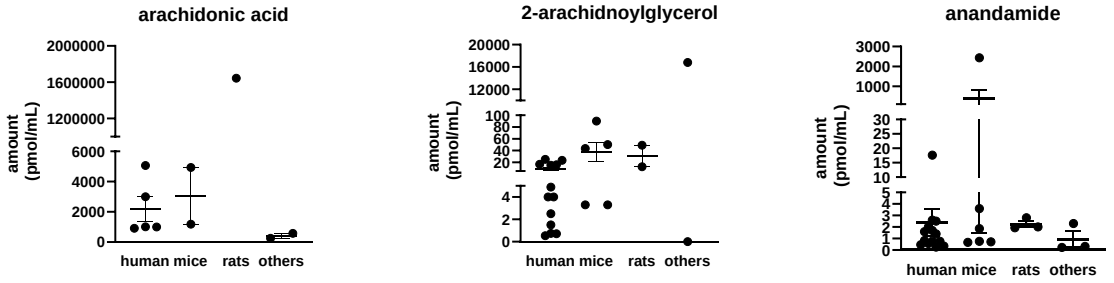


Figure 18: reported amount of arachidonic acid, 2-arachidonoylglycerol, and anandamide in plasma depending on the animal species (based on the Appendix tables)

When analysing more in details the reported levels of prostaglandin derivatives, it seems there is not only species-dependent differences as reported for their precursors (**Figure 18**) but also inside the same species (levels described for mice tissues are reported at the **Figure 19**), tissue-dependent differences as well as a report dependent variability. Altogether, those differences increase the complexity to set up a single run quantification method when considering prostaglandin quantification.

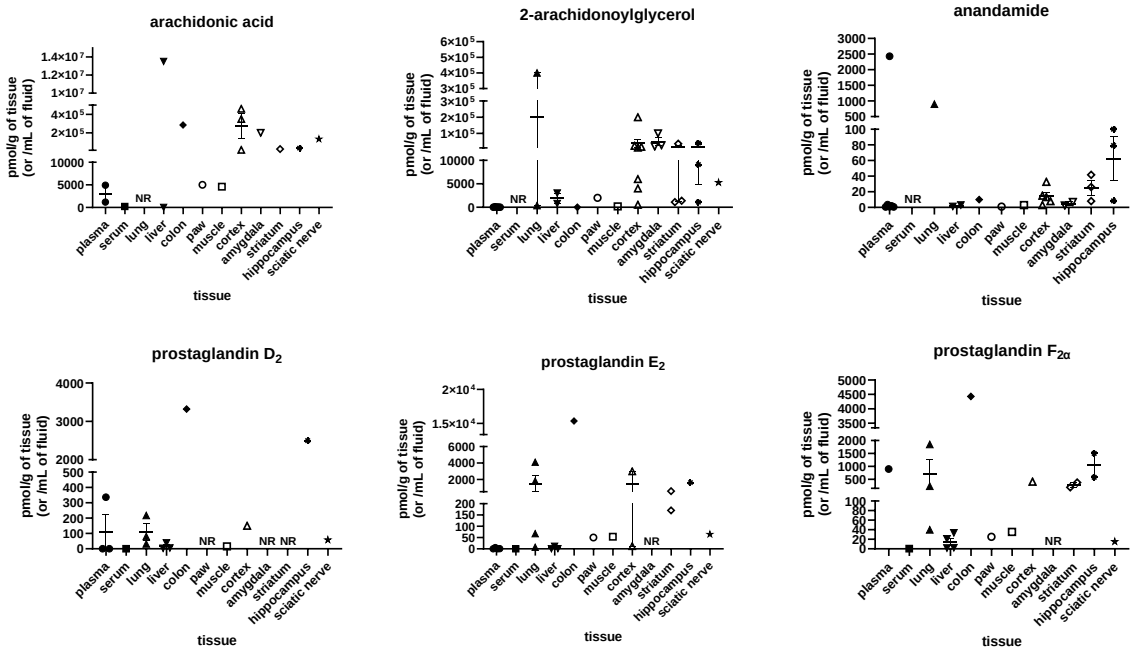


Figure 19: reported amount of the three precursors and prostaglandins depending to mice tissue (NR: not reported). Figure based on the data reported in Appendix tables.

When considering the methodology used, for instance for the clinical studies, most of the data come from LC-MS/MS methods. The limited number of studies reporting data obtained with other analytical methods does not allow a proper comparison between methods **(figure 20)**.

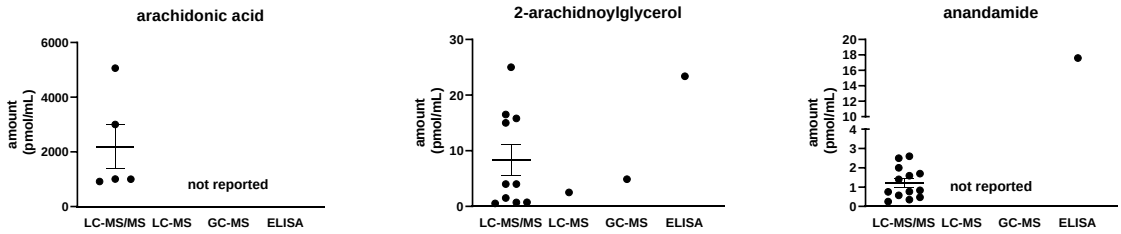


Figure 20: reported amount of the three precursors depending of the analytical technique used (within human plasma reports). Figure based on the data reported in Appendix tables.

When considering the glycerol ester and the ethanolamide derivatives of the prostaglandins, the number of reports is much more limited (compared to endocannabinoids or prostaglandins) (**Figure 17** and **Appendix tables**). This could be due to the fact that the field is

rather new (compared to the prostaglandin and endocannabinoid fields), as well as to the higher sensitivity probably required to quantify these molecules. For instance, levels of 100 fmol/g were reported for $PGF_{2\alpha}$-EA in mouse liver [250]. The detection of such amounts requires the use of LC-MS/MS instruments and an important optimization of the complete analytical process. As for human samples (see **Appendix tables**), no report has been published yet regarding the quantification of the glycerol esters and ethanolamide derivatives except the mention of a case report where PGE_1-EA has been quantified in human plasma after sepsis [251]. Even if several samples were considered, only one sample allows the detection of this metabolite and unfortunately the patient died the day after. This can point out the extreme difficulty for now to develop, validate and efficiently apply a quantification method in the case of these three families of compounds.

In the first part of the result section, I will describe how we tried to answer to these different challenges in order to develop, validate and apply to biological samples a method allowing the detection and quantification of femtomole range prostaglandins, prostaglandin glycerol esters, prostaglandin ethanolamides as well as their respective precursors (paper published in June 2023).

Part III. Short chain fatty acids, citrate cycle metabolites and ketone bodies analysis

III.1 SCFA, citrate cycle metabolites and ketone bodies shared metabolic pathways

SCFA are small fatty acyl derivatives exhibiting one to six carbon chains **(figure 21)**. They are, most of the time, produced by commensal gut bacteria after fibre digestion. The interest in these metabolites keeps increasing as they seem to be, at least in part, responsible for several so-called “bacteria-host” axes [252-256]. In fact, SCFA are linked to several pathophysiological processes [257-259]. They participate to the control of the secretion of gut hormones such as the glucagon like peptide-1 [260]. SCFA can also modulate the immune system as well as brain function [252]. Part of their effects come from their binding to G protein-coupled receptors such as GPR41 and GPR43 [257, 261, 262].

For those reasons, as it was the case for the gut microbiota, the number of quantification methods for SCFA increased steadily since the beginning of the 21th century (13 reports on pubmed between 1966 and 2000 for “short chain fatty acid AND quantification” and 1074 reports between 2000 and 2023). Several methods were then developed to extract, purify, analyse and quantify SCFA in biological samples [263-266].

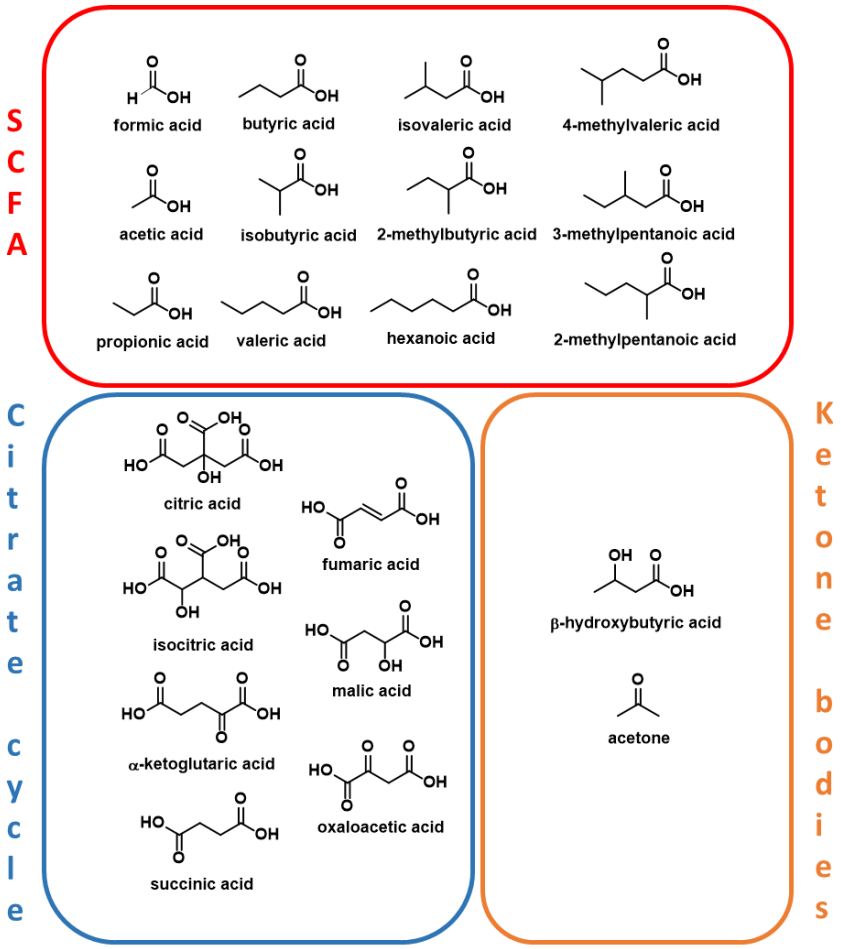


Figure 21: Structures of the main SCFA, citrate cycle metabolites and ketone bodies that are the focus of the analytical method described in the second chapter of the result section.

On the other hand, even if scientists focused only on the main metabolites of the family (i.e. acetate, propionate, butyrate and isobutyrate) there is a clear need to have a more global view of metabolite level changes to better characterize microbiota's effects in health and disease. Indeed, besides the "usual suspects" mentioned above, several other members of this family of lipids could be interesting to analyse (e.g. formate and valerate).

Besides, it is also important to consider other metabolites linked to SCFA synthetic pathways such as citrate cycle metabolites and ketone bodies. In fact, it is now described that depending on the microbe species [261, 267, 268] but also on the dietary fiber type [269, 270], or the

pathophysiological status [261, 268], one or another synthesis pathway is more active [271]. This will lead to the preferential production of one or the other SCFA.

It is therefore important to consider these complex interrelated pathways when studying SCFA as key players in the context of microbiota – host interactions. These include the synthetic pathways leading to SCFA, branched chain fatty acids (BCFA) which are obtained through the metabolization of branched chain amino acids by bacteria, citrate cycle metabolites (CCM) as well as ketone bodies which are terminal metabolites derived from acetyl-CoA (**figure 22**). After their syntheses, SCFA can also be oxidized by acyl-CoA oxidases which lead to the formation of acyl-CoA derivatives [272]. These newly generated compounds are involved in energy metabolism regulation [273] and chromatin regulation [274].

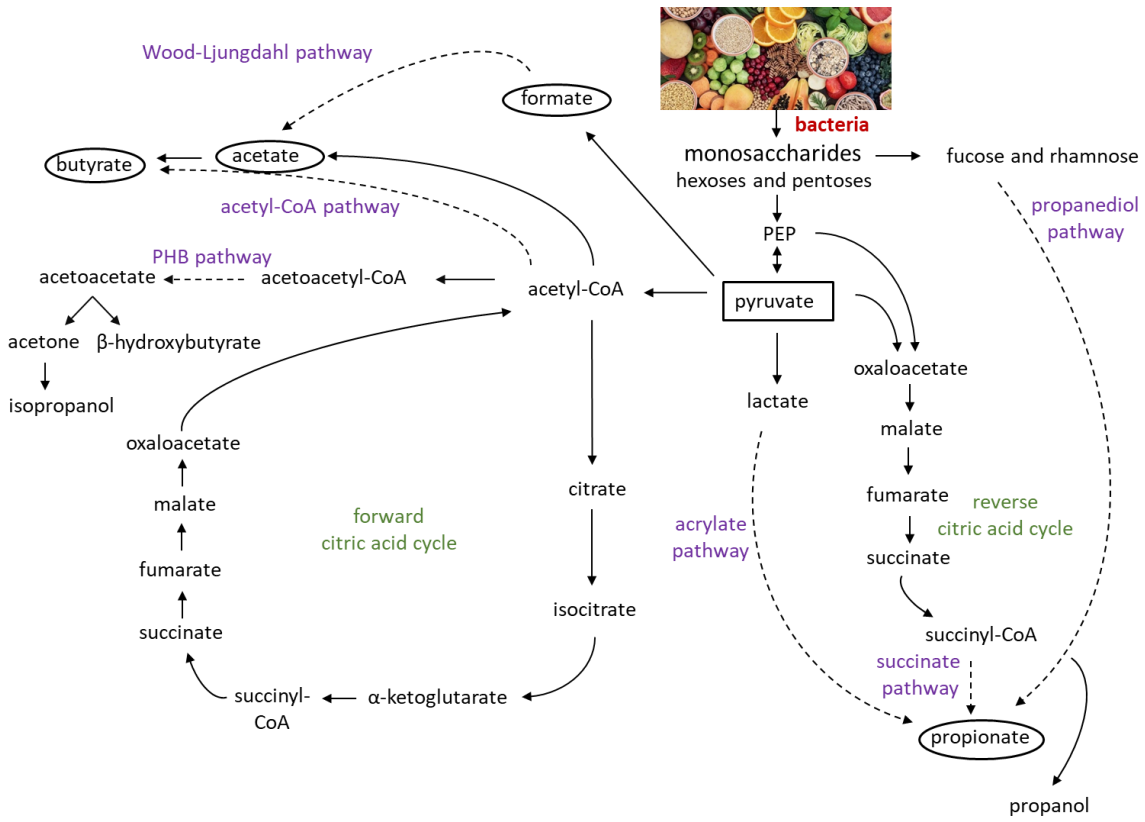


Figure 22: Simplified view of the metabolic pathways leading to SCFA quantification (adapted from [261]).

The scheme reported in **figure 22** is a simplified view of the complete metabolic pathways linked to microbiota as they appear in the KEGG pathway database (**figure 23**). In fact, microbiota metabolism is extremely complex, and even more so when considering the interplay between microbiota and host metabolism as observed for instance for bile acids [275, 276]. In the following sections, I will focus on the pathways leading to SCFA and BCFA as they are the focus of the second part of the results reported in this manuscript.

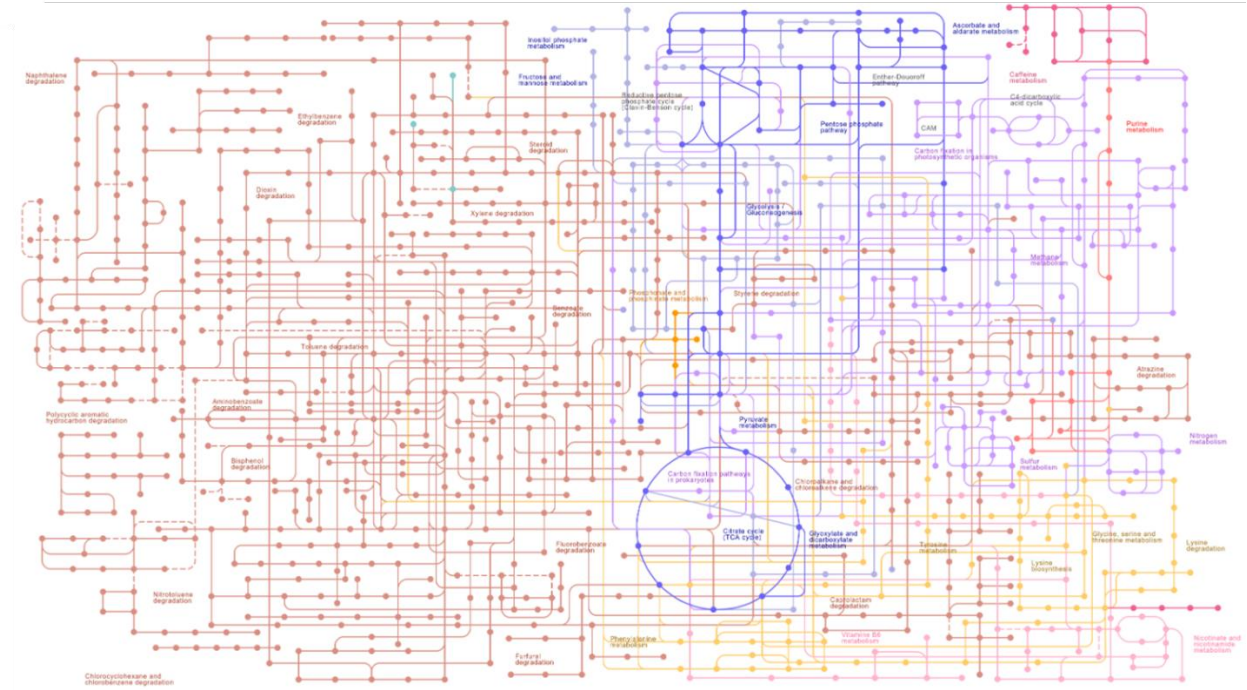


Figure 23: Complete microbial metabolism as reported on the KEGG pathway database.

As summarized in **figure 22**, there are several pathways leading from dietary fibres to SCFA. Besides, the citrate cycle plays an important role in the diversification of the metabolome coming from bacteria. Indeed, some of these metabolites also serve as precursors in the synthesis of other types of bacteria metabolites [277-279]. For instance, α-ketoglutarate takes part to the citrate cycle but can also serve as precursor in the synthesis of L-glutamate and L-proline, two important amino acids. Moreover, the citrate cycle uses acetyl-CoA, one of the key intermediates in the synthesis of SCFA, to produce energy. On the other hand, the reverse

citrate cycle produces succinyl-CoA which is the precursor of the succinate pathway that leads to the generation of propionate. Another described pathway for the generation of propionate is the acrylate pathway that uses lactate as an important precursor [280]. Through lactate metabolism, it is then possible to obtain propionate that could also be generated through the propanediol pathway. This pathway uses fructose or rhamnose as monosaccharides, obtained by fibre digestion, as primary substrates for propionate synthesis [281].

Acetyl-CoA, one of the first-rate limiting metabolites in the synthesis pathways, can also be converted into acetoacetyl-CoA [282]. The latter can then enter the ketone bodies synthesis pathway resulting in acetone and beta-hydroxybutyrate as final products.

Acetate can participate to the synthesis of butyrate via a transferase that uses butyryl-CoA and converts acetate into acetyl-CoA [282, 283]. Acetate can also be obtained through the Wood-Ljungdahl pathway which uses folate as cofactor to produce acetate from formate [284]. This alternative pathway can then overpass the need of acetyl-CoA for the synthesis of acetate (or butyrate).

The branched chain amino acids obtained by protein digestion can follow a catabolic pathway leading to branched chain fatty acids such as isobutyrate (from valine), isovalerate (from leucine) and 2-methylbutyrate (from isoleucine) [285, 286]. Finally, the longer SCFA (e.g. hexanoate) can be obtained *de novo* through the use of lactate by bacteria [287] but can also be the result of acyl chain elongation process [288-290]. This process (**figure 24**) consists in the generation of longer chain fatty acids (and longer branched chain fatty acids) from SCFA and BCFA.

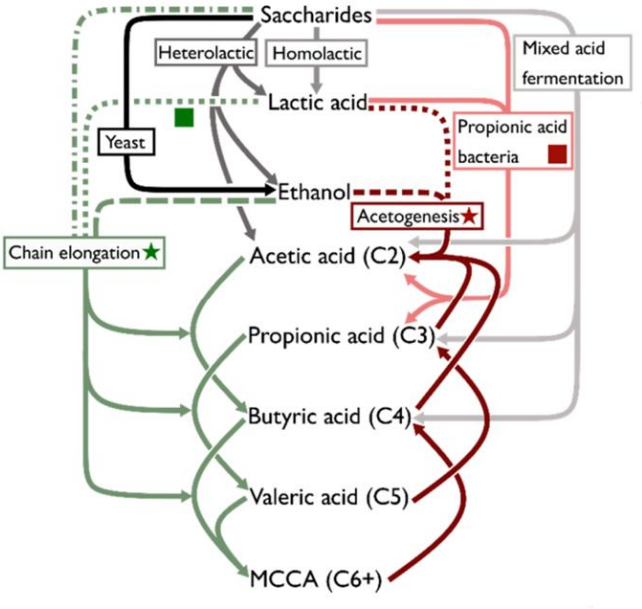


Figure 24: Schematic description of the chemical reactions occurring in microorganisms (adapted from [290]).

These numerous and intricate biochemical pathways result in the production by microorganism of a broad diversity of short chain and branched chain fatty acids (**figure 21**) that should ideally be quantified in the same sample. These metabolites are the result of biochemical pathways aiming at harvesting energy from food intake. However, an increasing number of reports point to SCFA and BCFA as important mediators in the crosstalk between microbiota and host. In the next sections I will summarize several important aspects of SCFA as lipid mediators.

III.2 SCFA as bioactive lipids

Due to their major site of production, many reports describe the involvement of SCFA in gut-linked disorders. However, their effects do not seem to be limited to the gut as SCFA were also associated to health and disease in the nervous, respiratory, urinary and vascular systems, as well as in immunity. These numerous effects are linked to their capabilities to bind several

GPR receptors (**Table 2**). I will describe in the next section some of the main pathophysiological effects of those bacteria metabolites.

	acetic acid (µM)	**propionic acid (µM)**	**butyric acid (µM)**	ref
GPR41 (FFA2)	35-431 250 120 1020 /	14-290 257 10 2.1 127	28-371 11 158 / /	[291] [292] [293] [294] [295]
GPR43 (FFA3)	>1000 12 50-200 431	6-127 11.7 50-200 290	42-158 371 / /	[291] [292] [293] [295]
GPR109a (HCA2)	/ /	/ /	700-1600 1600	[291] [296]
Olfr78	2350	920	/	[297]

Table 2: Reported EC_{50} values (µM) for SCFA on the most described SCFA receptors

III.2.1 Gut

SCFA play several roles in the gut going from homeostasis maintenance to the prevention of several disorders.

A key aspect of gut homeostasis is the mucus layer integrity. It has been shown that the consumption of a high fibre diet, resulting in higher SCFA production, is important to maintain a high mucus layer thickness. This in turn helps keeping the bacteria population far from the mucosa, reducing the risk of diseases [298]. Supplementation with fibres prevents the development of high fat diet-induced obesity notably by mechanisms linked to the mucus layer integrity [299]. SCFA were also reported to play a role in the maintenance of tight junctions in epithelial monolayer thus improving gut barrier function [300].

Given their role in the homeostasis of the gut barrier, dysregulation in SCFA levels can lead to increased gut permeability, a phenomenon known as “leaky gut” [301]. This in turn results in an increase of endotoxin (e.g. LPS) influx into the blood circulation causing a low-grade inflammation condition. This metabolic endotoxemia is an important factor in the development of obesity [302, 303]. Conversely, improving the gut barrier function has been suggested to counteract the development of obesity [304].

From a metabolic perspective, acetate and propionate play an important role in the secretion of glucagon like peptide-1 in the gut [305]. It was also reported that butyrate is able to induce the secretion of peptide YY by enteroendocrine cells in the distal intestine [305, 306]. GLP-1 and PYY playing important roles in the dietary intake and insulin production. Actually, type II diabetes was linked to changes in the microbiome and SCFA levels [307-309].

Inflammatory bowel diseases (IBD) were also linked to alterations in gut microbiota population [310]. SCFA are described to prevent IBD by controlling several immunological effectors that play roles in IBD initiation. These effects are linked to the binding of SCFA to GPCR (as mentioned above) but also due to their capacity to act as histone deacetylase inhibitor (i.e. butyrate)[311]. Moreover, most of the studies investigating these aspects have found that development of IBD is accompanied by dysbiosis. The dysbiosis favours a persistent alteration of the gut homeostasis with persistent immune activation and inflammation.

In addition to the evident link between gut microbiota and gut diseases, some papers showed over the years the importance of gut microbiota and microbiota metabolites (e.g. SCFA) to prevent several cardiovascular events (e.g. stroke, heart failure) (**figure 25**)[312-317]. It is not well understood how microbiota metabolites are involved in these effects but several

correlations were showed between both. For instance, beneficial effects of butyrate administration to prevent ischemic stroke were observed on several mice models [318].

III.2.2 Brain

Although its mechanisms are not fully understood, the existence of a gut-to-brain axis is largely described. This axis in involved in the control of satiety, learning processes, or mood and stress. SCFA are suggested to be involved in this axis as lipid mediators either directly (e.g. acetate is able, by direct brain activation, to induce satiety [319]) or indirectly.

SCFA have been linked to several central nervous system disorders such as depression, anxiety, Alzheimer's disease or Parkinson's disease [252, 320]. Indeed, as it was reported for gut barrier integrity, SCFA contribute to the brain blood barrier (BBB) integrity by affecting the tight junction and therefore the expression of inflammatory mediators. Interestingly, inflammation is thought to be involved in a broad range of nervous system disorders. Butyrate and propionate have been shown to reduce beta amyloid depots, a key point in the emergence of Alzheimer's disease [321] whereas propionic acid was suggested to impair social behaviour and lead to cognitive dysfunction similar to the ones observed in autism disorders [322].

One of the pathways described for SCFA is the vagal nerve pathway. Indeed, SCFA produced in the gut can either stimulate the secretion of hormones (i.e. insulin, leptin, ghrelin) or directly be transported through the vagal nerve to act on the CNS. Moreover, it is reported that SCFA are able to use transporters as entry point to the central nervous system [252]. It is for example the case for the SLC16A1 ubiquitously expressed in brain tissues. Interestingly, this transporter is also able to bring ketone bodies and citrate cycle metabolites inside the

central nervous system tissues [323, 324]. SCFA being substrates of these transporters opens the possibility of direct effects of SCFA transported in the blood stream.

Given the number of studies associating CNS diseases and gut microbiota alterations one can speculate on the importance of SCFA in these diseases. However, causative studies are needed to better link changes in SCFA intestinal or circulating levels and CNS disorders.

III.2.3 Immunity

Another “hot area” in microbiota’s effects is represented by the immune system. Even if bacteria-derived indole derivatives have been put forth in these effects [260], numerous reports support a role of SCFA. This is notably due to the fact that SCFA can bind to cell surface receptors expressed by immune cells (e.g. macrophages and neutrophils) as well as epithelial cells [325-327]. SCFA have been shown to decrease the inflammatory response in several models (e.g. DSS-induced colitis model of IBD [325], RSV infection [328], Methionine- and Choline-Deficient Diet-induced NASH [329]). Again one can speculate that decreased SCFA levels, due for instance to dysbiosis, can have important repercussions on immune and inflammatory processes [330]. The effect of SCFA on the immune system can in part explain the plethoric effects observed for SCFA.

As this is not an exhaustive review on SCFA’s effects, we focused here on selected organs. Of course SCFA are also described to act on the kidney [331, 332], the lungs [253, 333-335], the liver [261], …

Nevertheless, to better characterize the implications of SCFA in health and disease two things appear necessary (i) pharmacological/molecular tools to allow causative studies and (ii) the development of reliable, sensitive, and specific analytical methods to characterize metabolite

variation in various biological matrices. The latter point is the focus on the next section. Indeed, while numerous papers report SCFA levels the challenges to overcome in order to be as close as possible to the actual levels are numerous.

III.3 SCFA analysis

III.3.1 SCFA relevant matrices: sampling and special care

Caecal content and faeces

Caecal content and faeces are the most analysed matrices for the determination of SCFA [264, 265, 336]. Indeed, changes in SCFA levels in those matrices give a relatively direct idea of the consequences of changes in the gut microbiota communities [337-339]. Faeces are also interesting as a non-invasive collection of human samples is possible. However, several difficulties, related to the sampling and normalization, exist when quantifying SCFA in these matrices. As the heterogeneity inside the faecal samples is important, these two steps are crucial. Even if masses ranging from 0.03 g to 2 g of test sample were reported in the literature [264, 265, 340-343], Hsu and colleagues showed that an initial 1 g of human faecal sample is optimal to minimize sample variability. This is if appropriate mixing of the whole sample is performed. However, nothing was reported on the optimal faecal sample amount when working with mice samples [344]. Concerning the storage conditions, samples need to be kept at least at -20°C for SCFA quantification as they are known to be volatile compounds [344-346]. Moreover, as shown by the Bristol stool scale [347], it is important to consider differences in water content between the cohort of samples studied. To take this element in consideration some groups set up a freeze-drying procedure to normalize to the dry mass of sample [344, 346]. However, as SCFA are volatile this step is not straightforward. For example, we assessed and determined recently that it is better to apply this step separately to SCFAs

analysis to avoid possible earlier degradation that could occur by doing it as initial step. This point will be discussed further in the result section.

Plasma

Next to caecal content and faeces, plasma is the main matrix used for SCFAs analysis. As in direct contact with the gastrointestinal tract (especially in the portal vein), it is one supposed biofluid for the SCFA-driven effects [252, 261]. Compared to caecal content or faeces, normalization is easier in the case of plasma. Nevertheless, with mouse plasma, depending of the site of collection, changes in SCFA level could appear due to hepatic first pass. It is important to take that into consideration when comparisons while clinical samples are done (considering that the portal blood is an uncommon matrix in humans). Plasma remains a relevant matrix to SCFA variations even if the reported turnover for these molecules is in the order of minutes [348].

Solid tissues (brain, liver, lung, …)

The link between gut microbiota and neurological or hepatic disorders is now well established and could be mediated, at least in part, by SCFA [252, 258, 261]. More recently, the microbiota communities in the lung seem to show similar role than in the gastro-intestinal tract for the respiratory system physiology [253, 349, 350]. Thus, it seems important to consider solid tissues, such as brain, liver or lung, for SCFA quantification. This could be a challenging task due to the lower abundance of SCFA in those tissues [351, 352] (and result section II), but could be crucial to determine whether SCFA are present in the tissue at levels compatible for instance with receptor activation. For now, a few papers mentioned quantification in solid

tissues but this will increase in interest in the next years and the improvement of the analytical methods will help to solve the current sensitivity limitations.

Bronchoalveolar lavages fluid (BALF)

With the emergence of the interest for SCFA in the lung, considering bronchoalveolar lavages fluid (BALF) for the analysis of SCFA is important. To the best of my knowledge, only Ying-Ying Jin et colleagues described and validated a method applicable to their quantification in BALF samples [351]. To solve the issues related to the difference of volume obtained when recovering BALF from mice, they applied a freeze-drying procedure to resuspend the samples in identical volumes. However, this could lead to quantification bias due to loss of analytes during the process (see result section II). Thus, more investigations are clearly needed to determine appropriate procedures to normalize the data obtained from BALF samples. Finally, translational studies between mice and human are complicated as recovering BALF from human using invasive sampling methods is more difficult to implement.

Other biological fluids

Other relevant biofluids are gaining interest in the field of SCFA analysis. Among them, we find urine, milk or even saliva. Here again, the normalization is a challenging task. Creatinine clearance is increasingly used in metabolomic studies to normalize the data [353], thus this could be also assessed for SCFA quantification. For saliva and milk alternative strategies need to be tested and validated.

Besides the normalization method, these matrices are known to be dependent on the time of sampling [354, 355]. Again, this is a big hurdle when considering human studies where sampling is not always efficiently time-controlled.

III.3.2 SCFA extraction methods

Several extraction methods have been described for SCFA analysis. As mentioned above, this procedure is complex as no evaporation step can be implemented due to the volatility of most SCFA [355, 356]. For the same reason, the risk of cross contamination between high and low concentrated samples during processing is important to keep in mind. Finally, it is important to limit the SCFA contamination through the solvent used during the extraction procedure by using the smallest volume possible.

As polar and water-soluble compounds, SCFA extraction from biological matrices is highly challenging. To increase the lipophilicity of the SCFA, some methods described an acidification step allowing to keep the SCFA under protonated form [343, 357]. This improves their extraction by organic solvents however it increases their volatility. The addition of HCl (or other acids) could also lead to the hydrolysis of several compounds esterified with SCFA. Besides, the choice of the solvent is critical due to the frequent contamination by SCFA (see also the result section). For instance, ethyl acetate is described in the literature to contain acetate and has to be excluded in the extraction procedure if no blank samples are analysed simultaneously [358]. Acetonitrile is another chemical precursor of acetate, thus increasing the risk of contamination if this solvent is used. The majority of the described methods use protein precipitation as extraction procedure to efficiently extract SCFA from complex matrices [263, 359-361]. The advantage of this method is the applicability to solid and liquid matrices as well as the short duration of the procedure. Moreover, as mentioned above, no evaporation step is then needed. Other methods such as liquid-liquid extraction can also be used, again keeping in mind the increased risk of contamination linked to the volume of solvent employed.

III.3.3 Sample preparation methods

Once extraction is achieved, the samples need to be prepared differently depending on the method of analysis used. We will focus here on GC and LC as they are the two most described methods for SCFA analysis. If coupled to mass spectrometry, it is important to consider the ionisation pattern of the derivatized products. When most of them satisfy from an ESI ionisation source, some can require an APCI ionisation source.

GC-compatible sample preparation

The use of GC as analytical method requires samples adapted for analysis in gas phase. For that, derivatization methods are often used [362, 363] however as SCFA are volatile compounds, several groups described their analysis without any derivatisation [364]. Practically speaking, after their extraction, SCFA need to be dissolved in appropriate solvent such as hexane. The issues of using GC for SCFA analysis without derivatisation are linked to the complexity to discriminate SCFA generated from sample or generated by contamination (solvents, air, …). Moreover, depending on the type of detector (e.g. FID), coelution problems can occur without the implementation of longer analytical run.

For those reasons, other strategies consisted in using derivatisation procedures. Several methods proper to GC analysis are widely described and applicable to carboxylic containing molecules such as SCFA [362, 363]. Nonetheless, several criteria are important to consider in the case of SCFA. Firstly, even if ultimately the analyte needs to be injected in a volatile enough solvent (e.g. hexane or octane), the derivatization procedure needs to be suitable to water containing samples (faeces, plasma, tissue homogenate, …) to minimize the addition of potentially contaminated organic solvents. Secondly, all the derivatization reagents are not

suitable for GC analysis as their vaporization temperatures are too high. Finally, it is important to consider the matrix added during the derivatization procedure. A description of several derivatization methods efficiently applied to GC analysis of SCFA are reported in **Table 3**.

derivatization reagent	cofactors	method description	advantages	inconvenients	sources
N-methyl-N-t-butyldimethylsilyltrifluoroacetamide (MTBSTFA)	/	80 °C during 20 min and then room temperature for 24h	- easy to execute - products stability	- excessive heating - time-consuming - large quantities of reagents	[365]
propyl chloroformate	pyridine	mixture of propanol/pyridine added with the reagent, ultrasonication during 1 min and twice liquid/liquid hexane extraction. Sodium sulphate finally added to sampling vial to remove water residues	- easy to execute - fast	- toxicity	[366]
ethyl chloroformate	pyridine	mixture of ethanol/pyridine added with the reagent, ultrasonication during 1 min and twice liquid/liquid hexane extraction. NaOH added to adjust the pH and sodium sulphate added to remove water residues	- easy to execute - fast	- toxicity	[367]
Isobutyl chloroformate	pyridine	mixture of isobutanol/pyridine added, mixed thoroughly, gas release during 1 min, HS-SPME strategy	- easy to execute - fast	- toxicity	[368]
2, 3, 4, 5, 6-penta-fluorobenzyl bromide (PFBBr)	/	PFBBr added together with acetone in 0.5 M phosphate buffer, mixed during 1 min and then incubated in a water bath at 60°C for 90 min, liquid/liquid hexane extraction	- easy to execute - products stability	- excessive heating - time-consuming - toxicity	[369]
Benzyl chloroformate	pyridine	mixture of benzyl alcohol/pyridine added with the reagent, left 1 min without cap, closed and vortexed for 3 min. Then liquid/liquid cyclohexane extraction	- easy to execute - fast	- toxicity	[363]
Boron trifluoride (BF_3)	toluene	liquid/liquid lipid extraction, boron trifluoride in MeOH and toluene added to each tube, incubated at 100°C for 60 min, then liquid/liquid extraction by hexane/chloroform (4:1;v/v)	- easy to execute	- excessive heating - time-consuming - large quantities of reagents - toxicity	[370]
N-methylbenzylamine (MB)	N-[(Dimethylamino)-1H-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-N-methyl methaneminium hexafluorophosphate N-oxide (HATU)	MB in HATU, triethylamine, acetonitrile mixture was added to extracts, heated at 37°C for 20 min	- easy to execute - fast	- toxicity - cost (specific deuterated standards using d3MB)	[371]
tetramethylammonium hydroxide (TMAH)	/	added to extraction vial, then vortexed for 1 min, water added and vortexed 1 min more	- easy to execute - fast		[372]

Table 3: Derivatization methods applied to SCFA suitable to GC.

As reported in **table 3**, the derivatization methods suitable for GC targets the carboxylic group of the SCFA for the reaction. Some of them use electron acceptors to increase the reactivity with the SCFA, while some of them use strong acidic condition to favour the reaction. The reactions using chloroformate have the advantage of being fast and not requiring a heating step, a crucial point in the case of SCFA. However, they need an extraction procedure to recover products from the reaction mixture. The fatty acyl methyl esters (FAME) derivatives are less used in the newly described procedures due to their limitations in term of longer reaction times.

Despite these hurdles, the addition of derivatization step in the analysis of SCFA by GC is important to: 1) increase detection yield; 2) avoid potential further cross contamination.

LC-compatible sample preparation

In the case of LC, the advantage is that a broad diversity of sample is injectable on the LC system. However, as it is the case for GC, the analysis of SCFA without pre-treatment comes with increased risk of contamination due to the mobile phases and pH modifiers used. This is the reason why, even if, in theory, this is not strictly required, derivatisation methods are often applied when analysing SCFA by LC-based methods [265, 373, 374]. The second advantage in the case of those small molecules is to increase the m/z ratio of the analysed compound as this allows to analyse them in spectral regions less affected by the background noise (higher in the lower m/z values). Moreover, some derivatization methods used in GC can easily be transposed to LC analysis (e.g. fatty acid methyl esters generation) and some water compatible derivatization procedures not suitable to GC analysis as forming products with low vaporization capacities will be suitable for LC analysis [373, 375, 376]. For example, this is the case for 3-nitrophenylhydrazine, widely used for SCFA derivatization. The other advantages of

the 3-nitrophenylhydrazine are its compatibility with water containing samples, such as biological tissues, as well as the improvement of the ionisation for the derivatized products. Several derivatization methods suitable for LC analysis are summarized in **Table 4**.

derivatization reagent	cofactors	method description	advantages	drawbacks	sources
3-nitrophenylhydrazine (3-NPH)	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) / pyridine	SCFA extract put in a glass vial, 3-NPH added together with EDC/pyridine mixture, heated at 40°C for 30 min, reaction quenched by addition of acidified water, supernatant recovered	- easy to execute - products stability - mild heating conditions	- toxicity - post extraction procedure	[375]
2-bromoacetophenone	Na_2CO_3	2-bromoacetophenone in acetonitrile added to extraction mixture, heated at 40°C for 20 min. The mixture was centrifuge for 5 min and then an aliquot recovered	- easy to execute - products stability - mild heating conditions	- isomer separation	[376]
O-benzylhydroxylamine (O-BHA)	EDC	mixture of EDC/O-BHA was added to the extraction mixture, heated at 23°C for 10 min. Then liquid/liquid extraction using ethylacetate was used	- easy to execute - fast - mild heating conditions	- toxicity	[373]
N, N-dimethyl-6,7-dihydro-5H-pyrrolo [3,4-d] pyrimidine-2-amine (DHPP)	EDC, 2-mercaptoethanol and succinic acid	DHPP and EDC in methanol were added to the dried extract, heated at 30°C for 3 min until dryness under nitrogen stream. Reaction was quenched with 2-mercaptoethanol and succinic acid	- easy to execute - fast	- several evaporation steps - cost (specific deuterated standards using DHPP-d6)	[377]
2-picolylamine (2-PA)	EDC / 3H-1,2,3-triazolo [4,5-b] pyridin-3-ol (HOAt)	2-PA added to the extraction supernatant, together with EDC and HOAt, heated at room temperature for 10 min. Sample diluted in MeOH/water before injection	- easy to execute - fast	- toxicity	[378]
4-aminomethylquinoline (AMQ)	O-(7-azabenzotriazol1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) / N,N-Diisopropylethylamine (DIPEA)	HATU was added with AMQ:DIPEA (1000:1;v/v) in acetonitrile and shaken for 20 min, PBS and MTBE were added and shaken at 25°C for 20 min, upper organic layer was taken	- easy to execute - stability	- numerous steps	[379]
4-Acetoamido-7-mercapto-2,1,3-benzoxadiazole (AABD-SH)	Triphenylphosphine (TPP), 2,2'-dipyridyl disulfide (DPDS)	reagent in CH_2Cl_2 added to the supernatant extract, then vortexed at room temperature for 5 min, dried and reconstituted in MeOH	- easy to execute - fast	- toxicity	[380]
Aniline-^{12}C6 and Aniline-^{13}C6	EDC	supernatant extract derivatized by aniline in presence of EDC at 4°C for 2h, then reaction quenched using succinic acid and 2-mercaptoethanol	- easy to execute - fast	- toxicity - cost (specific internal standards using Aniline-13C6)	[381]

Table 4: Derivatization methods applied to SCFA suitable to LC.

The aim when using derivatization methods in the case of LC methods differs from the aim when GC is used. Indeed, in the latter, derivatization is used to increase volatility and/or increase the interaction with the stationary phases. In the case of LC-based methods, the derivatization reagent can increase the retention of the analyte by adding polar functions or aromatic rings (conferring hydrogen bond interactions and pi interactions, respectively) and/or add an ionizable moiety conferring a better ionization yield when MS is used as detector (resulting in improved sensitivity compared to underivatized SCFA).

Of note, in the case to MS detectors (for both GC and LC) derivatizing allows to increase the molecular mass. This is required for the analysers with lower limit for m/z values above the m/z of acetate or formate. Besides, it allows further fragmentation in the analyser thus facilitating the use of MS/MS analysers and the possibility of fingerprinting for the identification of the derivatized compounds of interest.

III.3.4 Sample clean up procedure

Most of the validated methods described for SCFA derivatization do not report the use of post reaction clean-up procedure [263, 381]. Nonetheless, depending of the type of method used, this could have some advantages (e.g. [382] and result section II)

Even if this step increases the time of sample preparation, it comes with several advantages. The main one is the decrease of matrix charge resulting from the derivatization procedure. This excess of matrix increases (i) the risk of decreasing the lifespan of the chromatographic (GC or LC) columns, (ii) the risk of clogging of the MS instrument used (if MS is used as detector) and comes with the substantial risk of co-elution or increased matrix effects during the analysis.

The challenge of applying a sample clean up procedure is the structure similarity existing between derivatized products and the unreacted reagent(s) due to the small size of the SCFA. Usually, liquid-liquid extraction, was the chosen method to clean-up the samples. By modifying the pH of the solution or using some salt exclusion techniques simultaneously to the choice of solvent with different polarity, it is possible to efficiently remove the reagent excess from the SCFA product solution (see chapter 2 of the result section). Other procedures involve SPE to efficiently remove the excess reagent(s) [382, 383]. Even if the purification capacity and selectivity are higher with this type of technique, to the best of our knowledge, no report of this use was present in the literature.

III.3.5 Analytical methods for the quantification of SCFA

Several analytical methods have been reported to efficiently measure SCFA. Even if methods as capillary electrophoresis or nuclear magnetic resonance were also described in the

literature, GC and LC remain the two most largely used methods [380-383]. Thus, in the next sections I will focus only on those two methods. For each of them, the use of internal standard(s) is important for data normalization. Depending of the type of analysis, the choice of internal standard is a crucial step. Similarly to the majority of analytical methods, the selection of an analogous bearing a stable isotope (e.g. deuterium) of the molecule of interest is the best analytical (if not economical) choice.

Gas chromatography separation

Described as the method of choice for a long time, several limitations of GC-based methods and improvement in LC methodologies resulted in a shift from GC to LC in the past few years. Nonetheless, GC remains one of the most used techniques to quantify SCFA. The first limitation linked to GC is the potential thermal degradation occurring during analysis [384]. Another possible limitation depending of the detector used, is the potential co-elution between SCFA and solvent front or other low molecular weight biological metabolites [385]. On the other hand, and contrary to LC, separation of SCFA without derivatization is easy to be implemented on GC [372, 386]. SCFA are indeed well adapted to capillary GC separation, including at the level of isomers. Naturally, the appropriate choice of the capillary column is crucial to achieve isomer separation (**Table 5**).

Liquid chromatography separation

For LC, the choice of an adequate stationary phase is crucial to allow isomer separation. Together with the column selection, determination of mobile phase composition and gradient are the second important parameters to optimize to achieve SCFA separation. It is important to consider the small size of the analytes as well as their relative polarity (even if SCFA are

considered lipids). In fact, SCFA have a similar behaviour during separation due to the common polar head (i.e. the carboxylic acid moiety), and a rather poorly discriminating lipophilic tail due to its limited size. Yet, the presence of the acyl chain results in difficulties to use polar stationary phases (such as the one used in normal phase LC). Interestingly, the group of Watson used a HILIC column to achieve SCFA separation [387].

Nevertheless, the use of reversed phase chromatographic columns is now widespread for SCFA analysis, even if retention times remain short due to the limited interaction between analytes and column. This is one of the reasons for the increased interest in derivatization methods focusing on the carboxylic group.

Table 5 summarizes several stationary phases used for GC and LC separation of SCFA [266, 361]. Of course, the choice of solvent (or temperatures for GC analysis) remains critical to obtain adequate separation of those compounds on the selected column. As suggested from **table 5**, the choice of stationary phases to efficiently separate SCFA in complex matrices is quite large. Regarding the different reports in the literature, it seems that mid-polar strategies with carbon acyl chains grafted to one aromatic/hydrogen bound acceptor moieties are the most used (e.g. pentafluorobenzyl column). It can be a grafted (or ungrafted) phenyl as well as ether links, smaller acyl chains links, …

column name	stationary phase	affinity	sources
gas chromatography columns			
Trajan™ BP1	polydimethyl siloxane	aromatic compounds, amines, hydrocarbons	[388]
Trajan™ BP10	dimethyl siloxane with 14% cyanopropylphenyl siloxane	medium polar compounds	[388]
Trajan™ BPX70	70% cyanopropyl polysilphenylene-siloxanes	fatty acyls, carbohydrates	[388]
Agilent DB-23	50% cyanopropyl-methylpolysiloxane	fatty acyls, carbohydrates	[389]
Agilent HP-5	5% phenyl-methylpolysiloxane	non polar compounds, aromatics	[390]
liquid chromatography columns			
Ascentis Express Phenyl-Hexyl	phenyl-hexyl	aromatic rings, slightly polar compounds	[391]
Restek Raptor C18	octadecylsilane (C18)	lipophilic and slightly acidic compounds	[263]
Kinetex Evo C18	core-shell octadecylsilane (C18)	lipophilic and slightly acidic compounds	[373]
Acquity UPLC HSS T3	octadecylsilane (C18) with T3 bonding	polar and apolar organic compounds	[392]
ACE C18-AR	octadecylsilane-phenyl (C18)	lipophilic compounds with aromatic ring	[393]
Kinetex XB-C18	core-shell silica/C18 with iso-butyl chains	lipophilic compounds with peak shape improvement	[394]
Waters BEH C18	highly grafted C18 column	lipophilic compounds	[379]
Synergi Hydro-RP	octadecylsilane-phenyl with ether link (C18) and polar endcapping	lipophilic, polar and aromatic compounds	[360]
Kinetex-PFP	pentafluorobenzyl engraftment	aromatic rings and slightly polar compounds	[395]

Table 5: representative gas and liquid chromatography columns with their respective stationary phases

Short chain fatty acids detection and analysis

Several detectors were efficiently used for SCFA detection following GC and LC separation. These include FID [340, 385, 396], ultraviolet detector (UV) [397, 398] and MS detector [265, 343, 373, 399]. Unfortunately, some shared limitations exist between FID (for GC analysis) and UV (for LC analysis) detector. The main could be the absence of compound specificity for those type of detection, when considering SCFA isomers, as well as with other endogenous compounds. The other main issue observed with those detectors is the relatively low

sensitivity towards SCFA. As long as the focus of the research field is on SCFA-rich matrix (e.g. caecal content) the poor sensitivity is of limited concern, but with the broadening of the scope of SCFA research sensitivity is an increasing issue. This can explain why FID and UV are much less used nowadays.

Clearly MS is the gold standard for SCFA detection (for both GC and LC analysis). Additionally, with the derivatization that potentiate SCFA detection, sensitivity can reach the femtomole or even lower levels [341, 393, 400]. For the same reasons, recent works mentioned the use of tandem- or triple quadrupole detectors for SCFA analysis in multiple reaction monitoring as these detectors exhibit better sensitivities than the ones obtained with single traps, or other detectors (e.g. orbitrap, TOF detectors, …) [265, 373, 393].

III.3.6 Short chain fatty acids quantification in biological matrices

Physiological relevance of SCFA quantification in tissues

When discussing the potential biological significance of an endogenous mediator, it is important to take into consideration its levels and its affinity for the relevant biological targets (e.g. receptors) (see **Table 2**). For instance, while butyrate is found at rather high levels in the gut, it is present at much lower concentration in the plasma raising the question of its potential effects outside the gut. Besides the reports in the GI tract and circulation, SCFA levels in other biological compartments (e.g. sputum of patients [401]) could also reach levels compatible with physiological significance. Clearly SCFA have been less studied in other tissues than the gut environment, and more studies are needed to have a complete picture of their presence. This further supports the relevance of developing sensitive methods [401, 402].

An overview of SCFA and BCFA abundance is available in **appendix table 3 and 4** for mice and human respectively. As for the prostaglandin derivatives, it exists a heterogeneity in the

reported values depending to the method used, the variety of processes as well as the sample management. Faeces is the most studied biological sample in the case of short and branched chain fatty acids. It is probably due to the relevance of this sample in the study of bacteria-driven effects and the availability of such samples.

Short chain fatty acids as potential biomarkers

As an association between SCFA and several pathological conditions are more and more established [300, 305, 310, 320, 325, 330, 402], the idea to use SCFA as biomarkers emerged in the past [403-405]. For example, Farup et al. proposed the use of the relation "propionic acid minus butyric acid" level as an IBD diagnostic biomarker [403]. Sarah et al. reported preliminary data on a possible link between reduced psychological well-being of patients suffering of congestive heart failure and plasmatic concentrations of SCFA [404].

However, as SCFA levels also depend on the microbiota and dietary sources [406], the use of SCFA alone as biomarker do not really appears as conceivable. Interestingly, Mayerhofer et al. suggested to consider SCFA levels together with other physiological parameters to fine-tune the diagnostic [407].

The development of new, more sensitive quantification methods together with the consideration of multivariate analysis combining SCFA levels with bacteria population, cell barrier integrity and other bacteria-related features could potentially overcome some of the limitations mentioned here. In this case, SCFA levels could be an interesting tool in the way to characterize health and disease.

AIMS

As mentioned in the introduction, prostaglandin derivatives and SCFA are two important families of metabolites having many roles in health and disease.

Prostaglandins are strongly linked to inflammation. This is notably due to the fact that the rate limiting enzyme of this pathway is the enzyme COX-2, a well-established inflammation-inducible enzyme. As summarized above, the interplay between the pathways linked to COX-2 is complex and a lot remains to be done before reaching a full understanding of the parameters linked to changes in prostaglandin, and prostaglandin derivative, levels. Moreover, these molecules are very low in abundance increasing the challenge for a complete pathway characterization.

Short chain fatty acids, are bacteria-derived metabolites for which the interest continue to grow in parallel with the growing demonstration of microbiota-induced effects. As many bacteria species within the microbiome remain to be characterized, one can assume that the interest for the SCFA will remain high. While several methods to quantify SCFA have already been described, most of the time they only focus on the more abundant SCFA. Besides, as discussed in the Introduction, the understanding of SCFA metabolism and role will benefit from methods allowing their quantification together with closely related metabolites.

Thus, the main goal of this thesis is the development of two quantification methods:

- In result section I, I describe our work aiming at developing a method for the quantification of prostaglandins, prostaglandin glycerol esters, prostamides as well as their respective precursors.

- In result section II, I describe our work aiming at developing a method allowing for the quantification of short chain fatty acids, branched chain fatty acids, citrate cycle metabolites and ketone bodies.

Both methods require a single run analysis and several validation criteria were assessed in order to provide reliable methods allowing for the quantification of the metabolites of interest in diverse biologically-relevant matrices.

As explained in the Introduction, we faced challenges related to the poor endogenous abundance of several analytes, the presence of isomers, and the fact that we wanted to develop methods compatible with a large array of biological matrices.

Taken together, the two chapters of the result section will illustrate several of the challenges the analyst interested in lipid quantification in biological setting has to face.

RESULTS

Result section I

The data reported so far support the interest of studying prostaglandin-glycerol esters (PG-G) and prostaglandin-ethanolamides (or prostamides, PG-EA) in inflammatory conditions. However, there is only a handful of methods described for their quantification in biological matrices. Moreover, given the shared biochemical pathways for arachidonic acid, 2-AG and AEA, a method allowing for the quantification of these precursors and the corresponding prostaglandin derivatives appears as largely needed. Thus, in this section of the results (section I) I report the development and validation of a single run UPLC-MS/MS quantification method allowing the quantification of these endocannabinoids-derived mediators together with the classical prostaglandins. Moreover, we applied the method to the quantification of these lipids in vitro (using lipopolysaccharides-activated J774 macrophage cells) and in vivo in several tissues from DSS-induced colitis mice. This femtomole-range method should improve the understanding of the interaction between these lipid mediators and inflammation.

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Set up and validation of a sensitive method to quantify prostaglandins, prostaglandin-glycerol esters and prostaglandin-ethanolamides, as well as their respective precursors.

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I. INTRODUCTION

Prostaglandins are well known lipid mediators produced following the metabolism of arachidonic acid by cyclooxygenases (COX, also known as prostaglandin-endoperoxide synthases) followed by specific prostaglandin synthases (**Figure 1**). While arachidonic acid is the prototypical COX-substrate, COX-2 (or PTGS2) is able to use other lipids as substrate, including the endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide (AEA) [167, 408]. When these two endocannabinoids are the substrates, the PGH_2 derivative resulting from the COX-mediated bis-dioxygenation (i.e. PGH_2-G and PGH_2-EA) is then taken up by prostaglandin synthases to produce the glycerol ester (prostaglandin-glycerol ester, PG-G) and ethanolamide (prostaglandin-ethanolamide or prostamide, PG-EA) derivatives of PGD_2, PGE_2 and $PGF_{2\alpha}$ [408-410] (**Figure 1**).

It is well established that numerous inflammatory stimuli increase the expression and activity of COX-2 resulting in increased production of prostaglandins (e.g. PGD_2, PGE_2, …) which play a role in controlling inflammation [141, 157, 411-413]. Less is known on the role of the 2-AG derivatives (PG-G) and AEA derivatives (PG-EA) in this context but their study is gaining interest. Reports from our lab and from others point to the biological interest of these lipid mediators [184, 187]. For instance, we have shown that PGD_2-G decreases TLR4-mediated macrophage activation in vitro as well as DSS-induced colitis in vivo [162, 176]. Hu and co-workers showed that intraplantar administration of PGE_2-G induced mechanical allodynia and thermal hyperalgesia [181]. In similar models we have shown that PGD_2-G decreases carrageenan-induced hyperalgesia [184]. Di Marzo's group reported the effects of $PGF_{2\alpha}$-EA on inflammatory pain while Rotondo and co-workers described the effect of PGE_2-EA on monocyte activation [414, 415] . Moreover, Woodward and colleagues put forth the role of prostamide derivatives (e.g. $PGF_{2\alpha}$-EA) in controlling intraocular pressure. These studies

resulted in the $PGF_{2\alpha}$-EA analogue Bimatoprost being commercialized for the treatment of glaucoma [182].

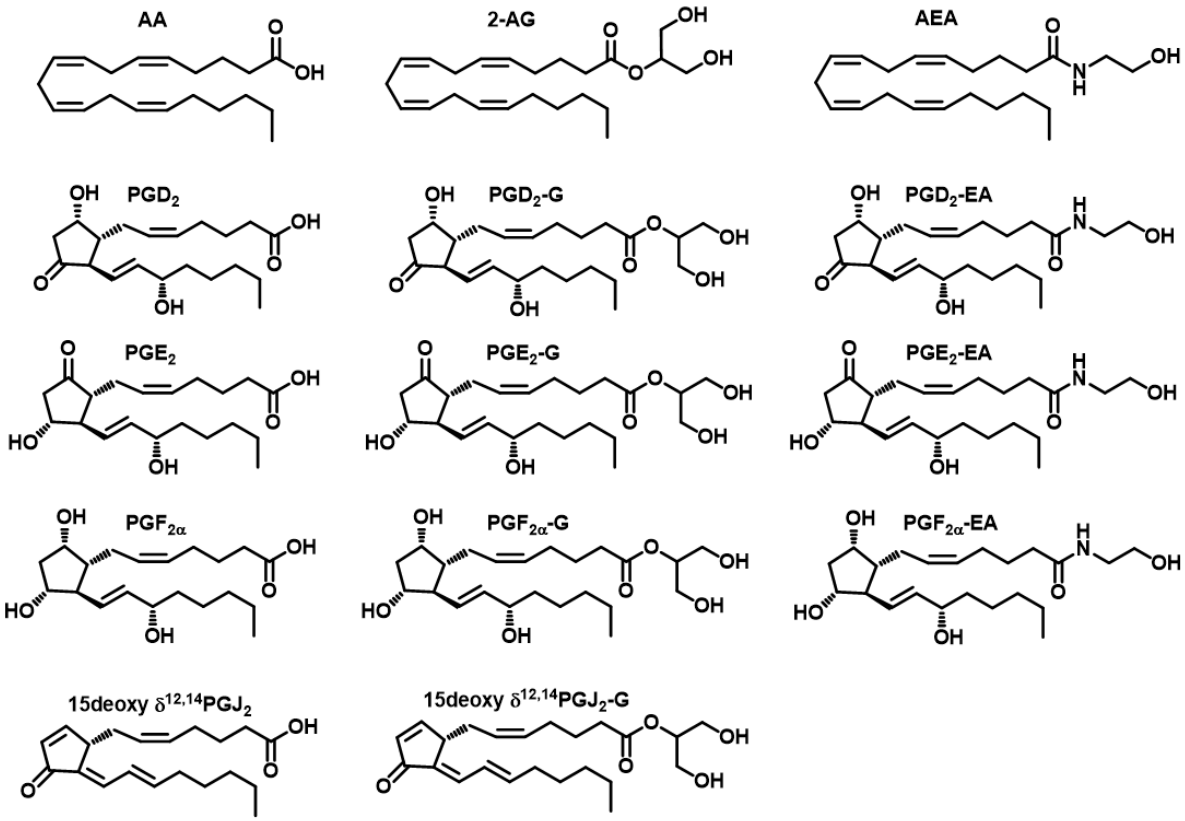


Figure 1: Chemical structure of the lipid mediators quantified by the validated method. Chemical structure of the prostaglandins, prostaglandin-glycerol esters, prostaglandin ethanolamides as well as their respective precursors (top row).

While the administration of these molecules (or analogues) or drugs interacting with their metabolism or activity (e.g. COX inhibitors, receptor antagonists) is essential to unravel their biological properties [133, 410, 416, 417], a quantification method would bring important complementary information such as the consequences of a given pathophysiological condition on the levels of these lipid mediators. In this line, the quantification of PG and endocannabinoids by HPLC-MS is well established [213, 418-420]. However, only a very limited number of methods are described to quantify PG-G and PG-EA derivatives. This can be due to the challenges inherent to their quantification, such as their closely related structures

(**Figure 1**), and the low levels in biological tissues, especially in uninflamed conditions, for several of these mediators [245].

Gouveia-Figueira et al. reported the quantification of PGE_2-EA in brain tissue from elk, cow and pig using however rather large amounts of tissue (80 mg to 180 mg) when considering the overall lipid content of a brain [244]. We previously detected 15-deoxy$\delta^{12,14}$prostaglandin J_2-glycerol ester (15deoxy$\delta^{12,14}PGJ_2$-G) in the colon of mice using the whole colon (around 200 mg) [176]. More recently, Simone and colleagues used ten mouse dorsal root ganglions to achieve the measurement of PGE_2-G [421]. Finally, Morgan and colleagues detected PG-G in whole mice brain by inhibiting monoacylglycerol lipase (using the inhibitor JZL-184) in mice overexpressing COX-2 [246]. Nevertheless, to our knowledge there is no report of a method allowing the simultaneous quantification of these three families of bioactive lipids and their precursors (i.e. arachidonic acid, 2-AG and AEA).

As an increasing number of reports support the interest of these lipid mediators in pathophysiological situations, we decided to develop a method for the simultaneous extraction, purification, detection, separation and quantification of the PG-G and PG-EA. Moreover, we reasoned that the understanding of the biological phenomena at play would gain from the concomitant quantification of their precursors, namely 2-AG and AEA, as well as from the quantification of the corresponding prostaglandins. Indeed, as we previously reviewed, these biologically relevant lipid mediators share metabolic pathways [133, 410].

Given the low abundance of several of these lipid mediators [133], besides the optimization of the UHPLC-MS/MS method, as for the other methods we developed, we also focused on the setup of the liquid-liquid extraction and solid-phase extraction (SPE) pre-purification of the analytes [98, 422]. Following the setup of the method and its validation, we demonstrated

its applicability to the detection of the lipid of interest using lipopolysaccharides (LPS)-activated J774 cells as well as a mouse model of colitis.

II. MATERIALS AND METHODS

II.1 Chemicals

Prostaglandins (prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 15-deoxy$\delta^{12,14}$prostaglandin J_2 (15deoxy$\delta^{12,14}$-PGJ_2)), prostaglandin-glycerol esters (prostaglandin D_2-glycerol ester (PGD_2-G), prostaglandin E_2-glycerol ester (PGE_2-G), prostaglandin $F_{2\alpha}$-glycerol ester ($PGF_{2\alpha}$-G), 15-deoxy$\delta^{12,14}$prostaglandin J_2-glycerol ester (15deoxy$\delta^{12,14}$$PGJ_2$-G)), prostaglandin ethanolamides (prostaglandin D_2-ethanolamide (PGD_2-EA), prostaglandin E_2-ethanolamide (PGE_2-EA), prostaglandin $F_{2\alpha}$-ethanolamide ($PGF_{2\alpha}$-EA)), as well as their respective precursors (arachidonic acid, 2-arachidonoylglycerol (2-AG), anandamide (AEA)) were purchased from Sanbio (The Netherlands). Deuterated internal standards (d_8-arachidonic acid, d_5-1-AG, d_4-AEA, d_4-PGE_2, d_5-PGE_2-G and d_4-PGE_2-EA) were also purchased from Sanbio (The Netherlands). HPLC- and MS-grade solvents (chloroform ($CHCl_3$), methanol (MeOH), hexane (Hex), isopropanol (iPOH) and acetonitrile (ACN)) and MS-grade acetic acid were from VWR (Belgium). Butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) and LPS (*Escherichia coli*, O55:B5) were from Sigma Aldrich (Belgium). Dextran sodium sulfate (DSS) was purchased from TdB labs (Sweden).

II.2 Sample preparation and purification

For tissues, frozen samples were homogenized in $CHCl_3$ (8 mL). Subsequently, MeOH (4 mL, containing 10 µg of BHT), H_2O (2 mL, containing 20 ng of EDTA) and internal standards (d_8-arachidonic acid 15 pmol, d_5-1-AG 15 pmol, d_4-AEA 5 pmol, d_4-PGE_2 15 pmol, d_5-PGE_2-G 5 pmol

and d_4-PGE_2-EA 5 pmol/sample, respectively) were added to the vials. For the analysis of the lipids in J774 cell cultures, we prepared a series of vials for the analysis of the cell culture media containing a mixture of $CHCl_3$-MeOH (8:4; v/v) and the internal standards and a series of vials for the analysis of the cells containing $CHCl_3$ and the internal standards. Firstly, media was recovered and transferred to the corresponding vial. Then cells were scraped from the petri dishes with 2 mL PBS (+EDTA) and transferred to the corresponding vial. The petri dishes were then rinsed with 4 mL MeOH (+BHT) that were transferred to the corresponding vial.

Vials were sonicated thoroughly in an ice bath and then centrifuged 10 min at 700*xg* at 4°C. The organic layer was recovered and brought to dryness under a nitrogen stream. Samples were reconstituted in $CHCl_3$-MeOH (99:1; v/v) and transferred on solid phase extraction (SPE) columns packed with silica (SiOH) and conditioned with $CHCl_3$. Loaded SPE were washed with $CHCl_3$ and then the fraction of interest was recovered with 3.5 mL of $CHCl_3$-MeOH (7:3; v/v). The eluent was evaporated under a nitrogen stream and the final purified sample was reconstituted in MeOH (30 µL) for injection.

II.3 Lipid analysis by UPLC MS/MS

The reconstituted samples were injected (2 µL) on an acquity UPLC® class H system (Waters) coupled to a Xevo TQ-S mass spectrometer (Waters) used in multiple reaction monitoring (MRM) mode. Mobile phase A consisted in H_2O-ACN-acetic acid (94.9:5:0.1; v/v/v) and mobile phase B in ACN-acetic acid (99.9:0.1; v/v) used in gradient mode. An acquity UPLC® BEH C18 column from Waters (150x2.1 mm; 1.7 µm) maintained at 40°C was used for lipid separation. Mobile phase gradient was as follows: 80 % of A to 62.5 % of A in 7 min; then 62.5 % to 28.8 % A in 2 min before reaching 95 % B at 17 min; a plateau was maintained during 3 min before re-equilibrating the column. A divert valve was used at the beginning of the run (during 3 min) and at the end of the

run (starting at 18 min) to decrease the amount of matrix sent on the mass spectrometer system. Source parameters were designed for PGE_2, PGE_2-G and PGE_2-EA in both positive and negative mode, thus a total of 6 parameter sets. Then the optimal parameters for the mass spectrometer were selected based on the signal to noise ratio obtained after analysis of each of the analytes of interest in the six parameter sets. The selected parameters are as follows: capillary voltage at 3.60 kV; cone voltage at 30 V; desolvation temperature at 550°C; desolvation gas flow at 1000 L/h; cone gas flow at 300 L/h; nebulizer pressure at 7 bar. MRM transitions were then optimized **(supplemental table 1)**. For each molecule, cone voltage was optimized on the quantification transition (Q) and subsequently collision energy was optimized for the three principal MRM transitions on the selected cone voltage value to choose the parameters giving the best detection. Finally, for each compound, two transitions were selected, a quantification (Q) transition used for the calibration curves and a qualification (q) transition to support the nature of the compound detected.

name	analyzed MRM transitions	cone voltage (V)	collision energy (eV)
arachidonic acid derivatives			
AA	Q: 305.3>120.9 q: 305.3>78.4	30 30	20 30
PGD_2	Q: 317.2>299.2 q: 317.2>90.86	30 30	10 37
PGE_2	Q: 317.2>299.2 q: 317.2>90.86	30 30	10 37
$PGF_{2\alpha}$	Q: 301.0>156.9 q: 301.0>128.7	30 30	25 30
15deoxy$\delta^{12,14}PGJ_2$	Q: 317.2>90.9 q: 317.2>299.2	30 30	5 35
2-arachidonoylglycerol derivatives			
AG	Q: 379.2>287.3 q: 379.2>90.9	30 30	10 40
PGD_2-G	Q: 391.3>299.3 q: 391.3>373.2	30 30	10 10
PGE_2-G	Q: 391.3>299.3 q: 391.3>373.2	30 30	10 10
$PGF_{2\alpha}$-G	Q: 393.2>275.2 q: 393.2>128.9	30 30	15 20
15deoxy$\delta^{12,14}PGJ_2$-G	Q: 373.2>299.2 q: 391.3>373.2	30 30	10 5
anandamide derivatives			
AEA	Q: 348.2>61.9 q: 348.2>287.3	30 30	15 10
PGD_2-EA	Q: 378.2>360.2 q: 378.2>342.2	30 30	10 10
PGE_2-EA	Q: 378.2>360.2 q: 378.2>342.2	30 30	10 10
$PGF_{2\alpha}$-EA	Q: 380.3>344.2 q: 380.3>362.2	30 30	10 10
internal standards			
d_8-AA	Q: 313.3>82.9 q: 313.3>111.0	30 30	30 20
d_5-1-AG	Q: 384.3>287.3 q: 384.3>269.3	30 30	12 16
d_4-AEA	Q: 352.2>65.9 q: 352.2>287.3	30 30	16 12
d_4-PGE_2	Q: 321.2>275.0 q: 321.2>148.9	30 30	14 30
d_5-PGE_2-G	Q: 396.3>299.3 q: 396.3>79.1	30 30	15 40
d_4-PGE_2-EA	Q: 382.2>364.3 q: 382.2>346.2	30 30	10 10

Supplemental table 1: Description of the MRM transition parameters. Description of the quantification (Q) and qualification (q) MRM transitions used for each analyte of interest. The cone voltage (V) and collision energy (eV) parameters used for each analyte are listed as well.

To normalize the data, the ratio between the area under the curve (AUC) of the analyte of interest and the AUC of the respective internal standard was used. To cover the chemical diversity of the molecules of interest we selected six different internal standards (i.e. d_8-

arachidonic acid, d_5-1-AG, d_4-AEA, d_4-PGE_2, d_5-PGE_2-G and d_4-PGE_2-EA). When cells were analysed the data were normalized to the number of cells or the volume of media analysed. When tissues were analysed the data were normalized to the weight (or volume for the plasma) of sample.

II.4 Method validation

II.4.1 Specificity

The specificity was evaluated by analyzing several matrices (colon, brain, plasma) with the procedure described above. For each molecule, we selected a quantification transition (Q) and a qualification transition (q) to increase the confidence of the identification.

II.4.2 Trueness

Trueness was evaluated on the three quality control levels of the calibration curves (low, middle and high). Bias was determined on the reference values of the calibration curves and trueness was express in relative bias (%).

II.4.3 Linearity, calibration curves, LOD and LOQ

11 points calibration curves were designed using an evenly-spaced calibration points strategy. The calibration points solutions were prepared in methanol and to ensure that the calibration curves were as close as possible from biological sample analysis, the whole analytical procedure was used to perform the calibration curves. Following the analysis, the ratios between the AUC of the analyte of interest and the AUC of its internal standard were determined and linear regression was selected as mathematical model.

The lower points of the calibration curves were selected as starting points to determine LOD and LOQ by further serial dilution. Thus, for each analyte we prepared 10 serial dilutions and

the LOD and LOQ were determined using signal to noise values. Based on FDA [423] and European Pharmacopoeia (Ph. Eur.) [424] guidelines, we compared the signal obtained for a low concentration sample with the signal obtained at the same retention time in a blank sample analysed in the same manner. We selected the amount providing at least a signal to noise (S/N) of 3.3 in nine different replicates obtained from three independent experiment in the case of LOD and one S/N of 10 for the LOQ.

Linearity was controlled regarding the parameters of the linear regression. For each compound, five points covering the entire calibration range were selected and linearity was determined by plotting the ratio obtained by analyzing known amount of the respective five points in the calibration curves. The slope and R square values of the new linear regressions were then used as linearity criteria in accordance with the guidelines.

II.4.4 Intra- and inter-day precision and accuracy

To determine intra- and inter-day precision, we selected, as recommended by the guidelines (i.e. FDA, Ph. Eur.), at least three quality control levels: the LOQ, low, middle and high levels as reported in the **supplemental table 2** [423, 424]. The four levels were analysed at day one (five replicates per level) and then newly analysed at day 1, 2 and 3 (three replicates per level) to determine the different precision criteria. The coefficient of variation (CV) was determined for each level and had to be comprise in the ±20 % of confidence interval recommended by the guidelines when considering endogenous compounds or impurities presence in tissue homogenates [425].

name	quality control level	amount (fmol)
AA	LOQ	400
	LOW	4000
	MIDDLE	6000
	HIGH	8000
PGD_2	LOQ	100
	LOW	400
	MIDDLE	600
	HIGH	800
PGE_2	LOQ	100
	LOW	400
	MIDDLE	600
	HIGH	800
$PGF_{2\alpha}$	LOQ	200
	LOW	400
	MIDDLE	600
	HIGH	800
$15deoxy\delta^{12,14}PGJ_2$	LOQ	100
	LOW	800
	MIDDLE	1200
	HIGH	1600
AG	LOQ	60
	LOW	4000
	MIDDLE	6000
	HIGH	8000
PGD_2-G	LOQ	100
	LOW	400
	MIDDLE	600
	HIGH	800
PGE_2-G	LOQ	50
	LOW	400
	MIDDLE	600
	HIGH	800
$PGF_{2\alpha}$-G	LOQ	40
	LOW	400
	MIDDLE	600
	HIGH	800
$15deoxy\delta^{12,14}PGJ_2$-G	LOQ	100
	LOW	400
	MIDDLE	600
	HIGH	800
AEA	LOQ	3
	LOW	400
	MIDDLE	600
	HIGH	800
PGD_2-EA	LOQ	30
	LOW	400
	MIDDLE	600
	HIGH	800
PGE_2-EA	LOQ	20
	LOW	400
	MIDDLE	600
	HIGH	800
$PGF_{2\alpha}$-EA	LOQ	8
	LOW	400
	MIDDLE	600
	HIGH	800

Supplemental table 2: Summary of the quality control levels. The table summarizes for each analyte of interest the different quality control levels (LOQ, LOW, MIDDLE and HIGH) used for the validation of the method. Values are expressed in femtomoles

Accuracy was determined using the same quality control levels by comparing the bias of each values with the reference ratio of each level. Accuracy values were determined based on three

days of analysis. By computing the average values of each level, we determined the accuracy of the method.

II.5 Quantification of the lipids of interest in naïve and activated J774 cells

As we previously reported, J774 cells were cultured (37 °C in a humidified 5% CO_2 incubator) in RPMI 1640 medium containing fetal bovine serum (10%) and antibiotics (100 units/mL of penicillin and 100 µg/mL of streptomycin). For the experiments, cells ($18*10^6$ cells/sample) were incubated with LPS (*Escherichia coli*, O55:B5) at 100 ng/mL or vehicle during 24h [162, 426]. After the incubation, cells and the culture media were analysed separately as described above.

II.6 Quantification of the lipids of interest in tissues from control and DSS-induced colitis mice

Colitis was induced in C57BL/6 male mice as we previously reported [77, 176, 427]. To induce colitis, 5% DSS was added to the drinking water during 5 days, and then replaced with normal drinking water for the remaining two days of the protocol. Control mice received normal drinking water during the 7-days protocol. During the experiment body weight was monitored daily. On day 7, mice were anesthetized using isoflurane and blood collected from the cava vein and the portal vein. Mice were then euthanized by cervical dislocation and the tissues removed and snap-frozen in liquid nitrogen before being stored at -80°C. The tissues were processed for lipid quantification as described above. This study was performed in compliance with the European Directive 2010/63/EU, transformed into the Belgian Law of May 29, 2013 regarding the protection of laboratory animals. Lab agreement number LA1230365 and Université catholique de Louvain's animal studies ethics committee approval 2017/UCL/MD/024.

II.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism v9.1.2 Software (San Diego, CA). The significance was set at p value < 0.05. For each set of values, normality and variance were controlled. Student t-test (or Mann-Whitney test if distribution was non-parametric) was used to compare two conditions and ANOVA (or Kruskal-Wallis test if non-parametric) was used to compare three or more conditions. Grubb's test was used to identify potential outliers (when applicable). FDR assessment was based on Benjamini-Hochberg method.

III. RESULTS AND DISCUSSION

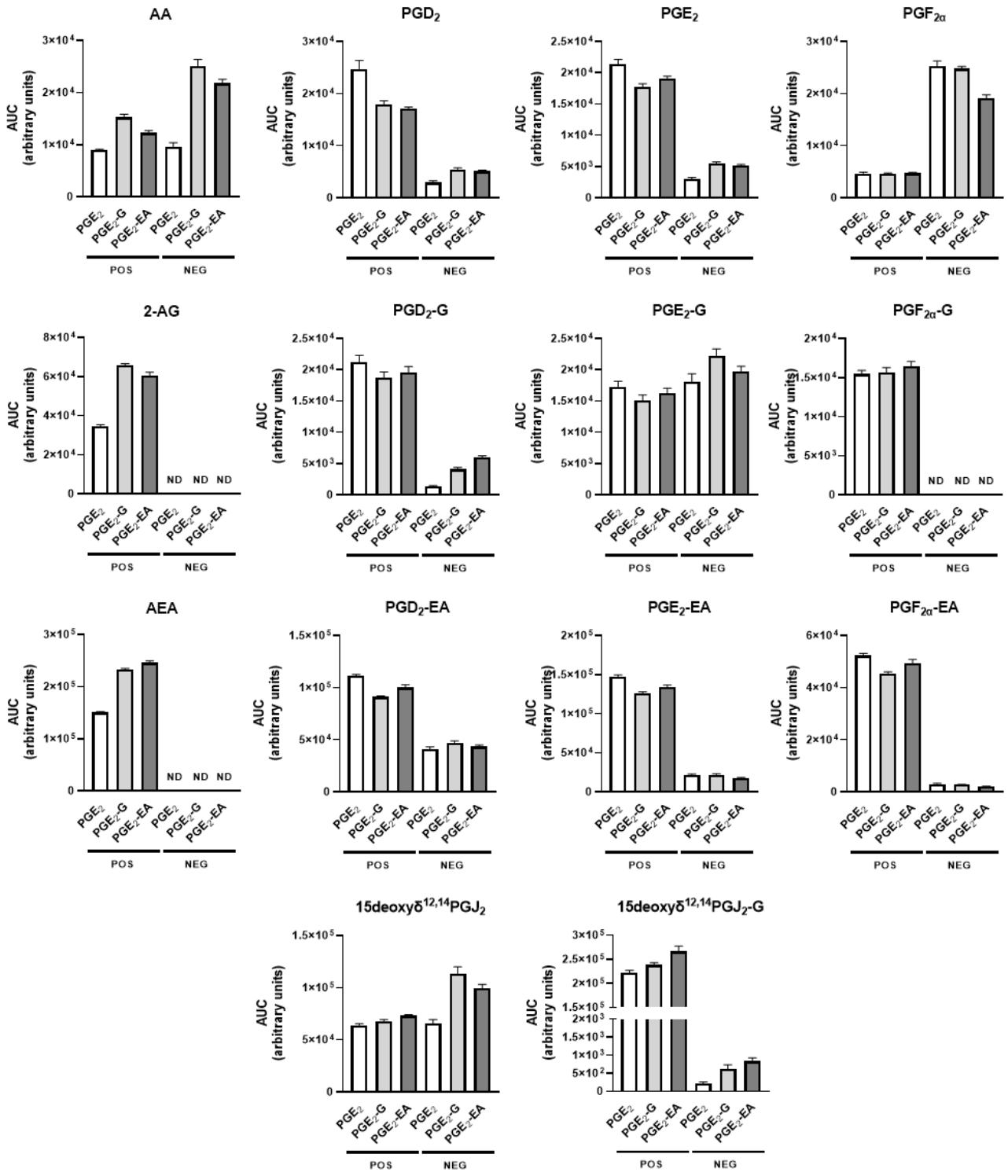
III.1 Method development

For the set-up of the extraction and purification procedures, we selected arachidonic acid, 2-AG, AEA, PGD_2, PGD_2-G and PGD_2-EA as representative compounds of their respective families. The mass parameters were determined and the validation was performed for the whole set of analytes of interest. Of note, while 2-AG is considered the most relevant isoform of arachidonoylglycerol, it is largely descried that 2-AG undergo fast interconversion into the more stable 1(3)-AG depending on the experimental conditions. Thus, most developed methods report 2-AG levels by considering the chromatographic peak of 2-AG and 1(3)-AG. Therefore, even if the chromatographic system we use resolved the two isomers, we followed a similar strategy for our method and quantified 2-AG as the signal from 2-AG and 1(3)-AG.

III.1.1 Optimization of the MS/MS detection and of the separation of the derivatives

We prepared standard solutions for each compound at 10^{-5} M in ACN-H_2O (1:1; v/v) to start the optimization process of the parameters of the tandem quadrupole mass spectrometer. This step allowed us to define MRM transitions as well as mass spectrometer parameters to use for each compound. Following Intellistart® (Waters)-guided design of transitions performed in positive and negative ionization mode, we fine-tuned the selection of the MRM transitions by implementing several transitions published in the literature [244, 428, 429], as well as additional potential transitions based on the structure of the compounds.

In parallel to the selection of the MRM transitions, we optimized the parameters of the mass spectrometer for the detection of the analytes of interest. For this we tuned one prostaglandin (PGE_2), one PG-G (PGE_2-G) and one prostamide (PGE_2-EA) in positive and negative modes. Then, we analysed an equimolar mixture of all the standards in the 6 different mass parameters. Of note, in our working conditions, we did not find any satisfying MRM transition for AEA, 2-AG and $PGF_{2\alpha}$-G in negative mode. As reported in **supplemental Figure 1**, the positive mode seems superior to the negative mode for our analytes of interest.



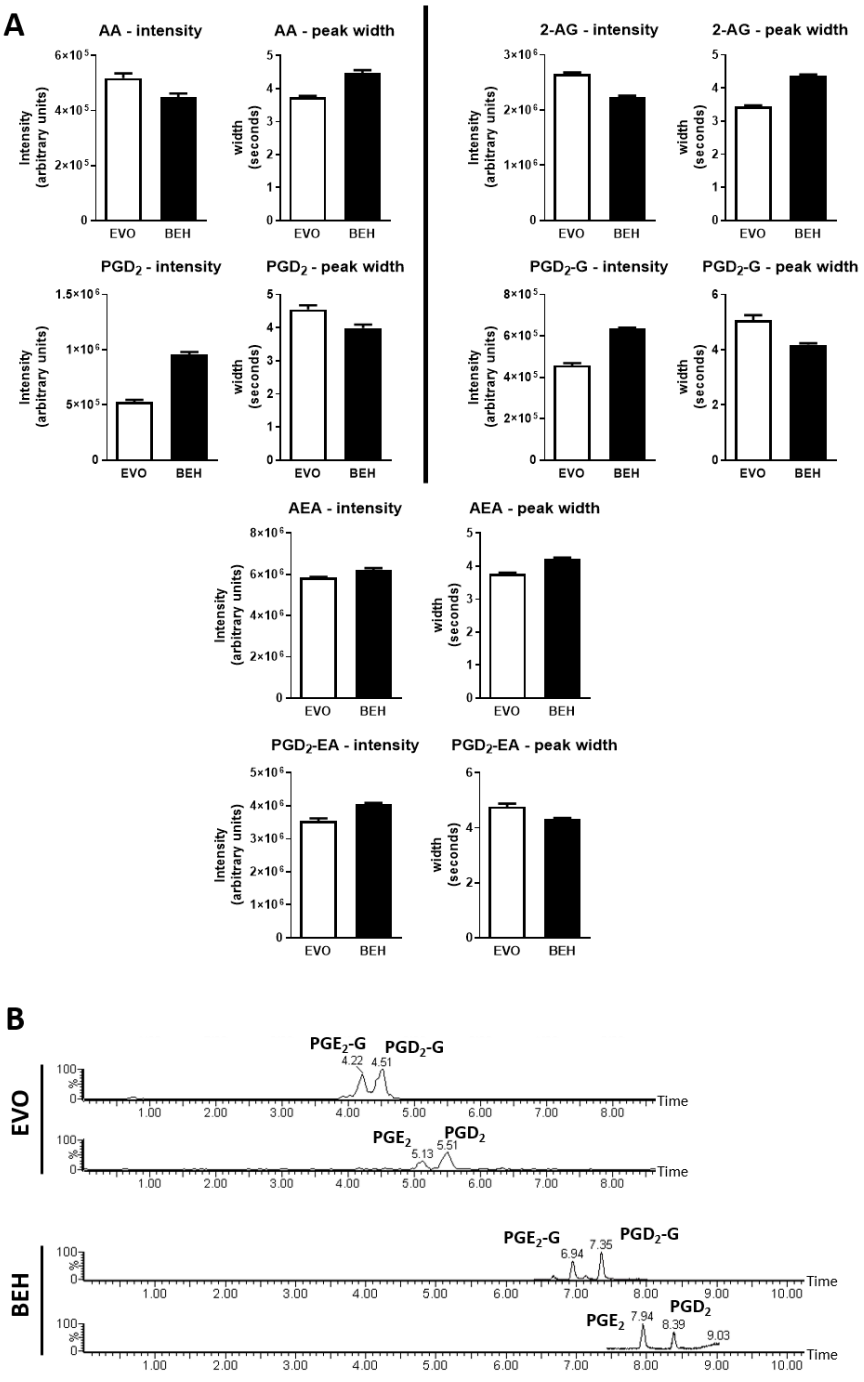
Supplemental figure 1. Selection of the appropriate source parameters. Comparison of the detection obtained for the derivatives using six different tune parameters. Mass parameters were optimized for one compound / family (PGE_2, PGE_2-G, PGE_2-EA) and then the determined parameters were applied for prostaglandin derivatives detection. In the case of 2-AG and AEA, we were not able to determined MRM transitions in negative mode leading to a not detected status. Area under the curve values were shown. N=2 in triplicate

Based on the data obtained, we selected the tune parameters obtained for PGE_2-G (in positive mode) as this gives us the best compromise when considering the whole set of analytes. Once the quantification and qualification transitions selected for each derivative (**Table 1**), we optimized the separation by testing several mobile phase compositions and gradients (**data not shown**) using two different UHPLC columns (the Kinetex® EVO C18 (100x2.1 mm; 1.7 µm) and the Waters acquity UPLC® BEH C18 (150x2.1 mm; 1.7 µm) columns) previously described in the literature to resolve several prostaglandin derivatives [244, 430, 431].

Name	abbreviation	molecular formula	MW [a]	analysed ion[b]	MRM transitions[c]	RRT[d]
arachidonic acid derivatives						
arachidonic acid	AA	$C_{20}H_{32}O_2$	304.5	$[M+H]^+$	Q: 305.3>120.9 q: 305.3>78.4	1.01
prostaglandin D_2	PGD_2	$C_{20}H_{32}O_5$	352.5	$[M+H-2H_2O]^+$	Q: 317.2>299.2 q: 317.2>90.86	1.06
prostaglandin E_2	PGE_2	$C_{20}H_{32}O_5$	352.5	$[M+H-2H_2O]^+$	Q: 317.2>299.2 q: 317.2>90.86	1.00
prostaglandin $F_{2\alpha}$	$PGF_{2\alpha}$	$C_{20}H_{34}O_5$	354.5	$[M+H-3H_2O]^+$	Q: 301.0>156.9 q: 301.0>128.7	0.95
15deoxy$\delta^{12,14}$prostaglandin J_2	15deoxy$\delta^{12,14}PGJ_2$	$C_{20}H_{28}O_3$	316.4	$[M+H]^+$	Q: 317.2>90.9 q: 317.2>299.2	1.39
2-arachidonoylglycerol derivatives						
2-arachidonoylglycerol	2-AG	$C_{23}H_{38}O_4$	378.6	$[M+H]^+$	Q: 379.2>287.3 q: 379.2>90.9	1.00
prostaglandin D_2 - glycerol ester	PGD_2-G	$C_{23}H_{38}O_7$	426.6	$[M+H-2H_2O]^+$	Q: 391.3>299.3 q: 391.3>373.2	1.07
prostaglandin E_2 - glycerol ester	PGE_2-G	$C_{23}H_{38}O_7$	426.6	$[M+H-2H_2O]^+$	Q: 391.3>299.3 q: 391.3>373.2	1.01
prostaglandin $F_{2\alpha}$ - glycerol ester	$PGF_{2\alpha}$-G	$C_{23}H_{40}O_7$	428.6	$[M+H-2H_2O]^+$	Q: 393.2>275.2 q: 393.2>128.9	0.96
15deoxy$\delta^{12,14}$prostaglandin J_2 - glycerol ester	15deoxy$\delta^{12,14}PGJ_2$-G	$C_{23}H_{34}O_5$	390.5	$[M+H-H_2O]^+$ and $[M+H]^+$	Q: 373.2>299.2 q: 391.3>373.2	1.52
anandamide derivatives						
Anandamide	AEA	$C_{22}H_{37}NO_2$	347.5	$[M+H]^+$	Q: 348.2>61.9 q: 348.2>287.3	1.00
prostaglandin D_2 – ethanolamide	PGD_2-EA	$C_{22}H_{37}NO_5$	395.5	$[M+H-H_2O]^+$	Q: 378.2>360.2 q: 378.2>342.2	1.10
prostaglandin E_2 – ethanolamide	PGE_2-EA	$C_{22}H_{37}NO_5$	395.5	$[M+H-H_2O]^+$	Q: 378.2>360.2 q: 378.2>342.2	1.01
prostaglandin $F_{2\alpha}$ - ethanolamide	$PGF_{2\alpha}$-EA	$C_{22}H_{39}NO_5$	397.5	$[M+H-H_2O]^+$	Q: 380.3>344.2 q: 380.3>362.2	0.98
internal standards						
d_8-arachidonic acid	d_8-AA	$C_{20}H_{24}D_8O_2$	312.5	$[M+H]^+$	Q: 313.3>82.9 q: 313.3>111.0	/
d_5-1-arachidonoylglycerol	d_5-1-AG	$C_{23}H_{33}D_5O_4$	383.6	$[M+H]^+$	Q: 384.3>287.3 q: 384.3>269.3	/
d_4-anandamide	d_4-AEA	$C_{22}H_{33}D_4NO_2$	351.6	$[M+H]^+$	Q: 352.2>65.9 q: 352.2>287.3	/
d_4-prostaglandin E_2	d_4-PGE_2	$C_{20}H_{28}D_4O_5$	356.5	$[M+H-2H_2O]^+$	Q: 321.2>275.0 q: 321.2>148.9	/
d_5-prostaglandin E_2 - glycerol ester	d_5-PGE_2-G	$C_{23}H_{33}D_5O_7$	431.6	$[M+H-2H_2O]^+$	Q: 396.3>299.3 q: 396.3>79.1	/
d_4-prostaglandin E_2 – ethanolamide	d_4-PGE_2-EA	$C_{22}H_{33}D_4NO_5$	399.6	$[M+H-H_2O]^+$	Q: 382.2>364.3 q: 382.2>346.2	/

Table 1: Chemical and analytical description of the bioactive lipids of interest. Description of the fourteen molecules (and their respective internal standards) analysed in the validated method. The table lists for each analyte the abbreviation used, molecular formula, molecular weight, analysed ions, quantification (Q) and qualification (q) transitions as well as the relative retention time based on the respective internal standard. [a] molecular weight for the neutral form; [b] analysed in positive mode; [c] quantitative (Q) and qualitative (q) transition used; [d] relative retention time (on the respective internal standard).

In our hands, when compared with the EVO C18 column, used in the same chromatographic conditions, the BEH column resulted in larger width and decreased intensity for the peak of 2-AG and others precursors. However, the opposite was observed for the peaks of PG-G and PG-EA (which are reported as less abundant in biological samples) (**Supplemental Figure 2A**). Moreover, when assessing chromatographic peak shape, the BEH column allows to separate the family isomers (e.g. PGD_2-G and PGE_2-G) but also the position isomers of the prostaglandin glycerol ester series (e.g. PGD_2-1-G and PGD_2-2-G) when compared to the EVO C18 column (**Supplemental Figure 2B**).



Supplemental figure 2. Selection of the UPLC column. Peak intensity (arbitrary units), width (seconds) and shape were used to select an optimal reversed phase column. Elution gradient was optimized on both columns and selection was performed after the injection of the same standard stock solution on both systems. (**A**) Comparison of intensity and peak width of six derivatives selected as representatives for the entire family of compounds. White bars refer to the use of a Kinetex® EVO C18 column (100x2.1 mm; 1.7 µm) and the black bars refer to the use of an acquity UPLC® BEH C18 column (150x2.1 mm; 1.7 µm). (**B**) Representative chromatographic separation obtained on both system after the injection of 500 fmol on the system. PGE_2, PGD_2, PGE_2-G, PGD_2-G were shown as representative for the separation and peak shape obtained for the entire family of compounds on both systems.

Based on these elements we selected the acquity BEH C18 column for our method. The resulting chromatographic method allows us to analyse the complete set of analytes within 15 min of run (**Figure 2**).

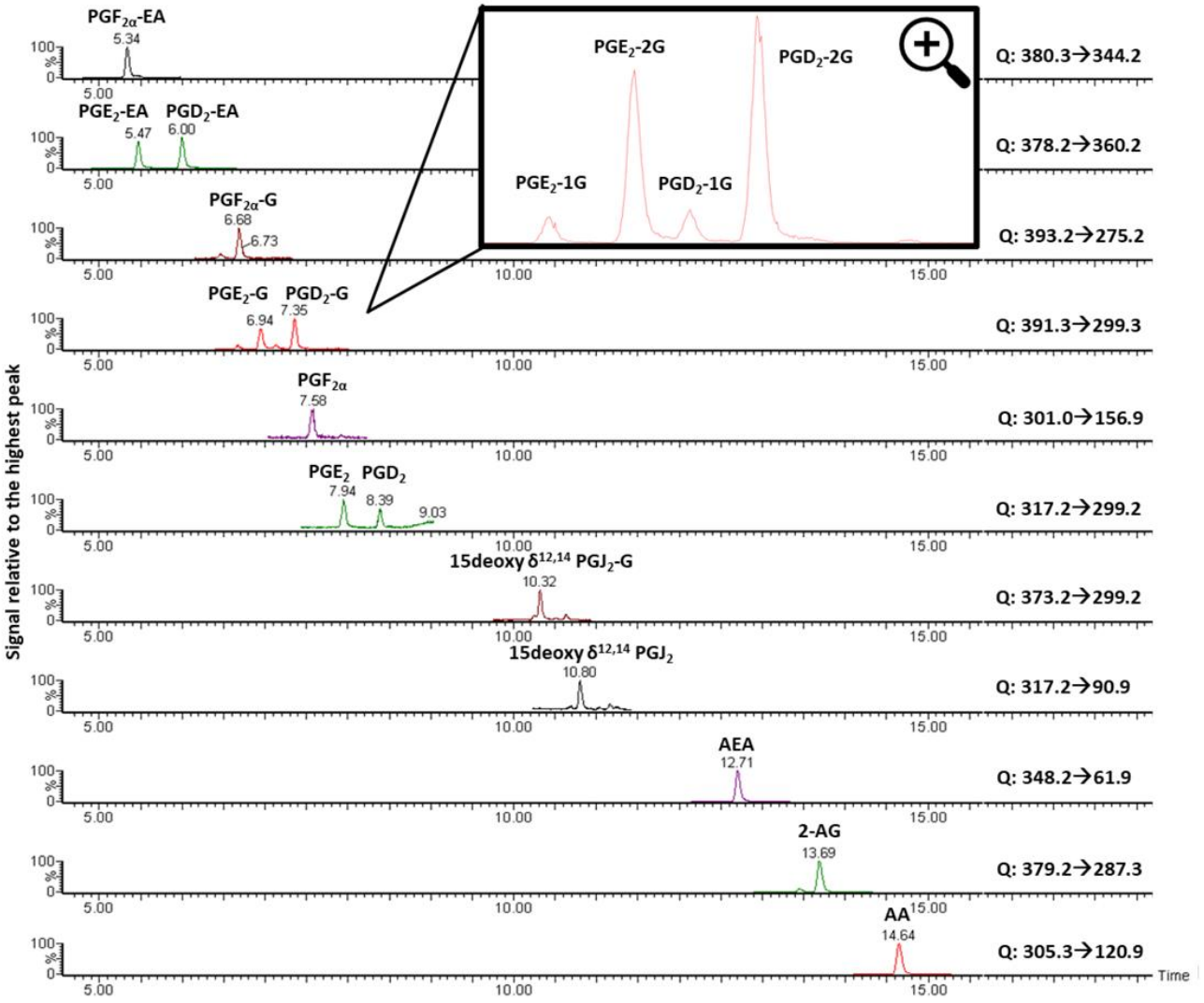
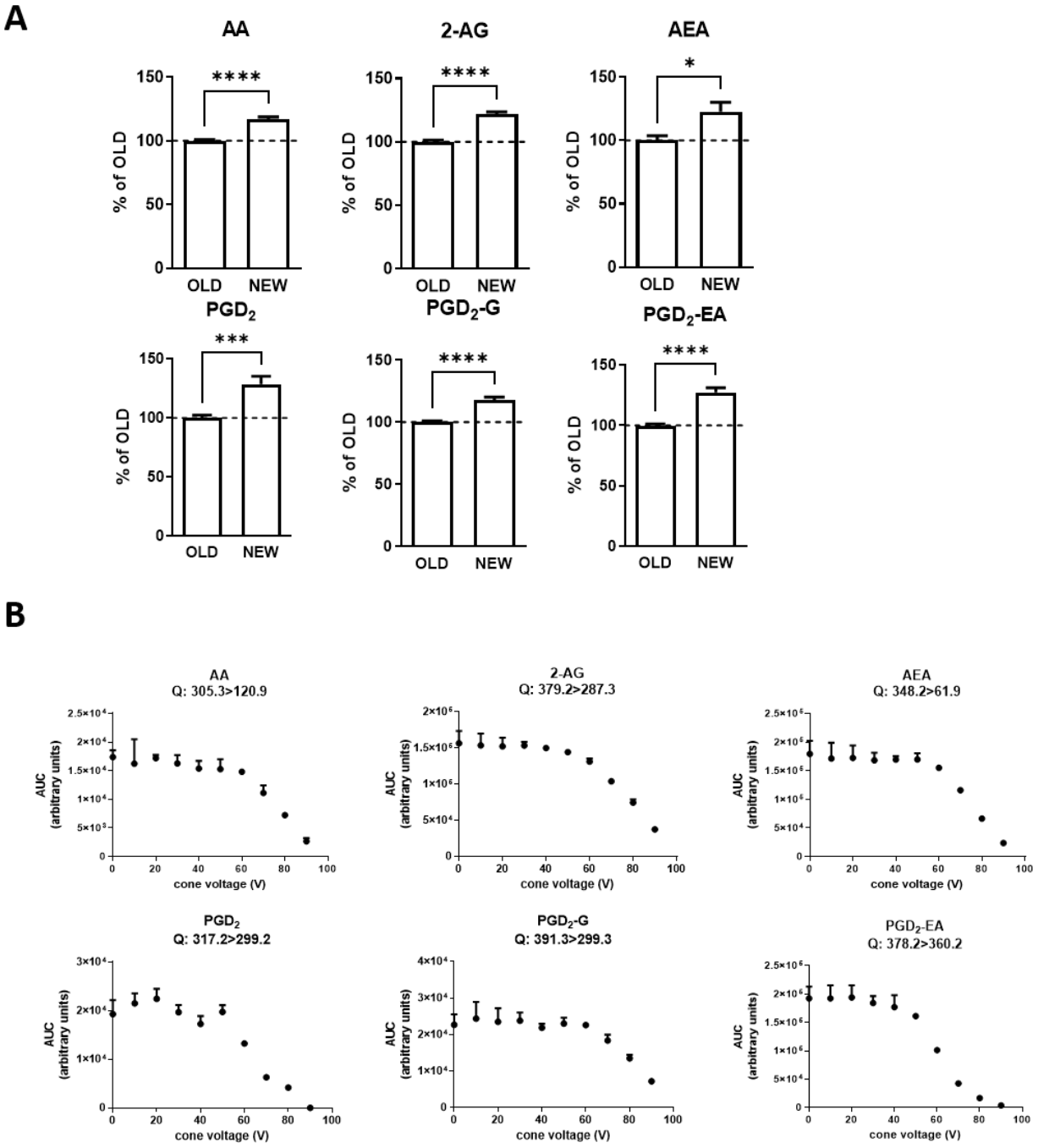


Figure 2: Typical chromatogram showing the analytical separation of the lipid mediators of interest. A mixture containing each standard (500 fmol) was injected on the UPLC-MS/MS system. For each standard, the signal corresponding to the quantitative transition is shown. The inset highlights the chromatographic separation between PGE_2-G and PGD_2-G as well as their respective position isomers (i.e. PGD_2-1-G, PGD_2-2-G, PGE_2-1-G and PGE_2-2-G).

III.1.2 Optimization of the parameters of the tandem quadrupole

As discussed above, we used the automatic Intellistart® module provided by the MassLynx® software to tune the molecules both in positive and negative ionization modes (**supplemental Figure 1**). Besides this initial set up, several others parameters can also be optimized to increase the detection of the analytes. First, with an incremental strategy, we decided to assess the source height, inclination, desolvation gas flow and cone gas flow together (**data not shown**). This led to an overall improvement of the detection of the analytes (spiked in a biological matrix) of 10-30% (**supplemental Figure 3A**).



Supplemental figure 3. Determination of source parameters. Compounds were injected using the complete analytical procedure. (**A**) Source parameters (electrospray height, electrospray inclination, cone gas flow and desolvation gas flow) were adjusted with an incremental strategy. The complete detection yield is represented as percentage of the "old", Intellistart determined, parameters. (**B**) Cone voltages (CV) were manually set between 0 and 90 volts and a mixture of standards was injected. Signals (AUC) obtained for the quantification transition (Q) were plotted function of the CV values. N=3 in triplicate. After normality assessment, student t-test was used. FDR assessment based on Benjamini-Hochberg method. Adjusted P values * p< 0.05; *** p< 0.001; **** p< 0.0001

We then assessed the influence of the cone voltage on the detection of the analytes of interest using the quantification MRM transition (**supplemental Table 1 and Figure 3B**). Based on the obtained data we selected a cone voltage value of 30 volts. Next, we assessed the effect of the collision energy (CE) on the signal obtained for the three selected transitions for each lipid. By varying the CE between 2 eV and 50 eV we selected for each of the 14 lipids of interest the most appropriate CE and transition (**Figure 3 and supplemental Table 1**).

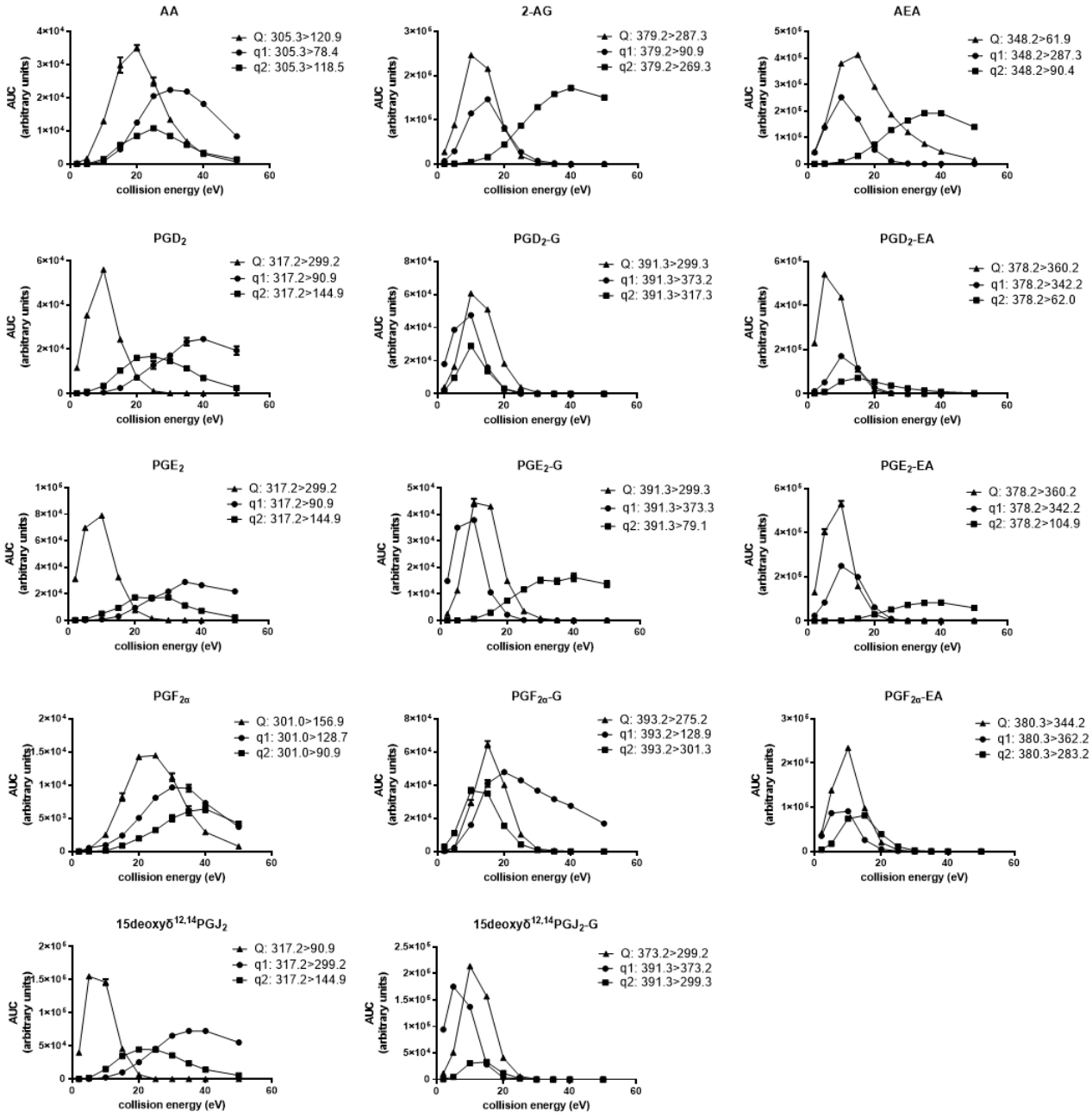
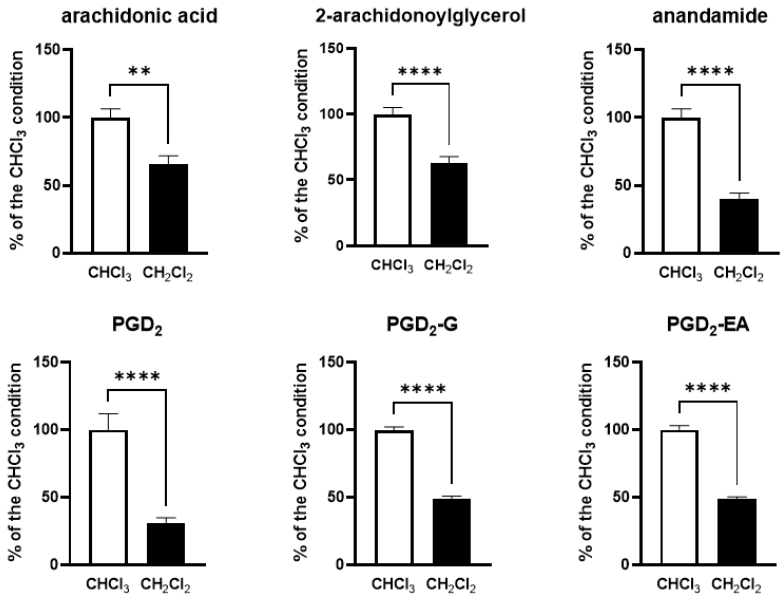


Figure 3: Determination of the MRM transition parameters. Standard solutions (500 fmol each analyte) were used for the set up. A fixed cone voltage of 30 V was used, while the effect of the collision energy of the three main MRM of each analyte was assessed between 2 eV and 50 eV. Signals (AUC) are plotted in function of the collision energy (eV) used. Q: quantitative transition, q1 and q2: qualitative transitions.

III.1.3 Optimization of the extraction procedure

Besides the MS parameters, quantitative analysis of low-abundance lipids in complex matrices requires a careful assessment of the extraction and purification procedures. Thus, based on previous protocols used in our lab [98, 422], we optimized the extraction and purification procedures. As with many methods for lipid analysis, we decided to use a Folch-type liquid-liquid extraction to obtain an efficient extraction from biological matrices [91]. While the original method used $CHCl_3$, we first decided to assess if replacing it with the less harmful dichloromethane (CH_2Cl_2) would be feasible (i.e. would not affect extraction yield). As reported in **supplemental Figure 4**, the extraction of the analytes in the presence of CH_2Cl_2 is significantly less efficient than in the presence of $CHCl_3$.



Supplemental figure 4. Selection of the extraction solvent. Folch's procedure was applied on standard solution using $CHCl_3$ or CH_2Cl_2. After the Folch's liquid-liquid extraction procedure, the lower organic phase was evaporated and reconstituted in MeOH followed by injection on the LC-MS system. Signal was evaluated as percentage of the $CHCl_3$-containing condition. N=3 in triplicate. After normality assessment, student t-test was used. FDR assessment based on Benjamini-Hochberg method. Adjusted P values ** p< 0.01; **** p< 0.0001

Although the chemical structures of the prostaglandin derivatives we are interested in are quite similar, the three families differ by the polar head (i.e. prostaglandins have an ionizable carboxylic group whereas PG-EA and PG-G have a small non-ionizable polar moiety) (**Figure 1**). Thus, we decided to assess whether acidification of the extraction mixture, composed of $CHCl_3$, MeOH and H_2O, would improve the extraction yield of the prostaglandins (due to the presence of a carboxylic moiety). In the presence of standards only, the acidification resulted in a slight decrease in 2-AG and PGD_2-EA signal, but did not improve the signal for arachidonic acid or PGD_2 (**Figure 4A**).

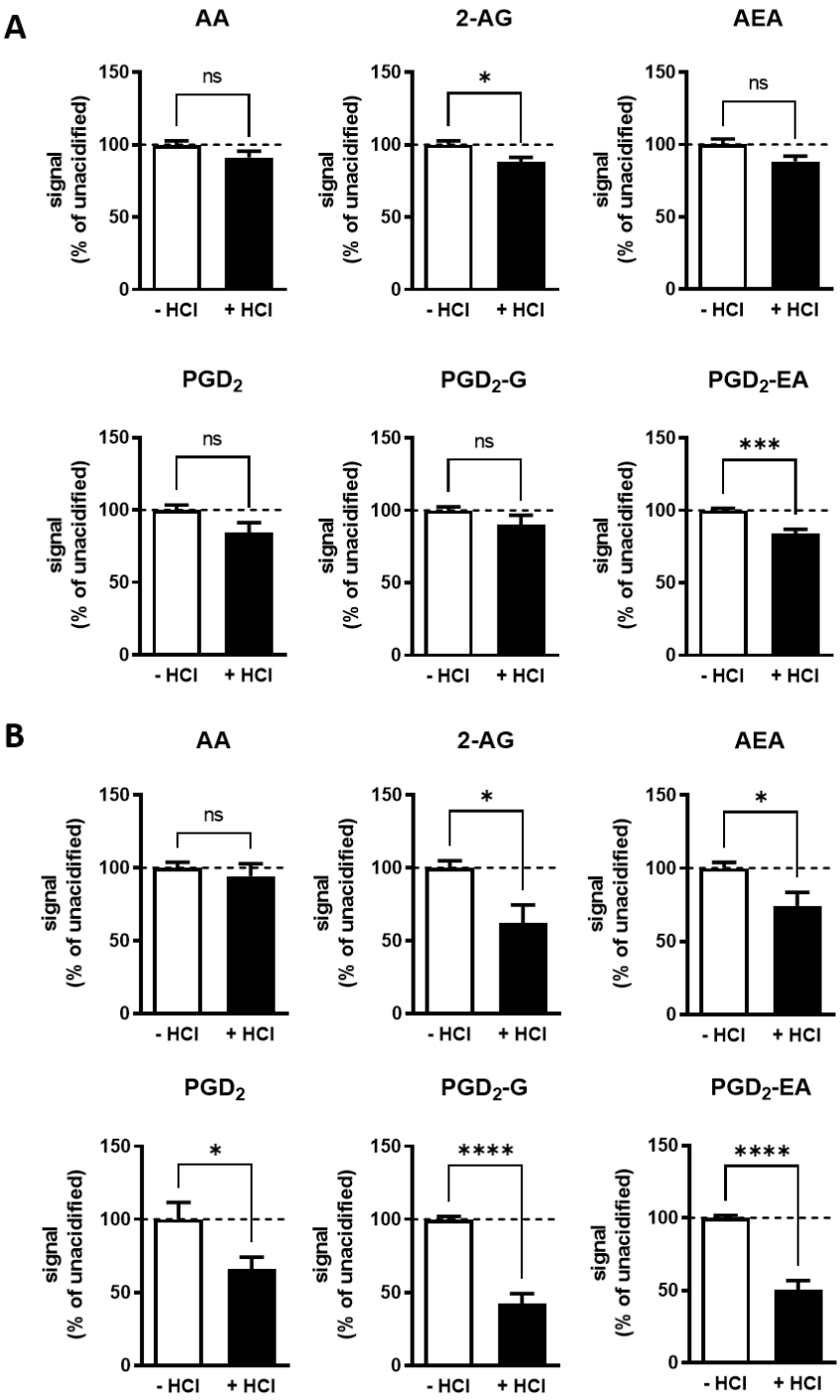


Figure 4: Assessment of the effect of the acidification during the extraction procedure. Six analytes (arachidonic acid, 2-AG, AEA, PGD_2, PGD_2-G, PGD_2-EA), selected as representatives of the entire analytes of interest, were chosen to set up the extraction procedure. The standards were extracted by liquid-liquid extraction in the absence (**A**) or presence (**B**) of matrix (10 mg of mouse brain). In each condition, the effect of the acidification (HCl 2N, 300µL) of the extraction mixture ($CHCl_3$-MeOH-H_2O, 8:4:2, v/v/v) was assessed. The data are reported as relative to the signal (AUC) obtained for the unacidified (- HCl) condition. After normality assessment, student t-test or Mann-Whitney non-parametric test was used. FDR assessment based on Benjamini-Hochberg method. Adjusted P values * p< 0.05; *** p< 0.001; **** p< 0.0001; N=3 in triplicate.

Strikingly, the acidification appeared as highly deleterious for the detection of most derivatives when assessed in the presence of a biological matrix (**Figure 4B**). Indeed, except for arachidonic acid, acidification decreased the detection of all the lipid of interest when extracted from the matrix. When comparing the data obtained in the absence and presence of matrix, one can speculate that the decreased signal is in part due to a higher extraction of other molecules present in the matrix [106, 422, 432]. Typically, phospholipids are known to be better extracted in acidic condition and to affect MS detection by ion-suppression effects [433].

The next step of the analytical method is a silica-based SPE purification procedure. This requires to select appropriate washing and elution steps. Thus, we looked for a solvent that would elute the more lipophilic compounds present in a biological matrix (typically cholesterol) while retaining the lipid mediators of interest on the column. Based on a previously validated method from our laboratory, we tested $CHCl_3$ (2 and 4 ml) as washing solvent [98]. As shown in **Figure 5A**, this resulted in the complete elution of cholesterol while retaining the lipids of interest on the column. Then we tested several solvent mixtures to determine the best eluent to recover the compounds of interest from the SPE. Based on preliminary data, we tested several mixtures of chloroform and methanol containing increasing proportions of methanol. This led to the selection of $CHCl_3$-MeOH (7:3; v/v) to effectively recover the analytes of interest (**Figure 5B**). Indeed, higher proportions of methanol would result in higher levels of phospholipids in the sample resulting in unnecessary matrix effects [422]. Of note, we decided to use 30% of methanol as lower methanol content yielded more variable results during the numerous assay we performed during the method development.

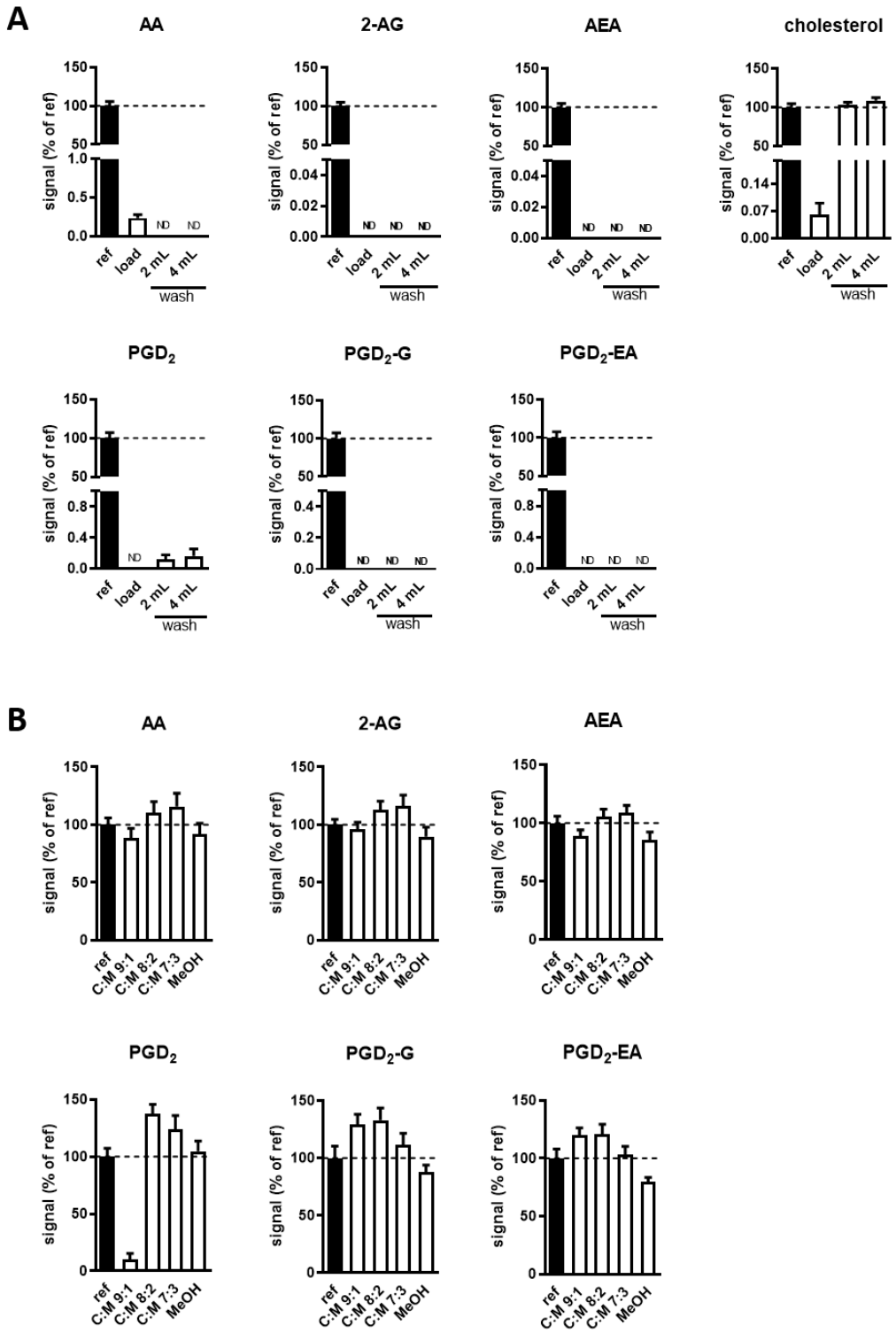


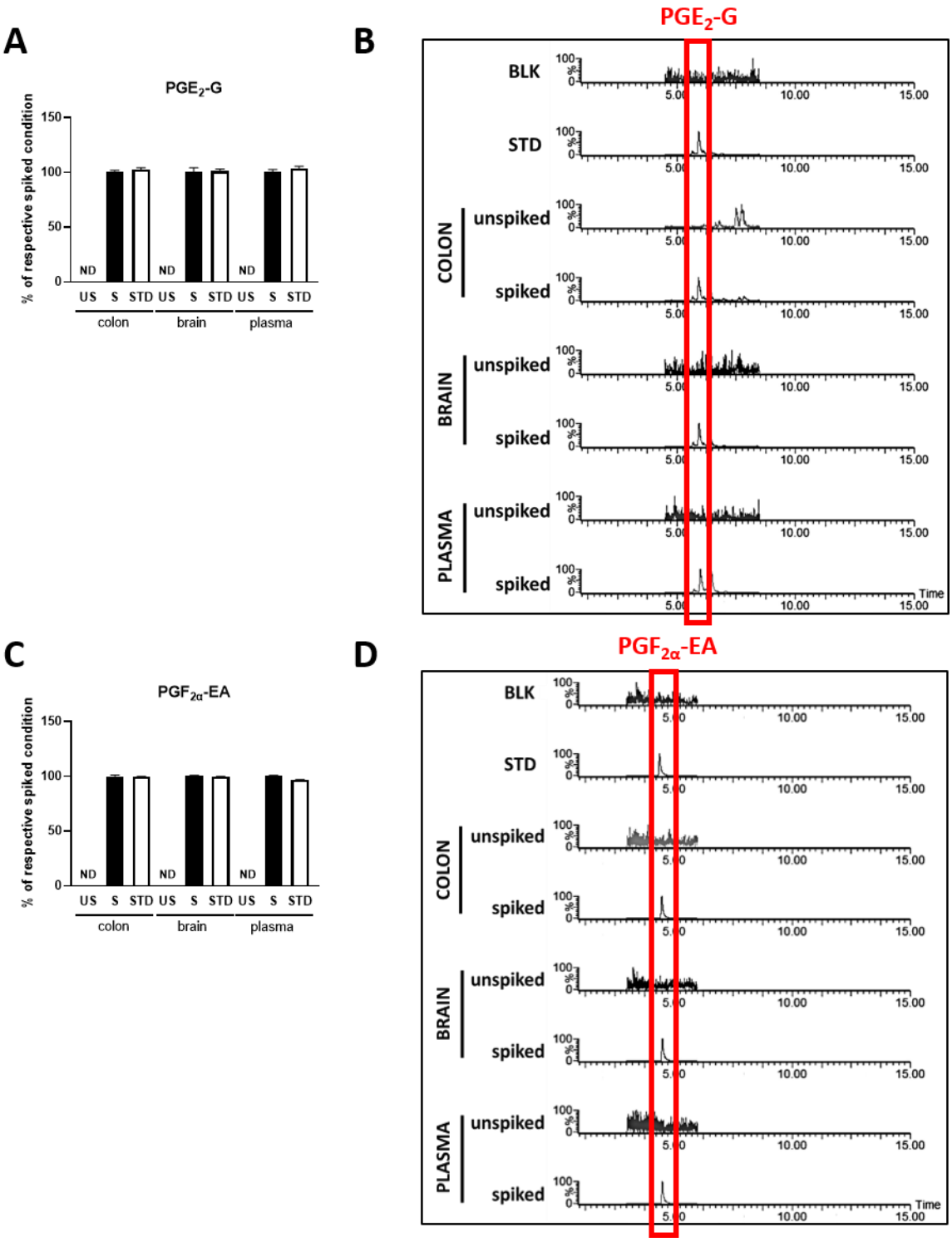
Figure 5: Optimization of the solid phase extraction procedure. Six analytes (arachidonic acid, 2-AG, AEA, PGD_2, PGD_2-G, PGD_2-EA), selected as representatives of the entire analytes of interest, were chosen to set up the SPE procedure. The standards were added to matrix (10 mg of mouse brain) before being extracted as reported above. (**A**) The lipid fractions were transferred on SPE silica columns using $CHCl_3$-MeOH (99:1; v/v). The flow through was recovered (load) as well as the eluting fractions obtained by using 2 mL or 4 mL of $CHCl_3$ as washing solvent. Following UPLC-MS/MS analysis, the data (AUC) are reported relative to the analysis of an extracted lipid fraction that did not went through the SPE procedure (ref). (**B**) To assess the elution of the analytes of interest, several mixtures of $CHCl_3$-MeOH were tested. The graph reports the data obtained following the elution using 3.5 mL of $CHCl_3$-MeOH (9:1, v/v), $CHCl_3$-MeOH (8:2, v/v), $CHCl_3$-MeOH (7:3, v/v), or pure MeOH. Following UPLC-MS/MS analysis of the eluted fractions, the data (AUC) are reported relative to the analysis of an extracted lipid fraction that did not went through the SPE procedure (ref). N=3 in triplicate.

III.2 Method validation

III.2.1 Specificity and selectivity

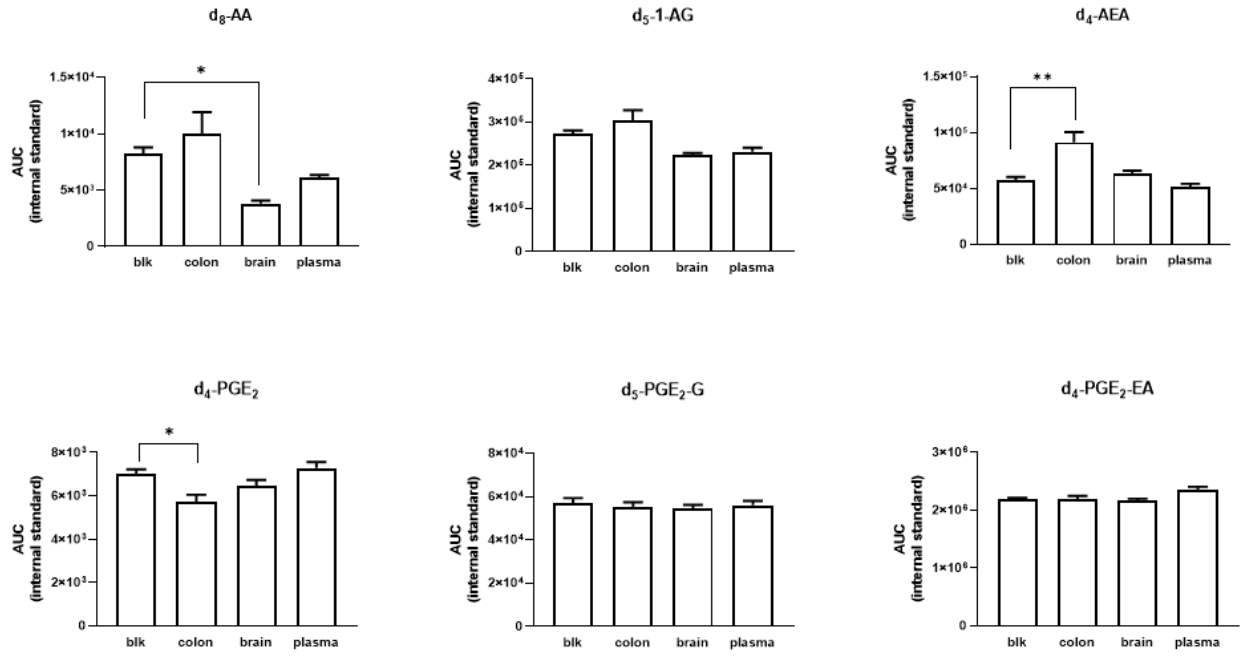
The selectivity is a critical parameter when analyzing prostaglandins as some of them are isomers of each other. Moreover, the glycerol ester series present a chromatographic double peak due to the interconversion of the glycerol position on the acyl chain (also identified with the 2-AG [99]). This was improved by selecting the best separation parameters after testing several UPLC columns as well as several mobile phases and gradients (data not shown). As mentioned above, an acquity UPLC® BEH C18 column from Waters (150x2.1 mm; 1.7 µm) was selected together with a gradient of H_2O-ACN-acetic acid (94.9:5:0.1; v/v/v) and ACN-acetic acid (99.9:0.1; v/v).

To go further we selected three representative matrices in term of lipid content (i.e. colon, brain and plasma) to examine whether the matrix content could affect compounds detection (e.g. coelution with others endogenous compounds or signal modification) or compounds separation (e.g. decrease in the resolution of peaks). For that, we applied the complete procedure to analyse the matrix alone, the standards alone and the matrix spiked with the standards. As illustrated in **supplemental Figure 5**, with PGE_2-G and $PGF_{2\alpha}$-EA used as example, none of the three selected matrices affects neither the detection nor the chromatographic separation of the analytes (as shown here for PGE_2-G).



Supplemental figure 5. Evaluation of the impact of the matrix on compound analysis. Unspiked matrices, spiked matrices and the standard amount used for the spiking were analysed using the entire developed analytical method. (**A,C**) Normalized ratios (PGE_2-G and $PGF_{2\alpha}$-EA) were compared as percentage of the spiked matrix set at 100% for the three selected matrices (colon, brain, plasma). US: unspiked, S: spiked, STD: standard. (**B,D**) Representative chromatograms obtained for PGE_2-G and $PGF_{2\alpha}$-EA for the blank sample, standard sample and for the unspiked and spiked conditions of the three selected matrices (colon, brain, plasma). Signal (%) relative to the highest detected peak for each chromatogram. Red squares show the analysed chromatographic peak.

Validation of quantification methods for endogenous compounds is made more complex by the absence of biological blank matrix (i.e. tissue or cells not containing the analytes of interest). Thus, to assess how the matrix would affect the detection of the analytes, we compared the signal obtained in the absence or in the presence of matrix (colon, brain, plasma) for the 6 deuterated standards used in the method (**Supplemental Figure 6**). For most of the conditions the signal of the deuterated standard was not altered by the presence of matrix. In the presence of colon, the signal of d_4-AEA was increased while the signal of d_4-PGE_2 was decreased. In the presence of brain as matrix, only the signal of d_8-AA was decreased compared to the signal obtained in the absence of matrix.



Supplemental figure 6. Evaluation of the matrix effect on the detection of the prostaglandin derivatives. The signal (AUC) for 6 deuterated standards, representative of the analytes of interest, obtained following extraction, SPE and LC-MS/MS analysis were compared between blank matrix and tissue matrices. Here three tissues were used as representative matrix, colon, brain and plasma from mice, N=3 in triplicate. After normality assessment, one-way Anova was used. FDR assessment based on Benjamini-Hochberg method. Adjusted P values * $p< 0.05$; ** $p<0.01$.

III.2.2 Calibration curves and response function

Based on the literature and on previous works of our research group, we expected the endogenous levels of PG-EA and PG-G to be low and likely near the instrument limits of detection. On the contrary, the endogenous levels of arachidonic acid and 2-AG are quite high in comparison with the PG-G and PG-EA. Based on the information gathered during the method development, we selected 900 fmol on column as the highest point of the calibration curve for the majority of the compounds. For arachidonic acid and 2-AG (9000 fmol on column) and for 15deoxy$\delta^{12,14}PGJ_2$ (1800 fmol on column) higher amounts were selected. To avoid leverage effect, we opted for the use of an evenly-spaced calibration points strategy and designed 11 points calibration curves. The lowest point of the calibration curve was 40 fmol on column, except for 15deoxy$\delta^{12,14}PGJ_2$ (80 fmol on column) and arachidonic acid as well as 2-AG (400 fmol on column). To consider the whole analytical process used for analyzing biological samples, the solutions prepared for the calibration curves were analysed by using the whole analytical process (i.e. extraction, SPE purification and LC-MS analysis).

To ensure the best fit between the experimental points and the calibration curve, several models were tested and the goodness of fit assessed [434]. Considering a 25% confidence interval between each value and the mathematical regression, a linear regression was selected (**Table 2**). This confidence interval was chosen as we are developing a quantification method for measuring endogenous compounds in biological matrices [425].

analyte	range[a]	Equation	LOD[b]	LOQ[c]	quality control level	trueness[d]	repeatability (% CV)[e]	intermediate precision (% CV)[f]	accuracy	linearity	
AA	400 - 9000	Y = 0.004586*X + 0.02251	100	400	LOQ		3.23	6.31	6.15	slope	0.99
					LOW	1.55	7.65	6.39	6.77		
					MIDDLE	-0.19	11.20	6.44	2.25	R^2	0.999
					HIGH	1.52	8.37	5.85	3.10		
PGD_2	100 - 900	Y = 0.004629*X - 0.1383	60	100	LOQ		8.29	8.89	4.17	slope	1.07
					LOW	-17.34	11.53	5.72	1.65		
					MIDDLE	-11.84	10.68	13.20	1.56	R^2	0.981
					HIGH	2.59	9.47	8.82	-2.50		
PGE_2	100 - 900	Y = 0.03153*X - 0.1345	60	100	LOQ		8.50	9.27	5.40	slope	1.01
					LOW	-4.46	10.78	6.35	4.44		
					MIDDLE	0.80	9.11	13.80	1.45	R^2	0.991
					HIGH	-8.01	7.22	8.17	-1.01		
$PGF_{2\alpha}$	200 - 900	Y = 0.006977*X - 0.1375	60	200	LOQ		9.32	8.19	5.58	slope	1.01
					LOW	-10.17	8.03	9.25	5.04		
					MIDDLE	-2.85	8.83	14.92	0.37	R^2	0.999
					HIGH	-0.25	9.14	11.05	1.48		
15deoxy$\delta^{12,14}PGJ_2$	100 - 1800	Y = 0.05947*X - 1.316	50	100	LOQ		8.15	9.77	2.98	slope	1.04
					LOW	-3.97	10.71	6.93	3.46		
					MIDDLE	-2.98	8.84	13.19	-1.60	R^2	0.998
					HIGH	-2.57	9.40	9.68	-1.85		
2-AG	400 - 9000	Y = 0.0009628*X - 0.04477	10	60	LOQ		6.10	3.21	0.74	slope	0.98
					LOW	0.88	3.30	4.05	-0.58		
					MIDDLE	0.36	4.48	6.82	-2.01	R^2	0.999
					HIGH	0.89	4.92	6.90	-1.78		
PGD_2-G	100 - 900	Y = 0.0005303*X + 0.004200	40	100	LOQ		7.63	8.26	3.06	slope	0.99
					LOW	-11.01	7.87	9.04	1.12		
					MIDDLE	-3.82	6.14	10.14	-1.01	R^2	0.993
					HIGH	-4.94	6.77	10.04	-0.79		
PGE_2-G	50 - 900	Y = 0.001314*X - 0.02856	15	50	LOQ		2.17	8.91	2.58	slope	1.03
					LOW	-8.25	5.88	9.49	2.47		
					MIDDLE	-1.35	9.12	11.89	0.76	R^2	0.999
					HIGH	1.35	6.71	9.48	0.81		

$PGF_{2\alpha}$-G	40 - 900	Y = 0.001459*X - 0.01017	15	40	LOQ		9.14	4.71	5.45	slope	0.97
					LOW	0.64	6.82	5.33	2.56		
					MIDDLE	0.25	8.68	9.22	0.56	R^2	0.995
					HIGH	0.64	6.75	8.64	1.01		
15deoxy$\delta^{12,14}PGJ_2$-G	100 - 900	Y = 0.002491*X + 0.005640	30	100	LOQ		6.93	6.74	-1.85	slope	1.02
					LOW	-4.74	5.94	9.99	-1.67		
					MIDDLE	-2.52	8.75	10.61	-3.40	R^2	0.993
					HIGH	1.96	8.60	8.23	-2.01		
AEA	40 - 900	Y = 0.009939*X - 0.03533	0.5	3	LOQ		11.45	16.57	0.26	slope	0.99
					LOW	1.81	4.51	5.62	-2.70		
					MIDDLE	-1.82	4.42	6.64	-3.10	R^2	0.999
					HIGH	0.12	5.36	8.17	-4.30		
PGD_2-EA	40 - 900	Y = 0.0001673*X + 0.0008319	10	30	LOQ		7.04	3.69	1.94	slope	1.01
					LOW	-3.87	6.33	6.25	2.64		
					MIDDLE	-0.90	8.88	9.91	-0.24	R^2	0.994
					HIGH	-3.21	7.76	8.11	-0.86		
PGE_2-EA	40 - 900	Y = 0.0005560*X - 0.01120	6	20	LOQ		8.73	6.34	2.28	slope	1.03
					LOW	-3.91	6.34	8.06	0.83		
					MIDDLE	-0.88	8.59	12.30	-2.06	R^2	0.996
					HIGH	1.41	6.33	7.77	-0.16		
$PGF_{2\alpha}$-EA	40 - 900	Y = 0.0002526*X - 0.001414	3	8	LOQ		7.69	14.19	15.32	slope	1.00
					LOW	2.01	6.10	6.94	1.40		
					MIDDLE	-0.87	7.78	10.28	-1.10	R^2	0.999
					HIGH	-0.50	7.14	8.56	-0.29		

Table 2: Summary of the validation parameters obtained for each analyte of interest. The table summarizes the main validation parameters. The range of calibration curves, the equation of the curves, the limit of detection (LOD), the limit of quantification (LOQ), the repeatability, the intermediate precision, the accuracy and the linearity are listed for each molecule. The equations reported correspond to the equation of the linear regression obtained for the different calibration curves. The LOD and LOQ are reported in femtomole on column. Repeatability and intermediate precision: CV (%) values obtained for each analyte after the assessment of the four quality control levels within the same day or on three different days. Accuracy: bias (%) obtained considering the four quality control levels analysed at three different time points. Linearity: slope and goodness of fit (R^2) of the linear regression line obtained when plotting the values (in femtomole) obtained for four different levels (quality control levels used in this case) with the theorical values used. [a-c] values expressed in femtomoles; [d] trueness (evaluated on the three calibration curve levels) expressed in relative bias (%); [e] intraday precision; [f] interday precision

III.2.3 Trueness

Trueness was evaluated on three quality control levels based on the calibration curves (i.e. low, middle and high) (**supplemental table 2**). The relative bias of each level was determined and all the values were found to be within the 20 % of acceptance criteria (**Table 2**). The highest value was observed for the lowest level of PGD_2 where -17 % of relative bias was obtained.

III.2.4 Limits of detection and quantification

Starting from the lowest point of the calibration curves, we prepared 10 serial dilutions to determine the LOD and LOQ of the method based on signal to noise values. We performed three experiments (in triplicate) and the LOD was determined as the amount giving a signal to noise of 3 for the nine replicates and the LOQ was the amount giving a signal to noise of 10 for the nine replicates (**table 2**). When comparing the effect of the "polar head", it appears that our method is more sensitive for the ethanolamide derivatives (PG-EA and AEA) when compared with the glycerol derivatives (PG-G, 2-AG) and with the carboxylic acid derivatives (PG and arachidonic acid). When comparing the prostaglandin series analysed here, it seems that the method is slightly more sensitive for the detection of the $F_{2\alpha}$ moiety compared to the D_2 and E_2 series.

III.2.5 Intra- and interday precision and accuracy

Based on the calibration curves, we selected four quality control levels to assess the precision criteria of the method (**supplemental table 2**). After determining the variation coefficient for the different precision levels, we assessed whether the values were comprised inside the confidence values of ± 15 % according with the guidelines [423, 435] (**Table 2**). For both

repeatability and intermediate precision, the determined values obtained were comprised in the confidence intervals allowed by the guidelines.

We used the same quality control levels, injected at least at three different time points, to determine the accuracy (bias) inherent to the method (**Table 2**). The method provided good accuracy values. Of note, we obtained for $PGF_{2\alpha}$-EA at the LOQ level a value of 15.3 % which is still acceptable when considering the fact that for low quality control level a confidence interval of 20 % is allowed.

III.3 Quantification of the prostaglandin derivatives in the J774 macrophage cell line

To test the applicability of our method, we decided to assess, in J774 cells, the effect of a pro-inflammatory stimulus on the levels of the PG, PG-G and PG-EA derivatives. J774 cells is a macrophage cell line largely used when studying lipid mediators in the context of inflammation. As we and others have shown, incubation of the cells with LPS results in the production of pro-inflammatory mediators (e.g. IL-6, TNFα, MCP-1) and the overexpression of several enzymes involved in inflammation, including COX-2 [162, 426, 436]. As we have previously used this cell line to study the anti-inflammatory mechanism of PGD_2-G [162], we thought it would be an interesting model to test our method.

Although we were not able to detect all the lipid mediators in the vehicle-treated condition (i.e. control condition), following incubation of the cells with LPS our method allowed us to quantify several of the lipid mediators of interest (**Figure 6**). As expected, prostaglandins are much more abundant than the corresponding PG-G, which in turn are more abundant than the PG-EA. Incubation of the cells with LPS resulted in decreased cell levels of arachidonic acid and increased cell levels of the prostaglandins. This increase was also apparent in the cell culture medium. For the endocannabinoid 2-AG we observed a similar trend with decreased

cell levels of 2-AG and increased cell levels of PG-G. For AEA we observed a different trend as cell incubation with LPS resulted in increased levels of the endocannabinoid, as we previously reported [426]. As for the PG-EA we observed a strong increase in their levels in the cell incubation media, but no detection (except for PGD_2-EA) in the cells.

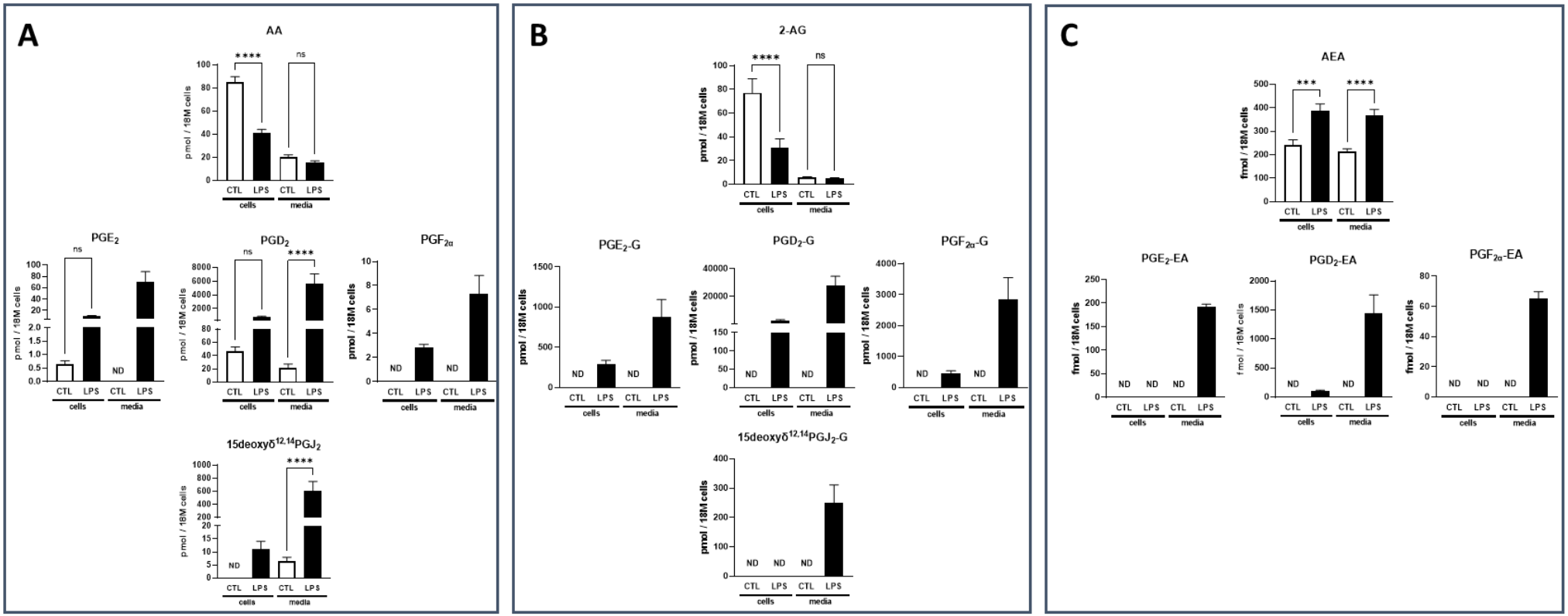
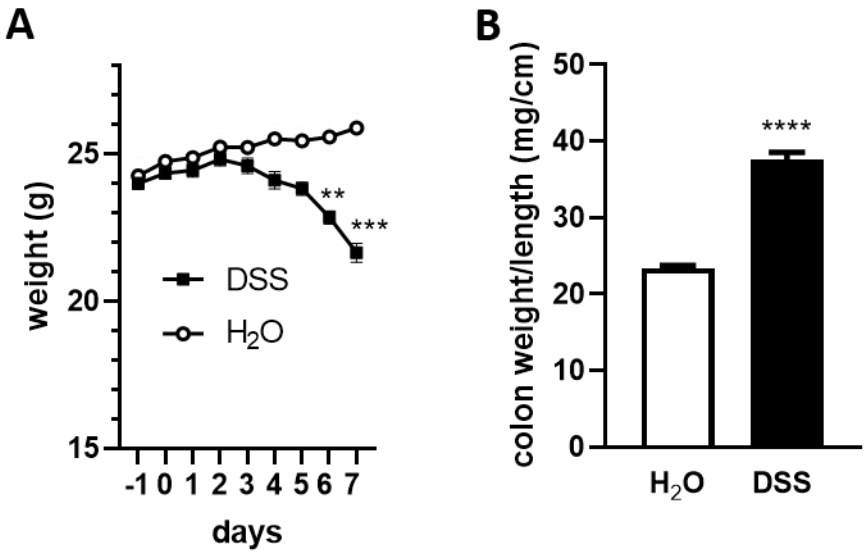


Figure 6: Quantification of the analytes of interest in J774 cells and their culture media following LPS activation. J774 cells were treated with vehicle (CTL) or with 100 ng/mL of LPS (LPS) for 24h. Then the cells and media were recovered and independently analysed using the developed method. (**A**) Quantification of arachidonic acid and derived prostaglandins. (**B**) Quantification of 2-AG and derived prostaglandin-glycerol esters. (**C**) Quantification of anandamide and derived prostaglandin-ethanolamides. The signal (AUC) of each analyte was normalized with the AUC of the corresponding internal standard and the calibration curves used to convert the ratio into analyte levels (pmol or fmol per 18M cells depending on the analyte). Data are expressed in mean ± SEM. N=3 in triplicate. After normality assessment, one-way Anova was used. *** p< 0.001; **** p< 0.0001 with Sidak's multiple comparison post-test.

Prostaglandins are described as secreted mediators, detecting them in the cell culture media in larger amounts than in the corresponding cell samples is therefore not surprising [129]. Less is known on the secretion of PG-G and PG-EA, however their presence in the cell culture media is coherent with the fact that they can act as biological mediators.

III.4 Quantification of the prostaglandin derivatives in a DSS-induced colitis mouse model

To test further the applicability of our method, we decided to assess the effect of a DSS-induced colitis (**Supplemental Figure 7**) on the levels of the lipid mediators of interest in several tissues of relevance in the context of colitis.



Supplemental figure 7. DSS-induced colitis. Colitis was induced by the addition of DSS in the drinking water (5%) for 5 days, followed by 2 days of normal drinking water. The control (CTL) group received only normal drinking water. Mice were euthanized on day 7. (**A**) Evolution of the mice body weight during the study. (**B**) Colon weight (mg) over length (cm) ratio, a reliable marker of colitis. 8 mice per group. Mean ± SEM. For (A) two-way ANOVA followed by Bonferroni post hoc test, for (B) student t-test ** p< 0.01 *** p< 0.001 **** p< 0.0001.

We chose this model as we have previously shown that administering or altering endocannabinoid and PG-G derivative levels can decrease colitis extent [176, 427, 437]. Moreover, our and other works have shown that the levels of these lipid mediators are altered

in the context of colon inflammation [176, 427, 437-440]. In this study, besides the colon, we also analysed the draining lymph nodes as well as the spleen that are important tissues in the immune response involved in the disease. Also, we analysed the visceral adipose tissue as it has also been shown to be subjected to inflammation during colitis [441-443]. Finally, we also measured the lipid levels in the blood from the portal and the cava veins, as well as the liver (**Supplemental table 3**).

tissue / metabolite	colon[a]		lymph node[a]		visceral adipose tissue[a]		spleen[a]	
	CTL	DSS	CTL	DSS	CTL	DSS	CTL	DSS
AA	1324,6 ± 236,5	638,6 ± 44,6 *	7420,4 ± 756,6	5101,4 ± 383,2 *	5221,1 ± 962,1	5913,5 ± 462,7	4544,4 ± 221,7	5792,6 ± 608,6
PGD_2	67,0 ± 18,3	215,7 ± 46,7 *	98,0 ± 19,6	400,1 ± 156,9	15,2 ± 2,6	125,0 ± 36,9 *	462,4 ± 70,8	1114,7 ± 127,1 ***
PGE_2	22,6 ± 2,1	212,1 ± 38,6 ***	18,4 ± 5,3	50,4 ± 14,4	9,5 ± 0,9	33,6 ± 7,2 **	39,2 ± 9,8	66,9 ± 8,9
$PGF_{2\alpha}$	14,4 ± 2,3	34,4 ± 5,6 **	21,9 ± 9,7	21,7 ± 4,9	4,4 ± 0,6	19,9 ± 6,9 *	9,6 ± 1,6	21,8 ± 4,3 *
15deoxy$\delta^{12,14}PGJ_2$	ND	1,31 ± 0,36	ND	ND	ND	ND	ND	ND
2-AG	3446,1 ± 304,9	2943,5 ± 139,3	883,9 ± 95,9	2042,4 ± 525,5	9967,9 ± 2895	19484,5 ± 2435,7 *	1097,7 ± 85,9	1281,5 ± 88,4
PGD_2-G	ND	ND	ND	ND	ND	ND	ND	ND
PGE_2-G	7,1 ± 0,7	4,8 ± 0,4 *	20,1 ± 2,9	23,3 ± 2,9	3,8 ± 0,4	5,7 ± 0,3 **	51,8 ± 6,1	49,9 ± 7,4
$PGF_{2\alpha}$-G	ND	ND	ND	ND	ND	ND	ND	ND
15deoxy$\delta^{12,14}PGJ_2$-G	ND	ND	< LOQ	< LOQ	ND	ND	ND	ND
AEA	1,19 ± 0,09	1,49 ± 0,04 *	2,9 ± 0,2	2,3 ± 0,1 **	2,5 ± 0,2	1,7 ± 0,2 *	1,02 ± 0,1	1,19 ± 0,1
PGD_2-EA	ND	ND	ND	ND	ND	ND	ND	ND
PGE_2-EA	ND	< LOQ	ND	ND	ND	ND	ND	ND
$PGF_{2\alpha}$-EA	ND	ND	ND	ND	ND	ND	ND	ND

tissue / metabolite	liver[a]		portal vein[b]		caval vein[b]	
	CTL	DSS	CTL	DSS	CTL	DSS
AA	1287,6 ± 157,6	2456 ± 363,8 *	1453,7 ± 103,7	960,3 ± 58,1 ***	1360,6 ± 130,3	927,7 ± 46,6 **
PGD_2	ND	ND	3,74 ± 0,5	7,5 ± 1,9	ND	ND
PGE_2	ND	ND	5,4 ± 0,7	15,8 ± 3,2 *	4,2 ± 0,6	2,0 ± 0,1 **
$PGF_{2\alpha}$	ND	ND	ND	ND	ND	ND
15deoxy$\delta^{12,14}PGJ_2$	ND	ND	ND	ND	ND	ND
2-AG	156,3 ± 10,5	227 ± 20,2 **	41,2 ± 3	35,9 ± 4,1	31,5 ± 2,4	26,4 ± 2,4
PGD_2-G	ND	ND	ND	ND	ND	ND
PGE_2-G	ND	ND	ND	ND	ND	ND
$PGF_{2\alpha}$-G	ND	ND	ND	ND	ND	ND
15deoxy$\delta^{12,14}PGJ_2$-G	ND	ND	ND	ND	ND	ND
AEA	0,24 ± 0,01	0,31 ± 0,03 *	0,43 ± 0,01	0,37 ± 0,01 *	0,57 ± 0,1	0,54 ± 0,04
PGD_2-EA	ND	ND	ND	ND	ND	ND
PGE_2-EA	ND	ND	ND	ND	ND	ND
$PGF_{2\alpha}$-EA	ND	ND	< LOQ	ND	ND	ND

[a] values are reported in pmol / g of tissue; [b] values are reported in pmol / mL of plasma

Supplemental table 3: Amounts of the prostaglandin derivatives in tissues of CTL and DSS induced colitis mice Tissues (colon, lymphoid nodes, visceral adipose tissue, spleen, liver, portal vein, cava vein) were stored at -80°C until analysis. The complete analytical procedure was applied to quantify the different prostaglandin derivatives. Values are expressed in pmol/g (or mL for plasma samples) of tissue. ND: not detected; <LOQ: values below the respective LOQ mentioned in the method. Eight mice per group. Data are expressed in mean ± SEM. After normality assessment, student t-test or Mann-Whitney non-parametric test was used. * p< 0,05; ** p<0,01; *** p< 0,001

As shown in **Figure 7**, in accordance with the literature [440], we observed an overall increase in prostaglandin levels due to colitis induction (the tissue levels are reported in **Supplemental table 3**). In the plasma, we observed that while PGE_2 levels increased in portal vein, they were decreased in the cava vein. Regarding the PG-G, only PGE_2-G was present in levels high enough to be quantified (i.e. above the LOQ of the method). Its levels were decreased in the colon and increased in the visceral adipose tissue due to the DSS-induced colitis. However, colitis did not change PGE_2-G levels in the lymph nodes and spleen. Unfortunately, we were unable to detect prostamide derivatives in the analysed tissues. The fact that AEA levels are much lower than 2-AG levels could explain the much lower levels of PG-EA which are below the LOD of our method. This is supported by our in vitro data as we found lower levels of PG-EA compared to PG-G levels. At the biological level we found quite marked variations in lipid levels in the visceral adipose tissue further supporting its sensitivity to colon inflammation. Further quantifications of bioactive lipids in this tissue are warranted in both preclinical and clinical studies on colitis.

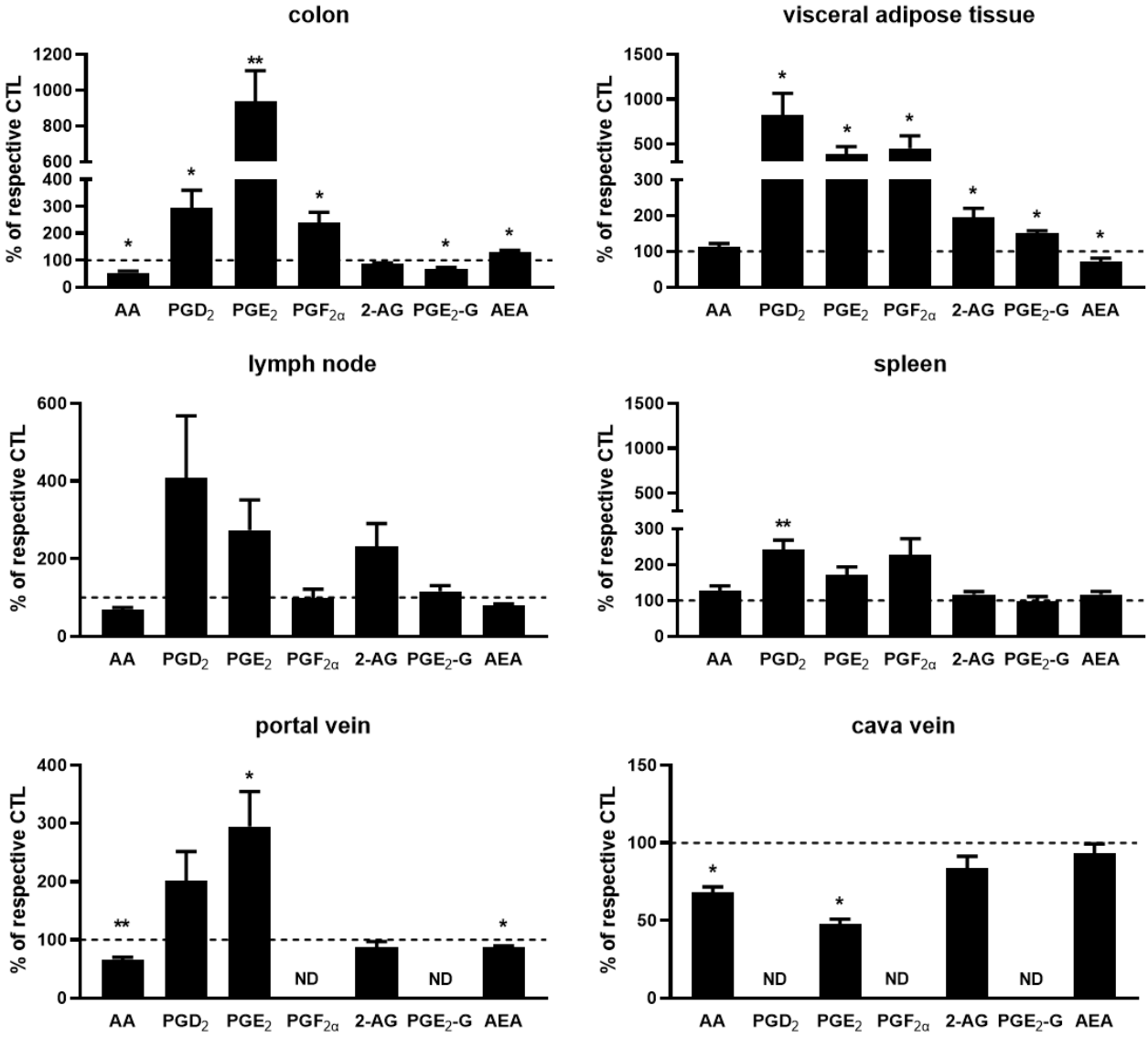


Figure 7: Quantification of the analytes of interest in DSS-induced colitis mice. Colitis was induced by adding DSS (5%) in the drinking water. Control mice (CTL) had access to drinking water throughout the study. On day 7, tissues (colon, lymphoid node, visceral adipose tissue, spleen, portal and cava vein plasma) were recovered. The levels of analytes were analysed in each tissue and are reported here relative to the control group set at 100% (dotted line). Data are expressed in mean ± SEM (8 mice/group). The levels measured in both groups are reported in supplemental table 3. After normality assessment, student t-test or Mann-Whitney non-parametric test was used. FDR assessment based on Benjamini-Hochberg method. Adjusted P values * p< 0,05; ** p<0,01.

IV. CONCLUSION

As mentioned, low endogenous abundance represents the main challenge when quantifying in biological matrices PG-EA, and to a lesser extent PG-G, derivatives. In this report we described an UHPLC-MS/MS method allowing the single-run quantification of prostaglandins and their glycerol ester and ethanolamide analogues, as well as their respective biochemical precursors. The use of this femtomole-range method will improve our understanding of the interactions between these lipid mediators that share biochemical pathways and receptors.

V. Acknowledgements

G.G. Muccioli is the recipient of subsidies from the Fonds Spéciaux de Recherches (FSR, Université catholique de Louvain) and from the FRS-FNRS, Belgium. The MASSMET platform is acknowledged for the access to the mass spectrometer. Prof. Mireille Alhouayek (LDRI, UCLouvain) is acknowledged for her expertise and help with the colitis model.

VI. Authors contribution

A. Paquot and G. G. Muccioli designed the research; A. Paquot performed experiments and analysed the data; J. Bestard-Escalas conducted the in vitro experiments; A. Paquot and G. G. Muccioli wrote the manuscript; G.G. Muccioli supervised the whole study; and all authors read and approved the manuscript.

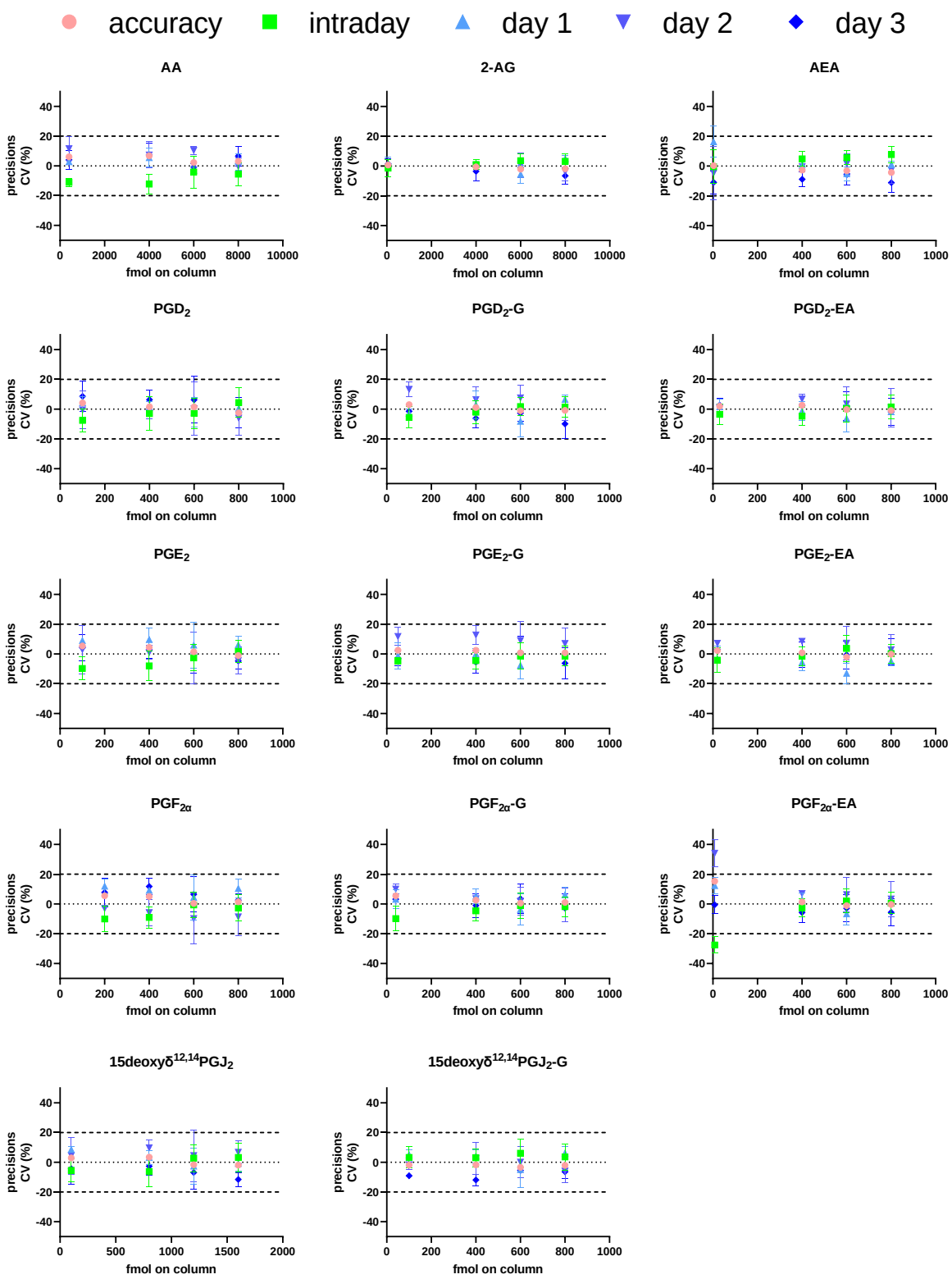
VII. Conflicts of interest

The authors declare that they have no competing interests.

Additional comments and potential limitations of the work

In work reported above, that the accuracy was determined using standard solutions rather than using matrix containing set amounts of standard as recommended by the ICH guidelines when developing and validating an analytical method for the measurement of endogenous compounds. This could result in a potential bias when analysing biological samples.

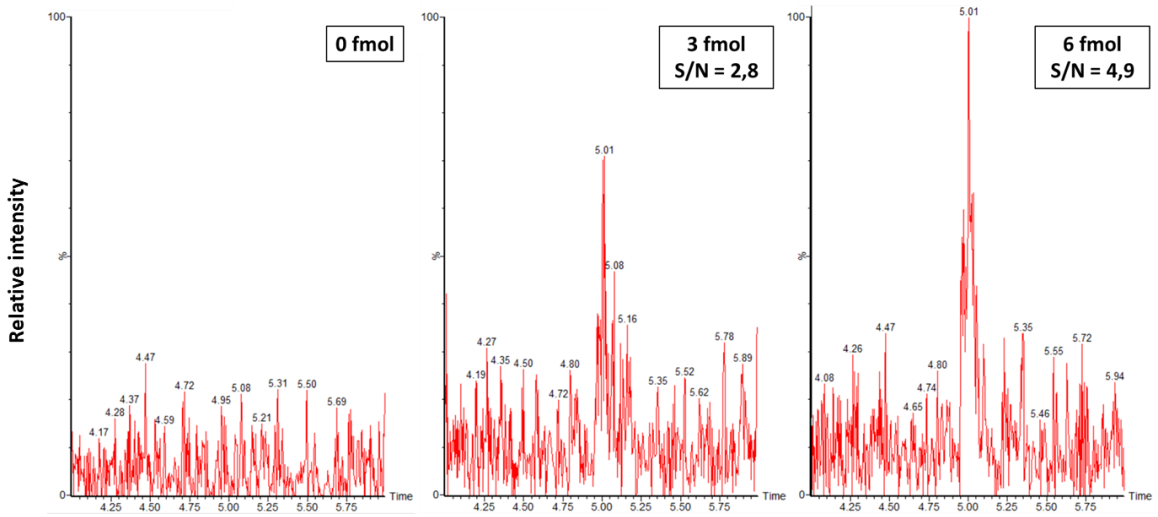
Besides, we report below graphs showing the precisions values for the compounds analysed by the method to help the reader having a better sense of this validation criteria (**supplementary figure 8**).



Supplemental figure 8: Precisions values obtained for the compounds analysed by the method For each prostaglandin related compound, the CV (%) of the repeatability, intermediate precisions and accuracy were reported for the four quality control levels. Dotted lines show the zero values, meaning there is no variation from the reference (theoretical) value. Dash lines show the ± 20% values set as range of acceptance for the validated method.

Another limitation lies in the determination of the specificity as the data shown in the published paper (see above) only partially discussed the specificity of the method for biological samples. Indeed, we only reported those data for $PGF_{2\alpha}$-EA and PGE_2-G selected as examples for all the analytes of interest.

The method used for the determination of the limits of detection and quantification was selected based on the European Pharmacopoeia guidelines. This requires to determine the signal to noise ratio obtained during analysis and select a S/N of 3.3 for the LOD and a S/N of 10 for the LOQ. However, graphical examples of such procedure were not reported in the present article which could be interesting to ensure the proper selection of the limits. As example, representative from the entire family of compounds, **supplemental figure 9** illustrates the MRM chromatograms obtained for the $PGF_{2\alpha}$-EA which allowed us to determine the LOD. The LOQ was determined by multiplying the determined LOD by 3.

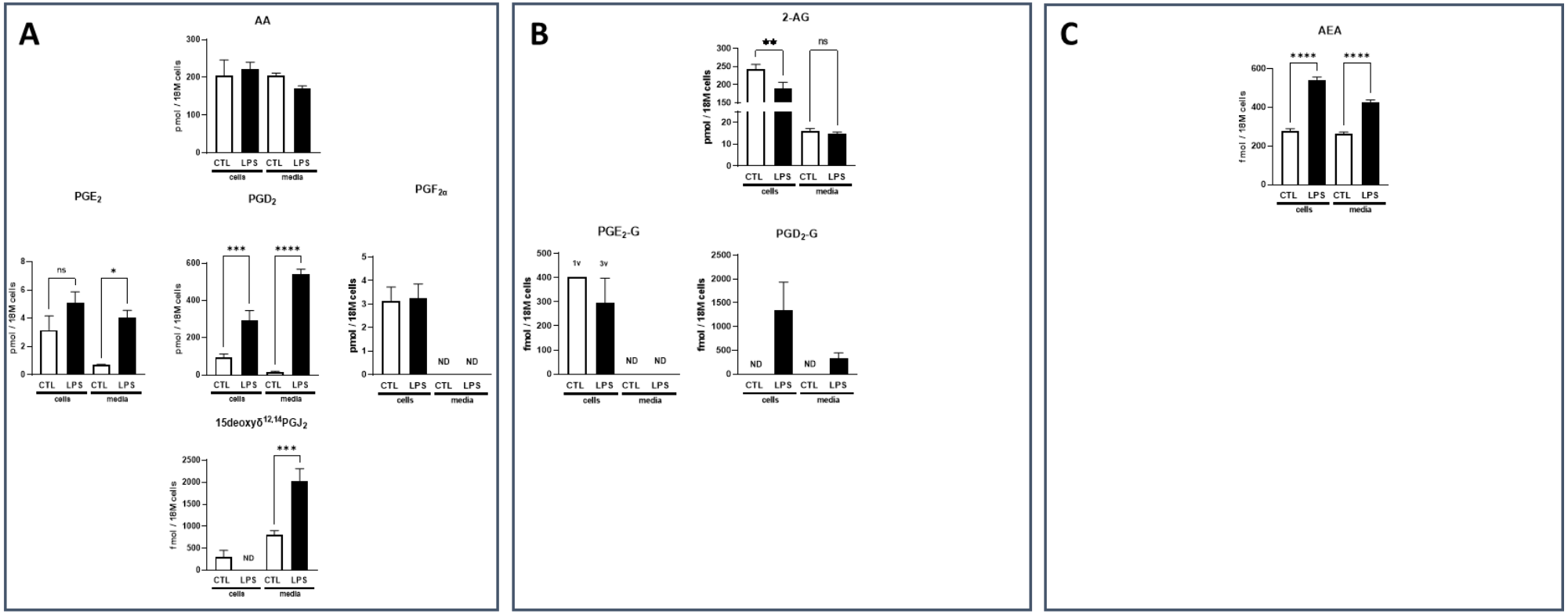


Supplemental figure 9: Chromatograms obtained after the injection of increasing amounts of $PGF_{2\alpha}$-EA. The chromatograms are representatives of the compounds analysed for method development. The three chromatograms represent injection of 0, 3 and 6 fmol of $PGF_{2\alpha}$-EA.

In addition to the data on J774 cells we published showing the effect of 24h of incubation with LPS, we also analysed the effect of 4h of incubation with LPS. Although it was not interesting on a validation point of view, this information is interesting from a biological point of view and supports the kinetic study proposed in the perspectives of this PhD thesis (see Perspectives section).

As reported in **Supplemental figure 10**, we were not able to detect several derivatives within the entire family after 4h LPS incubation. It was mainly the case for the ethanolamine derivatives although AEA was significantly increased upon LPS incubation. For the detected prostaglandin derivatives, the trends are similar to the ones observed after 24h of incubation even if the fold changes are less pronounced after 4h. This was notably marked for the conventional prostaglandins. Interestingly, as several PG-G and PG-EA derivatives were not detected after 4h but were detected after 24h, it seems that those derivatives are more

implicated in the late inflammatory process or that the metabolizing process is delayed for 2-AG and AEA. At the contrary, conventional prostaglandins are already induced earlier. Another view of the same system is that PG-G and PG-EA were not detected after 4h because they were below the limit of the detection of the method, even if exerting some biological effects on the system.



Supplemental Figure 10: Quantification of the analytes of interest in J774 cells and their culture media following LPS activation. J774 cells were treated with vehicle (CTL) or with 100 ng/mL of LPS (LPS) for 4h. Then the cells and media were recovered and independently analysed using the developed method. (**A**) Quantification of arachidonic acid and derived prostaglandins. (**B**) Quantification of 2-AG and derived prostaglandin-glycerol esters. (**C**) Quantification of anandamide and derived prostaglandin-ethanolamides. The signal (AUC) of each analyte was normalized with the AUC of the corresponding internal standard and the calibration curves used to convert the ratio into analyte levels (pmol or fmol per 18M cells depending on the analyte). Data are expressed in mean ± SEM. N=3 in triplicate. After normality assessment, one-way Anova was used. *** p< 0.001; **** p< 0.0001 with Sidak's multiple comparison post-test.

Result section II

In this section of the results (section II) I report our work aiming at developing a quantification method that would allow the quantification of SCFA and BCFA. As described in the introduction, as citrate cycle metabolites and ketones bodies are metabolically linked with short chain and branched chain fatty acids, we extended the scope of our method to those metabolites. We also tried to take into account the challenges linked to the analysis of SCFA and BCFA in biological matrices (e.g. isomeric structures, volatility, cross contamination, …). Moreover, we reasoned that a method would be even more interesting if we were able to show its applicability in a wide array of matrices.

As described below, the method that we developed will allow the quantitation of 19 compounds in a single run. We then applied the method to the quantification of those compounds in relevant matrices (i.e. faeces, caecal content, plasma, liver, colon and ileum from mice as well as plasma and faeces from human sample).

Set up and validation of an UPLC-MS quantification method for short chain fatty acids, branched chain fatty acids, citrate cycle metabolites and ketone bodies

Adrien Paquot, Julien Masquelier, Giulio G. Muccioli

I. INTRODUCTION

As discussed in the general introduction, SCFA gained in interest in the past years and there is a need to develop new tools to characterize this family of lipids. As summarized above, SCFA metabolic pathways are various and complex, thus we decided to develop and validate a quantification method that could quantify not only SCFA, but also branched chain fatty acids (BCFA), citrate cycle metabolites (CCM) as well as ketone bodies. Even if described as SCFA products playing interesting roles in health and diseases, we did not include acyl-CoA derivatives during the method development as we decided to focus on the "free" species.

For the quantification of SCFA, methods using GC coupled with mass spectrometry (MS) or a flame ionization detector (FID) are widely described in the literature [340, 363]. However, GC-FID has some issues related to the identification of peaks, thermic degradation of SCFA or co-elution with solvent or other endogenous compounds [444]. Derivatization followed by GC-MS analysis appears as an alternative to solve FID-related problems. However, the use of the GC technique requires higher running temperatures that could impact samples integrity [266]. Besides, few derivatization procedures suitable for the GC analysis are compatible with SCFA due to their high volatility and the potential compound loss that could happen during sample preparation [265]. Thus ultra-high performance liquid chromatography (UHPLC) coupled to MS emerged in the past years as a new strategy for the analysis of SCFA and related compounds often after derivatization [445].

Based on the different limitations observed with the GC-based methods, and the complexity that the combined analysis of SCFA, BCFA, CCM and ketone bodies represents, we decided to develop a UHPLC-MS method. As reported in the literature, derivatization strategies can increase the detection of compounds [376]. Recently, several derivatization procedures were described and successfully applied to LC-MS analysis [263, 336, 376]. Regarding the described methods, we selected 3-nitrophenylhydrazine (3-NPH) as derivatization reagent. The interest of this reagent is its suitability with water-containing matrices that should allow to avoid the use of additional solvent and simplify the extraction procedure that are both sources of bias in SCFA analysis. The second interest is that 3-NPH is a carboxylic group-derivatizing agent that can react with both SCFA and CCM. Moreover, Zeng et al., observed higher signal response for SCFA when using 3-NPH compared to three others carboxylic acid groups-derivatizing agents [341].

Recently, several groups described methods allowing the quantification of SCFA. The group of Simonetti described a method allowing the quantification of several SCFA and CCM [264] but, to the best of our knowledge, nobody described a method to detect and quantify the entire family as well as the different precursors. Here we report the development and optimization of a method allowing the separation and the quantification of the different SCFA, BCFA as well as the different CCM and ketone bodies. During the development, special attention was given to the optimization of the extraction, derivatization and cleanup procedures of the samples. Moreover, we took into consideration the potential contamination and early degradation of the samples. We also assessed the freeze-drying importance and optimal procedure in the case of SCFA. Altogether, these efforts resulted in a method that will allow us to analyse the metabolites of interest in several biological matrices from animals and humans.

II. MATERIALS AND METHODS

II.1 Chemicals

SCFA standards (analytical grade), specifically propionic acid (PA), isobutyric acid (*i*BA), butyric acid (BA), 2-methylbutyric acid (2-MeBA), isovaleric acid (*i*VA), valeric acid (VA), 2-methylpentanoic acid (2-MePA), 3-methylpentanoic acid (3-MePA), 4-methylvaleric acid (4-MeVA), hexanoic acid (HA), were purchased from Sigma-Aldrich. Acetic acid was bought from VWR. CCM (i.e. pyruvic acid (PyA), succinic acid (SA), lactic acid (LA), malic acid (MA), α-ketoglutaric acid (αKA), citric acid (CA)) as well as valproic acid and d_4-succinic acid (used as internal standards; IS) were all purchased from Sigma-Aldrich. β-hydroxybutyric acid (βHA) and acetone were bought from Alfa Aesar Chemicals and VWR, respectively. HPLC and MS grade solvents (acetonitrile (ACN), chloroform ($CHCl_3$) and methanol (MeOH)) and hydrochloride (HCl) were from VWR. Derivatization reagents (i.e. 3-nitrophenylhydrazine (3-NPH), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC) and pyridine were bought from Sigma-Aldrich. Double distilled H_2O was obtained by daily distillation of distillated H_2O.

II.2 Sample preparation

Approximatively 50-60 mg of wet caecal content was homogenized in double distilled H_2O (ratio of 20 mg caecal content / 50 µL of H_2O). For fecal sample, the ratio was 10 mg / 50 µL of H_2O. After shaking and sonicating 10 min in an iced bath, 50 µL of suspension was transferred in a microcentrifuge tube containing 200 µL ACN and 10 µL of valproate and d_4-succinate solutions (10^{-2}M) as internal standards. At the same time, 50 µL of the same suspension were transferred in a freeze-drying tube and directly stored (< -60°C) until the freeze-drying procedure on a Labconco® freeze-dryer 4.5 instrument. For liver, 50 mg were

homogenized and then ACN was directly added (together with the internal standards) to achieve protein precipitation. For plasma, 75 µL of plasma were directly added to a microcentrifuge tube with 200µL of ACN (containing the internal standards). Samples were mixed and then stored at -20°C (1 h). After protein precipitation and sample centrifugation at 15000x*g* on a Mikro 200R Hettich® centrifuge, the supernatants were transferred in 5 mL glass tubes.

II.3 Derivatization method and compound extraction

3-NPH was used as reagent for SCFA, BCFA, CCM and ketone bodies derivatization [265]. Briefly, 200 µL of a freshly prepared 3-NPH 0,14 M solution in H_2O-ACN (1:1; v/v) and 200 µL of an EDC 0,05 M solution in H_2O-ACN (1:1; v/v) containing 3 % v/v of pyridine were added to each sample. Samples were shaken and incubated at 40°C for 1 h with vortex-mixing each 20 min. At the end of the process, the reactions were quenched by transferring the tubes on ice.

To remove the excess derivatizing reagents, 1,5 mL chloroform, 750 µL distilled H_2O and 50 µL of HCl 2N were added to the mixture. Samples were shaken vigorously and then centrifuged 10 min at 700x*g*. The organic layer containing the derivatized products was recovered and the solvent removed by evaporation under a nitrogen stream. The dry residue was suspended in MeOH for UHPLC-MS analysis.

II.4 UHPLC-MS conditions

The analysis was carried out on an UPLC-LTQ Orbitrap XL from ThermoFisher scientific equipped with an APCI probe set in positive ionization mode. The source parameters were set as follows: Vaporizer temperature: 400°C; Sheath gas flow rate: 20 a.u.; Auxiliary gas flow rate: 5 a.u.; Sweep gas flow rate: 10 a.u.; Discharge current: 5 µA; Capillary temperature: 250°C; Capillary voltage: 2 V; Tube lens 35 V. Orbitrap parameters were as follows, Full AGC target :

50 000 ; Maximum injection time MS : 200 ms, 1 µscan ; Full MS mass range : 110-800 m/z; Multiple RF frequency: 2860.2; Main RF frequency: 1190.5; Resolution: 30000; no divert valve was used.

For compounds separation, a Kinetex F5 core-shell column (100x2,1 mm; 1,7 µm) from Phenomenex maintained at 40°C was used. Two mobile phases used in gradient mode were selected for elution and a flow rate of 200 µL/min was used. Mobile phase A was composed of H_2O-ACN-acetic acid (94,9:5:0,1; v/v/v) and mobile phase B contained ACN-acetic acid (99,9:0,1; v/v). We designed the gradient as follows: 5 % of B during 10 min followed by an increase to 15 % of B in 10 min then 30 % of B after 10 others minutes. Finally, the gradient reaches 100 % of B in 2 min and keeps this composition until the end of the run before re-equilibrating the system with the initial conditions. The temperature of the sample manager was kept at 15°C. The Orbitrap XL system provides 30000 m/z of mass resolution and allows a mass precision above four order of decimals. The Xcalibur© software was used for data processing and analysis. The ratio between the standard of interest and the internal standard was used to normalize the data. For the caecal content and fecal samples, normalization on the dry matter obtained after freeze-drying was used to normalize the results. In the case of tissue homogenate, weighted tissue was used for data normalization.

II.5 Method Validation

II.5.1 Selectivity and specificity

Selectivity was assessed by comparing retention time and mass precision for the different compounds and by evaluating potential interferences of the derivatization products together and with others biological compounds sharing similar masses using spiked matrices.

Moreover, MS^2 fragmentation was used to confirm the identity of the peak as well as ensuring no contaminant in the analysed peak.

II.5.2 Trueness

Trueness was evaluated on three quality control levels of the calibration curves (low, middle and high). Bias was determined on the reference values of the calibration curves and trueness was expressed in relative bias (%).

II.5.3 Calibration curves, LOD and LOQ, linearity

For the calibration curves, 11 concentration levels were selected for each compound. The range of the calibration curves was selected depending of the biological abundance of the metabolites (assessed before establishing the calibration curves). Valproate and d_4-succinate were selected as internal standards for data normalization. For each molecule, the detection observed for blank samples was subtracted from the others points to consider the detection of SCFA and BCFA due to different contamination sources. The complete procedure was used to build the calibration curves in order to mimic as much as possible the experimental conditions of the biological samples. A linear regression representation was selected. Based on these regressions and according to the FDA and European Pharmacopoeia guidelines [423, 424], the limit of detection (LOD) and limit of quantification (LOQ) were determined using the value at intercept method. Thus, based on the calibration curves, LOD and LOQ were determined using the following relations:

$$LOD = (3.3 * X_{blk}) / S_{y.x} \text{ and } LOQ = (10 * X_{blk}) / S_{y.x}$$

Where, X_{blk} is the value at the intercept and $S_{y.x}$ is the slope of the calibration curve

Samples with no biological matrix or standard (i.e. blank samples) were analysed with the same protocol used for the experimental samples and for each compound, the signal obtained

in the blank at the retention time of the injected standard was used as basal level. As SCFA are frequent contaminants in air or solvents, the mean of several independent blank samples coming from analysis performed at several moments was used. Then the value obtained by taking three times (and ten times) the mean value was used as the reference value for the LOD/LOQ experiments allowing some precautions in the determination of the LOD and LOQ.

To assess the linearity, for each compound five points covering the entire calibration range were selected and linearity was determined by plotting the ratio obtained by analyzing known amount of the respective five points in the calibration curves. The slope and R square values of the new linear regressions were then used as linearity criteria in accordance with the guidelines.

II.6 Quantification of the lipids of interest in biological samples

Several matrices were selected to assess the applicability of the method. The selection was based on the matrix relevance and on the potential for translational studies. For mice, caecal content, faeces and plasma were first selected as they are the most studied matrices in the case of SCFA. In addition, although less studied as matrices, liver, colon and ileum were selected because of their high relevance due to their proximity with the gut microbiota. For human matrices, we were limited by the accessibility of the matrices and decided to apply the method on the plasma and faeces.

Samples were prepared as mentioned above. For solid tissues (i.e. liver, colon and ileum), weighed frozen tissues were homogenized in water to reach a ratio of 50 mg/100 µL water. Directly after homogenization, acetonitrile was added to limit the risk of sample alteration. Then samples were analysed using the same methodology than used for faeces and caecal content.

II.7 Statistical analysis

Mann-Whitney and student t-test were performed using the GraphPad Prism v9.1.2 software (San Diego, CA). Data were expressed in nmol/mg of dry matter for caecal content and fecal samples, in nmol/50 mg for liver and in nmol / 50 µL of plasma for the others conditions. ND, < LOD and < LOQ stand for not detected, under the limit of detection and under the limit of quantification, respectively.

III. RESULTS AND DISCUSSION

III.1 Optimization of the extraction and derivatization procedures

As mentioned above, we developed here a quantification method for the analysis of SCFA, BCFA as well as CCM and ketone bodies (KB). The volatility of the molecules and the numerous contamination sources are the two main risks for sample alteration. For these reasons, we decided to use protein precipitation as extraction technique to minimize the risk of interference. As the presence of SCFA (and to a lesser extent of CCM) in many solvents is known [342], we compared the SCFA content (for acetate, PA and BA as they are the most described contaminant in solvents) in the solvents classically reported in the literature for protein precipitation [341, 374, 446]. Interestingly, when we used diethyl ether (often used for SCFA extraction procedures [340, 372, 446]) as precipitation solvent we observed an important detection of acetate and propionate as compared with the others solvents tested (**Fig. 1A**). Moreover, the use of diethyl ether did not allow us to detect valproate in the mixture, potentially due to the extensive use of 3-NPH by the contaminants present in diethyl ether. Based on these experiments, we selected acetonitrile as precipitation solvent.

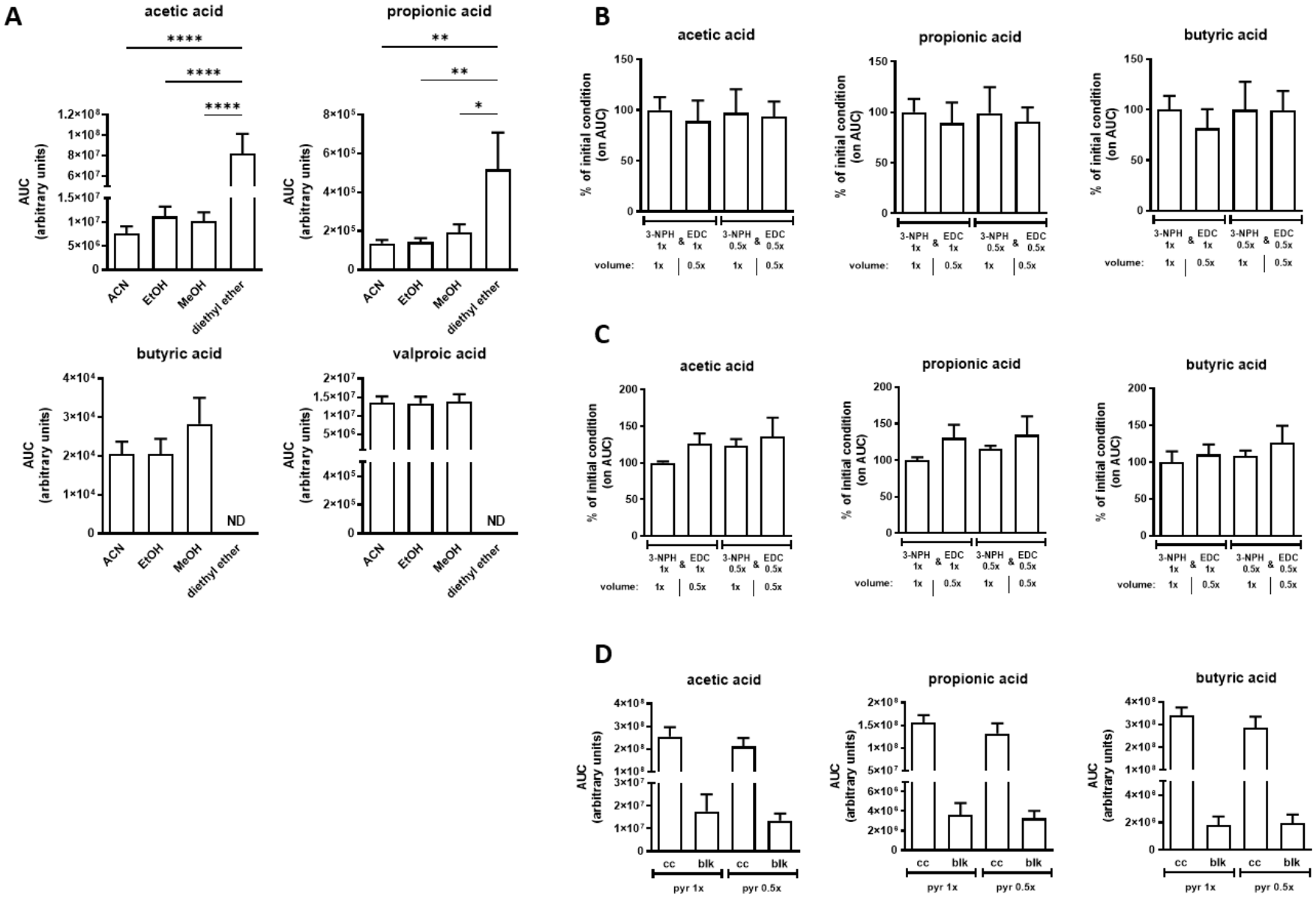

A
acetic acid
propionic acid
butyric acid
valproic acid
AUC (arbitrary units)
ACN
EtOH
MeOH
diethyl ether
ND
B
acetic acid
propionic acid
butyric acid
% of initial condition (on AUC)
3-NPH 1x & EDC 1x
3-NPH 0.5x & EDC 0.5x
volume: 1x | 0.5x 1x | 0.5x
C
acetic acid
propionic acid
butyric acid
% of initial condition (on AUC)
3-NPH 1x & EDC 1x
3-NPH 0.5x & EDC 0.5x
volume: 1x | 0.5x 1x | 0.5x
D
acetic acid
propionic acid
butyric acid
AUC (arbitrary units)
cc
blk
pyr 1x
pyr 0.5x

Figure 1: Assessment of the contamination by SCFA of the solvents used to precipitate the proteins. Solvent contaminant as well as the impact of reagent concentration and the volume on contaminant levels were assessed in blank samples and in presence of matrix. (**A**) acetate, propionate (PA), butyrate (BA) and valproate levels assessed in several widely used precipitation solvent. Blank samples were analysed with the complete procedure. Data expressed as the AUC of each peak. (**B**) acetate, PA and BA assessed in blank sample using literature-inspired derivatization conditions (3-NPH & EDC 1x and volume 1x) as well as half the concentration and the volume (see main text for more details). Data expressed as percentage of the initial condition (reagent 1x and volume 1x) (**C**) acetate, PA and BA assessed in presence of matrix (caecal content, 20 mg) using literature inspired derivatization conditions (3-NPH & EDC 1x and volume 1x) as well as half the concentration and the volume. Data expressed as percentage of the initial condition (reagent 1x and volume 1x). (**D**) acetate, PA, BA assessed in blank sample and in presence of matrix using literature inspired derivatization conditions (pyridine 1x) as well as half the concentration. Data expressed as AUC obtained. N=3 in triplicate. Data are expressed in mean ± SEM. After normality assessment, one-way Anova was used. * p< 0,05; ** p< 0,01; **** p< 0,0001 with Sidak's multiple comparison post test.

To minimize sample contamination during the extraction and derivatization procedures we decided to assess the impact of the derivatization procedure, and compared several conditions to select an optimal balance between contamination presence and derivatization achievement. The expected derivatization reaction is shown in **Figure 2A** with succinate as example.

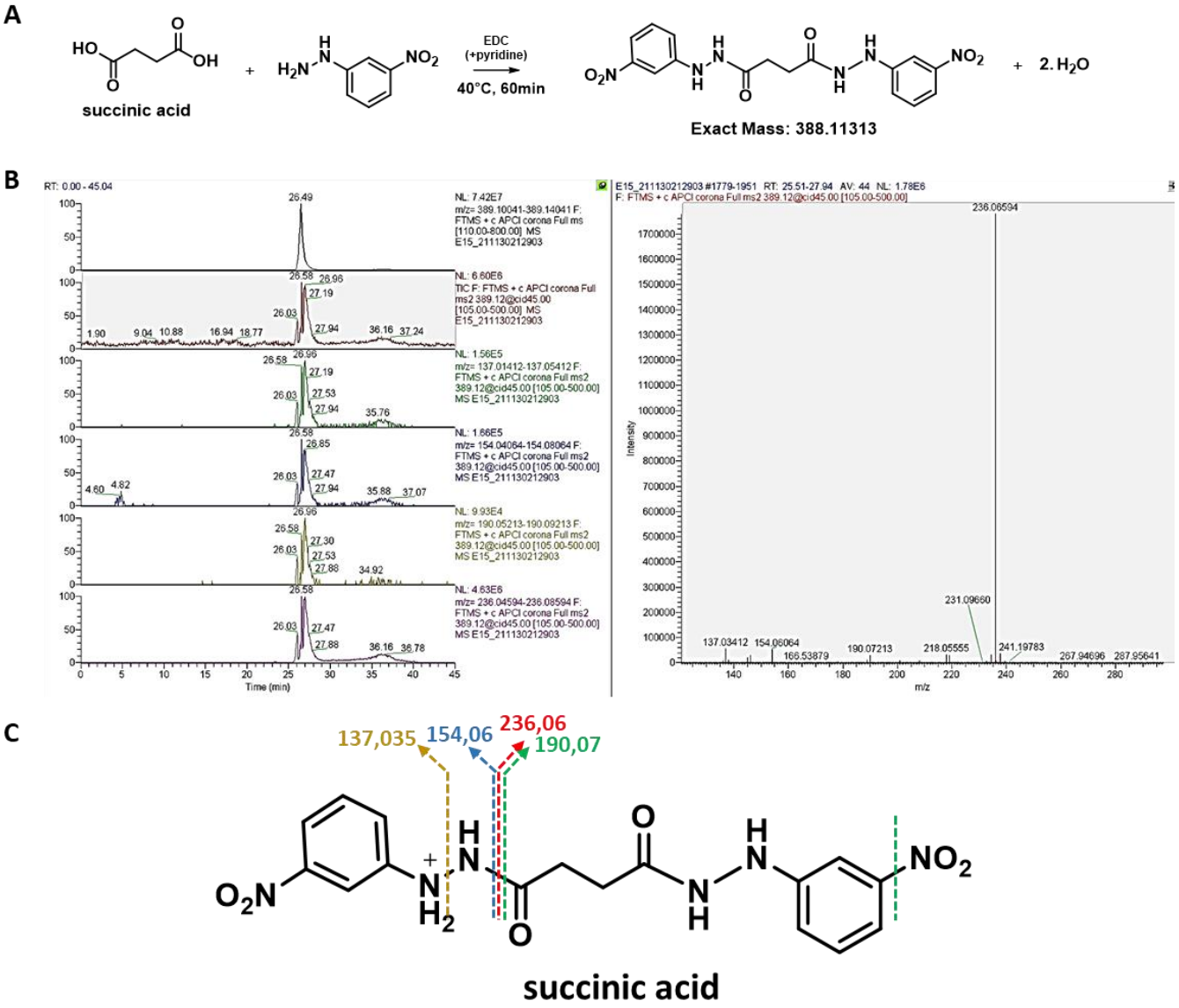


Figure 2: **Characterization of the derivatization products.** Succinate (SA) is used as representative molecule. Characterization of its derivatization product was achieved using the fragmentation capacity of the LTQ Orbitrap-XL. (**A**) Theorical derivatization reaction with the precursor, derivatization reagent, conditions, the product and its respective exact mass. (**B**) Extracted ion chromatogram for the analysed product as well as the total ion chromatogram obtained for the MS^2 linked to the analysed product as well as the ions pattern in the MS^2 peak and their respective extracted ion chromatogram. (**C**) Fragmentation pattern draw on the theorical analysed product with the respective masses of each fragment.

Thus, we compared (with and without matrix) the volume of the reactant mixture used as well as the quantity of reactants. As shown, lowering the volume or the content of reactants initially selected based on the literature [265] does not decrease the contamination level in our conditions (**Fig. 1 B-D**). As we aimed for an excess of reactant, we selected 200 µL (1x) of 0,14 M (1x) for the NPH solution and 200 µL (0.5x) of 0,05M (1x) for the EDC solution.

We then assessed the effect of the incubation temperature and time to maximize SCFA, BCFA, CCM and ketone bodies derivatization. As observed (**Figure 3**), incubating during 90 minutes at 40°C seems to be the best choice for the smaller derivatives (i.e. acetate and propionate). However, this does not seem to be the case for longer chain SCFA (i.e. hexanoate). Based on the relative endogenous abundance of the analytes, we selected an incubation at 40°C during 60 minutes as final condition.

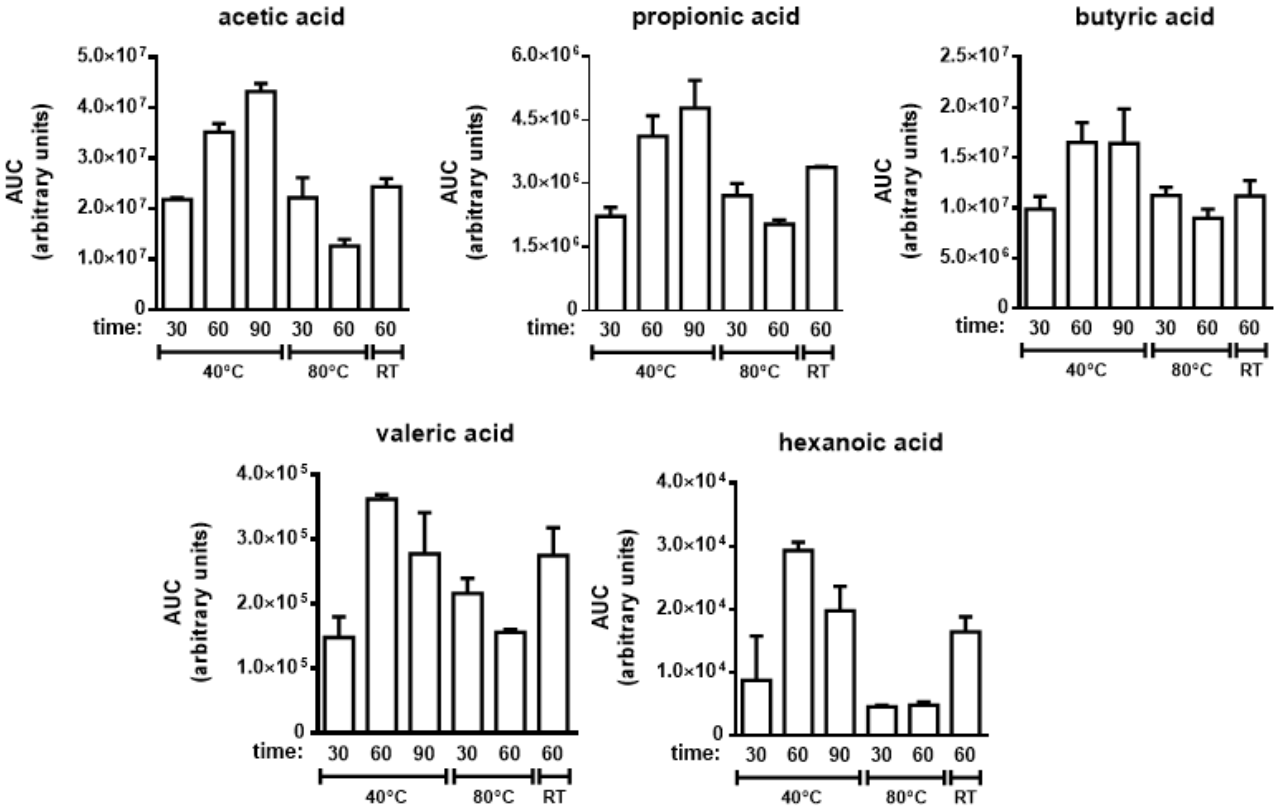


Figure 3: **Effect of the temperature and duration of incubation on SCFA derivatization** Caecal content homogenate (20 mg) was analysed following the complete derivatization procedure. Several timings and temperatures for the derivatization step were assessed. Data expressed as AUC obtained. N=1 in triplicate. Data are expressed in mean ± SEM.

III.2 Clean up procedure

To decrease the exogenous matrix load due to the derivatization, we decided to set up a cleaning procedure allowing us to extract the derivatized products from the reaction mixture while leaving the reagents and the underivatized polar compounds in the reaction mixture. Indeed, while the derivatization step is strategically important to quantify SCFA, it increases the risk of matrix effects and lowers the life span of the columns and instruments.

We based our strategy on previous methods developed in our laboratory based on Folch's liquid-liquid extraction [91, 422]. After testing several conditions **(data not shown)** we decided

to select 1,5 mL chloroform, 750 µL H_2O and 50 µL of HCl 2N as liquid-liquid extraction components **(Figure 4)**. Solvents were directly added to the final derivatization mixture to minimize potential losses linked to a transfer step. As shown in the figure, the liquid-liquid extraction procedure efficiently removes the excess of derivatizing agent **(Figure 4A)** while maintaining sufficient detection of the different derivatives as compared to the condition without liquid-liquid extraction **(Figure 4B)**. Based on these results, we selected the one-step extraction procedure for developing the method as the level of compounds of interest recovered is close to 100% and that a two-step extraction did significantly improved the extraction yield.

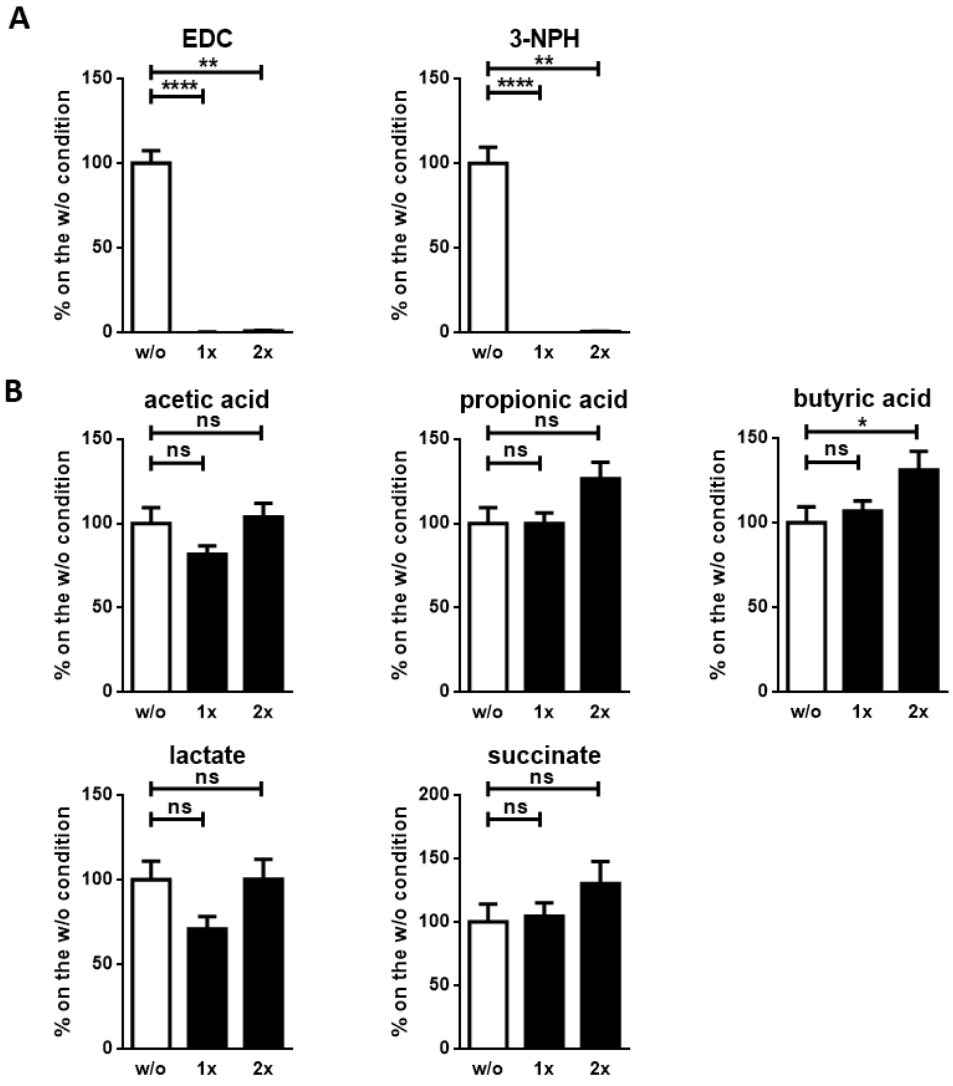


Figure 4: A final liquid / liquid extraction following the derivatization allows an efficient clean-up of the sample. At the end of the derivatization procedure, $CHCl_3$ (1.5 mL), H_2O (750 µL) and HCl 2N (50 µL) were added to the samples. After mixing and decantation, the lower organic phase containing derivatized products was removed and evaporated to dryness. Data are expressed as percentage of the "without liquid/liquid extraction procedure" (w/o) condition. N=3 in triplicate. Data are expressed in mean ± SEM. After normality assessment, one-way Anova was used. ** p< 0,01; **** p< 0,0001 with Sidak's multiple comparison post-test.

III.3 Set up and optimization of the LC-MS method

While the SCFA and BCFA have a single carboxylic moiety, CCM and ketone bodies have several moieties that, in theory, can react during the derivatization step. Using the Orbitrap we characterized for each analyte of the derivatization mixture in order to characterize the obtained product (**Table 1**).

name	ralative retention time[a]	molecular formula	molecular weight[b]	derivatized product formula	corresponding formula	derivatized product mass[c]
short chain fatty acids						
acetic acid	0,34	$C_2H_4O_2$	60,02113	$C_8H_{10}N_3O_3^+$	$[M + H + 3\text{-NPH}]^+$	196,07167
propionic acid	0,44	$C_3H_6O_2$	74,03678	$C_9H_{12}N_3O_3^+$	$[M + H + 3\text{-NPH}]^+$	210,08732
isobutyric acid	0,55	$C_4H_8O_2$	88,05243	$C_{10}H_{14}N_3O_3^+$	$[M + H + 3\text{-NPH}]^+$	224,10297
butyric acid	0,56	$C_4H_8O_2$	88,05243	$C_{10}H_{14}N_3O_3^+$	$[M + H + 3\text{-NPH}]^+$	224,10297
2-methylbutyric acid	0,68	$C_5H_{10}O_2$	102,06808	$C_{11}H_{16}N_3O_3^+$	$[M + H + 3\text{-NPH}]^+$	238,11862
isovaleric acid	0,70	$C_5H_{10}O_2$	102,06808	$C_{11}H_{16}N_3O_3^+$	$[M + H + 3\text{-NPH}]^+$	238,11862
valeric acid	0,73	$C_5H_{10}O_2$	102,06808	$C_{11}H_{16}N_3O_3^+$	$[M + H + 3\text{-NPH}]^+$	238,11862
2-methylpentanoic acid	0,84	$C_6H_{12}O_2$	116,08373	$C_{12}H_{18}N_3O_3^+$	$[M + H + 3\text{-NPH}]^+$	252,13427
3-methylpentanoic acid	0,85	$C_6H_{12}O_2$	116,08373	$C_{12}H_{18}N_3O_3^+$	$[M + H + 3\text{-NPH}]^+$	252,13427
4-methylvaleric acid	0,87	$C_6H_{12}O_2$	116,08373	$C_{12}H_{18}N_3O_3^+$	$[M + H + 3\text{-NPH}]^+$	252,13427
hexanoic acid	0,88	$C_6H_{12}O_2$	116,08373	$C_{12}H_{18}N_3O_3^+$	$[M + H + 3\text{-NPH}]^+$	252,13427
citrate cycle derivatives						
lactic acid	0,45	$C_3H_6O_3$	90,03169	$C_9H_{12}N_3O_4^+$	$[M + H + 3\text{-NPH}]^+$	226,08223
malic acid	0,92	$C_4H_6O_5$	134,02152	$C_{16}H_{17}N_6O_7^+$	$[M + H + 2*(3\text{-NPH})]^+$	405,11532
succinic acid	1,00	$C_4H_6O_4$	118,02661	$C_{16}H_{17}N_6O_6^+$	$[M + H + 2*(3\text{-NPH})]^+$	389,12041
citric acid	1,12	$C_6H_8O_7$	192,02700	$C_{24}H_{24}N_9O_{10}^+$	$[M + H + 2*(3\text{-NPH})]^+$	462,11353
pyruvic acid	1,43	$C_3H_4O_3$	88,01604	$C_{15}H_{15}N_6O_5^+$	$[M + H + 2*(3\text{-NPH})]^+$	359,10984
α-ketoglutaric acid	1,48	$C_5H_6O_5$	146,02152	$C_{17}H_{16}N_6O_7^+$	$[M + H + 3*(3\text{-NPH})]^+$	552,15859
ketone bodies						
β-hydroxybutyric acid	0,48	$C_4H_8O_3$	104,04734	$C_{10}H_{14}N_3O_4^+$	$[M + H + 3\text{-NPH}]^+$	240,09788
acetone	1,35	C_3H_6O	58,04186	$C_9H_{12}N_3O_2^+$	$[M + H + 3\text{-NPH}]^+$	194,0924
internal standards						
valproic acid		$C_8H_{16}O_2$	144,11503	$C_{14}H_{22}N_3O_3^+$	$[M + H + 3\text{-NPH}]^+$	280,16557
d_4-succinic acid		$C_4H_{10}O_4$	122,05571	$C_{16}H_{21}N_6O_6^+$	$[M + H + 2*(3\text{-NPH})]^+$	393,14951

[a] expressed with the ratio of retention time between the metabolite of interest and the respective internal standard

[b] exact mass from the neutral form

[c] exact mass from the positive ion of the derivatized product

Table 1: Chemical and analytical description of short chain fatty acids, branched chain fatty acids, citrate cycle metabolites and ketone bodies Description of the nineteen compounds (and their respective internal standards) (continued) analysed with the developed method. The table mentions the respective abbreviations, molecular formula, molecular weight, derivatized products formula, derivatization description as well as their respective masses and their relative retention time obtained as ratio with the retention time of respective internal standards.

Using the Orbitrap, we obtained the "exact mass" and fragmentation pattern of the derivatized product. These are important information regarding the identification of the compounds of interest (**Table 1 and Figure 2 B&C**). Once the MS detection set up, we optimized the chromatographic separation of the analytes. The chromatographic separation

is important here given the number of isomers among the compounds of interest. To determine the optimal separation parameters, we tested several UPLC columns (data not shown), mobile phases and chromatographic gradients before selecting a Kinetex® F5 100 Å (150x2.1 mm; 1.7 µm) column with mobile phases consisting to mixture of H_2O - ACN - acetic acid (94.9:5:0.1; v/v/v) and ACN - acetic acid (99.9:0.1; v/v) as the best compromise between compound separation and run length (**Fig. 5**). Due to the limited lipophilicities of the derivatization products, we designed a gradient characterized by a slow increase from the aqueous to the organic mobile phase to achieve isomers separation.

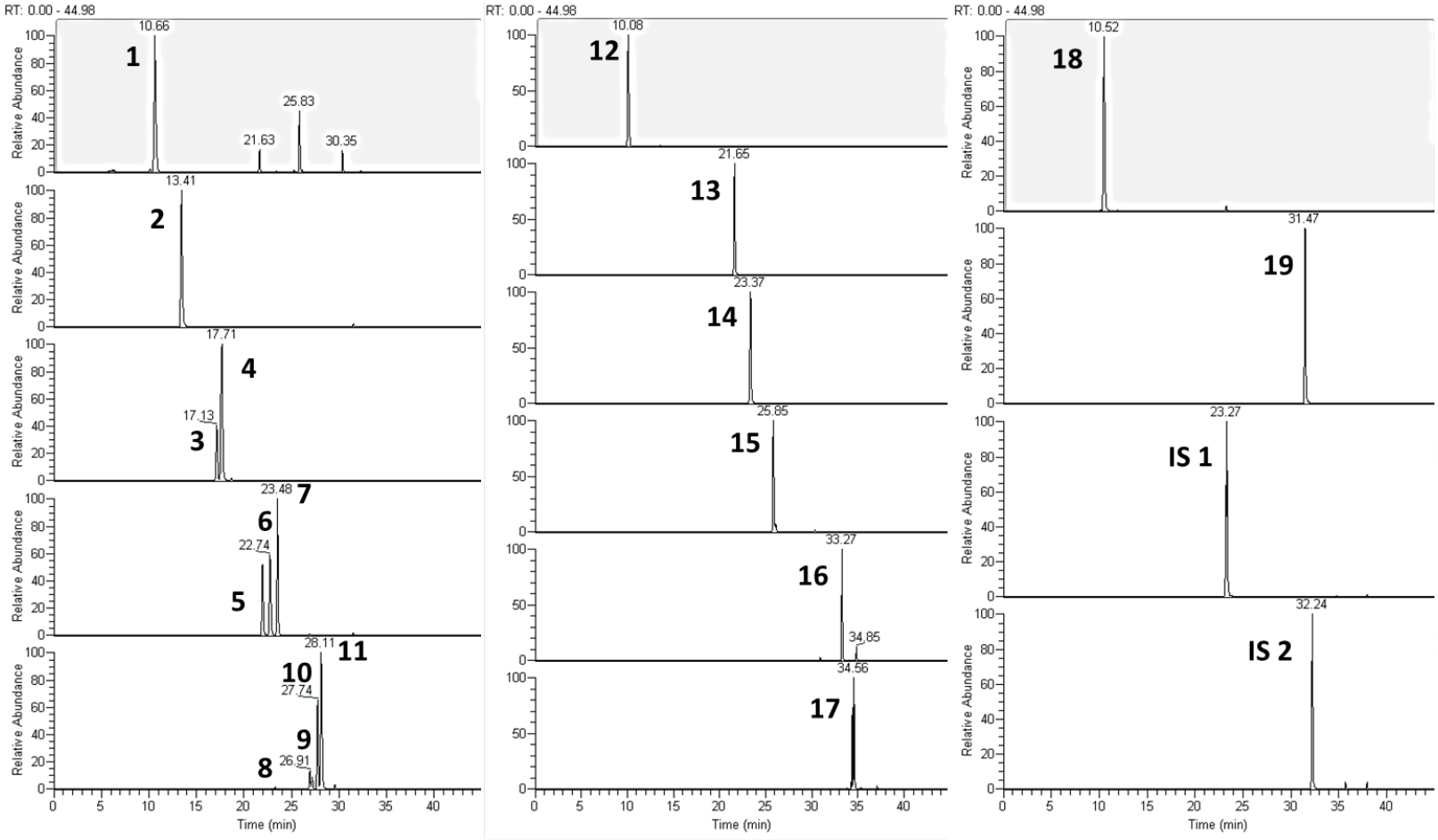


Figure 5: Typical UPLC-MS chromatogram of a mix of standards. The extracted ions are: 1. acetate; 2. propionate; 3. isobutyrate; 4. butyrate; 5. 2-methylbutyrate; 6. isovalerate; 7. valerate; 8. 2-methylpentanoate; 9. 3-methylpentanoate; 10. 4-methylvalerate; 11. hexanoate; 12. lactate; 13. malate; 14. succinate; 15. citrate; 16. pyruvate; 17. α-ketoglutarate; 18. 3-hydroxybutyrate; 19. acetone; IS1. d_4-succinate; IS2. Valproate.

III.4 Method validation

III.4.1 Selectivity, specificity and sensitivity

The use of extracted ion chromatograms together with the high resolution of the Orbitrap used allowed us to increase the selectivity and the specificity of the method. The comparison of the relative retention time (to the internal standard) obtained from both standards mixture and spiked matrix was used to determine the method selectivity (**Table 1 and Figure B&C**).

Before going further in the validation of the method, we assessed the potential sensitivity of the method by detecting the endogenous SCFA, BCFA, CCM and ketone bodies in a wide range of samples going from mice (plasma, caecal content, faeces) to bacteria media and human samples (plasma, faeces). In each matrix type, detection of the different metabolites was evaluated to adjust the size of the sample and to comfort us the potential final applicability of the method.

III.4.2 Calibration curves and response function

Next, we used commercially available molecules to obtain calibration curves. A global standard mixture was prepared with the different compound of interest. Based on the literature and on previous works of our research group, we expected the endogenous levels for SCFA, BCFA, CCM and KB to be quite different depending on the analyte as well as the matrices. For those reasons, we selected values going from 1 picomoles (e.g. 3-methylpentanoate) to 90 nanomoles (e.g. acetate) depending to the analyte (**Table 2**). To avoid leverage effect, we opted for the use of an evenly-spaced calibration points strategy and designed 11 points calibration curves. Moreover, we systematically realized four blank solutions, in addition to the zero level, to evaluate contamination specific to the experiment

day. Each standard solution was subjected to the same analytical procedure that the one used to analyse matrices.

Using the 11-points calibration curve, we assessed the goodness of fit to find the best mathematic relation for the calibration curves obtained. Considering a 25% confidence interval between each value and the mathematical regression, a linear regression was selected (**Table 2**). This confidence interval was chosen as we are developing a quantification method for measuring endogenous compounds in biological matrices [425].

III.4.3 Trueness

Trueness was evaluated on three quality control levels (i.e. low, middle and high) based on the calibration curves. The relative bias of each level was determined and all the values were found to be within the 20 % of acceptance criteria (**Table 2**). The highest values were observed for the lowest level of acetate and acetone where 20.2 and -24.9 % of relative bias, respectively, were obtained.

III.4.4 LOD and LOQ

The limits of detection and quantification were determined based on the calibration curves using the value at intercept method (**Table 2**). As several analytes exhibit rather high levels in the blank (e.g. acetate and propionate) we decided to use this strategy as the signal to noise method (another LOD & LOQ determination method) has some limitations here. We then removed blank signal from calibration curves performed for LOD & LOQ determination and used the Y-intercept as well the standard deviation at the intercept following the formulae reported in the method section.

As the abundance of the compound is different depending of the species and the matrix analysed, values of LOD and LOQ cover a broad range of levels even for compounds with close structures (e.g. butyrate and isobutyrate).

Interestingly, adding some challenges to the quantification of these compounds, blank levels may vary between experiments. We had the opportunity to assess for a quite long period of time (i.e. 4 years) the evolution of the blank levels for acetate, PA, and BA which are the three compounds most susceptible to sample interference. As shown in **figure 6**, values differ between experiments and more surprisingly between year period.

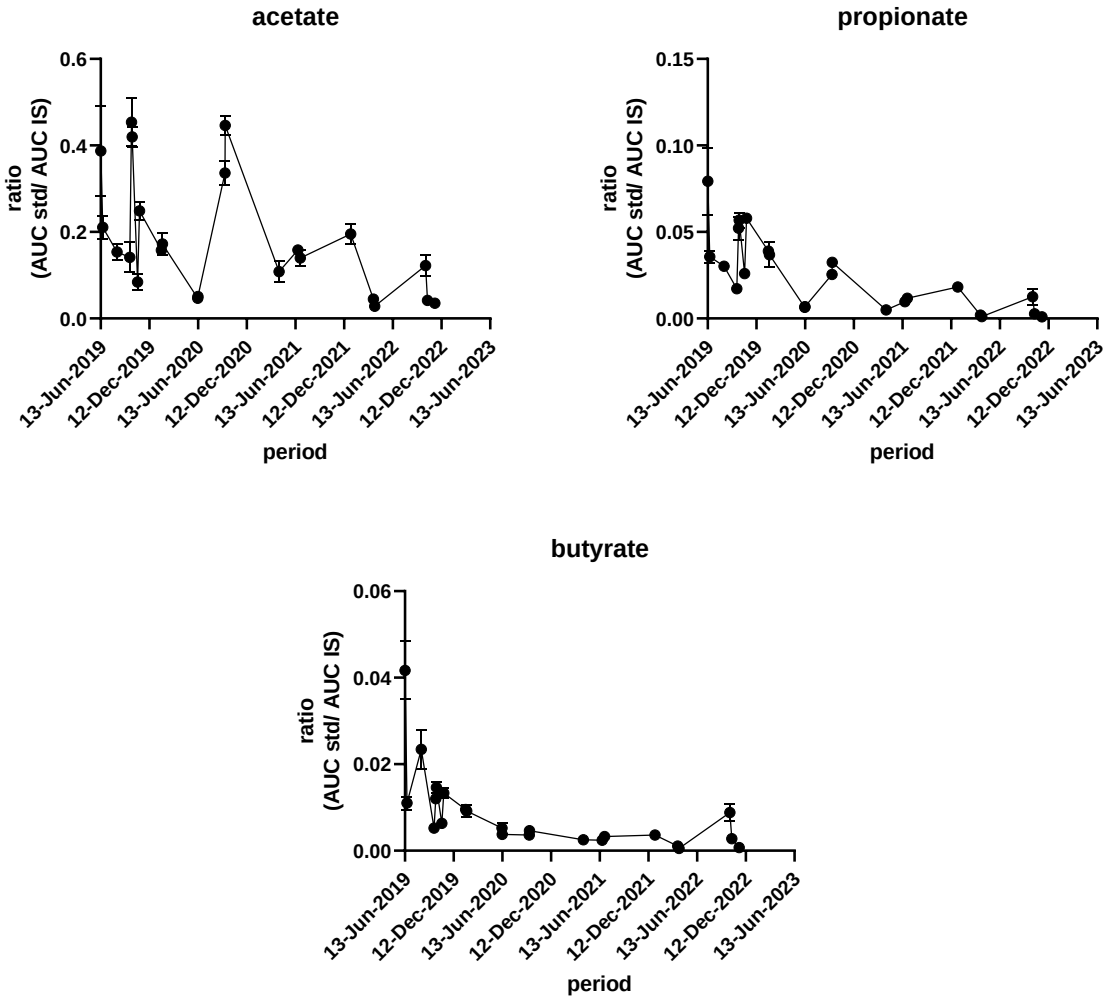


Figure 6: Evolution of the intensity of the signal corresponding to acetate, propionate and butyrate over time. Ratio were reported in function of the experiment date on a follow up period of four years. Data are expressed as mean ± SEM.

Interestingly, even if blank levels vary widely over time, the variations within the same experiment for replicates is limited. Based on these observations, we decided to use the blank levels of each experiment and to subtract them from the related samples [447-449].

name	range[a]	equation	LOD[b]	LOQ[c]	quality control level	trueness[d]	linearity	
AA	0,178 - 89,3	Y = 0,02036*X + 0,07217	1683	5100	LOQ		slope	0,86
					LOW	20,19	R^2	0,97
					MIDDLE	-11,23		
					HIGH	-9,33		
PA	0,089 - 44,63	Y = 0,02756*X + 0,01585	202	613	LOQ		slope	0,96
					LOW	6,93	R^2	0,97
					MIDDLE	-11,78		
					HIGH	-4,45		
isoBA	0,0178 - 8,88	Y = 0,05534*X + 0,005111	29,3	88,9	LOQ		slope	1,02
					LOW	-7,68	R^2	1,00
					MIDDLE	6,79		
					HIGH	-3,63		
BA	0,089 - 44,63	Y = 0,03273*X + 0,03097	154	466	LOQ		slope	1,01
					LOW	-3,04	R^2	1,00
					MIDDLE	1,02		
					HIGH	-1,79		
2-MeBA	0,0093 - 4,46	Y = 0,07345*X + 0,002833	10,7	32,4	LOQ		slope	1,00
					LOW	1,09	R^2	1,00
					MIDDLE	2,60		
					HIGH	-0,76		
isoVA	0,0178 - 8,88	Y = 0,04609*X + 0,004751	23,9	72,6	LOQ		slope	1,03
					LOW	-4,56	R^2	1,00
					MIDDLE	4,51		
					HIGH	-2,81		
VA	0,0178 - 8,88	Y = 0,04676*X + 0,0005655	60,6	184	LOQ		slope	0,97
					LOW	5,24	R^2	1,00
					MIDDLE	-0,35		
					HIGH	3,13		
2-MePA	0,00178 - 0,88	Y = 0,03792*X - 9,436e-005	3,4	10,3	LOQ		slope	0,96
					LOW	5,69	R^2	1,00
					MIDDLE	-3,28		
					HIGH	2,38		
3-MePA	0,00178 - 0,88	Y = 0,03143*X - 6,541e-005	4,1	12,4	LOQ		slope	0,99
					LOW	1,96	R^2	1,00
					MIDDLE	-3,95		
					HIGH	2,85		
4-MePA	0,0093 - 4,46	Y = 0,03143*X - 6,541e-005	14,1	42,7	LOQ		slope	1,10
					LOW	2,06	R^2	0,99
					MIDDLE	-2,16		
					HIGH	-0,58		
HA	0,0178 - 8,88	Y = 0,03697*X + 0,002235	84,9	257,3	LOQ		slope	1,04
					LOW	-0,29	R^2	1,00
					MIDDLE	-2,59		
					HIGH	0,10		
LA	0,178 - 71	Y = 0,001358*X - 0,0001715	1030	3123	LOQ		slope	1,11
					LOW	1,79	R^2	0,99
					MIDDLE	5,29		
					HIGH	0,43		
MA	0,044 - 22,31	Y = 0,03902*X - 2,842e-005	51,4	155,8	LOQ		slope	0,98
					LOW	7,86	R^2	0,99
					MIDDLE	-9,29		
					HIGH	4,87		
SA	0,044 - 22,31	Y = 0,1317*X + 0,01747	31,7	96	LOQ		slope	0,99
					LOW	0,91	R^2	1,00
					MIDDLE	1,45		
					HIGH	0,10		
CA	0,178 - 89,3	Y = 0,01101*X + 0,02448	519,5	1574	LOQ		slope	0,98
					LOW	5,12	R^2	0,99
					MIDDLE	-8,64		
					HIGH	4,00		
αKA	0,089 - 44,63	Y = 0,02126*X - 0,002763	229,8	696,5	LOQ		slope	1,02
					LOW	-0,14	R^2	1,00
					MIDDLE	-3,77		
					HIGH	0,89		
PyA	0,044 - 22,31	Y = 0,003868*X - 0,0008744	146,8	444,9	LOQ		slope	1,03
					LOW	4,83	R^2	1,00
					MIDDLE	-7,94		
					HIGH	3,63		
3-OHBA	0,044 - 22,31	Y = 0,02108*X + 0,001143	57,9	175,5	LOQ		slope	0,96
					LOW	3,77	R^2	0,97
					MIDDLE	-14,07		
					HIGH	7,67		
acetone	0,044 - 22,31	Y = 0,03457*X - 0,008128	147,4	446,7	LOQ		slope	1,18
					LOW	-24,91	R^2	0,99
					MIDDLE	-2,85		
					HIGH	1,98		

[a] values expressed in nanomoles and [b,c] values expressed in picomoles; [d] trueness (evaluated on the three calibration curve levels) expressed in relative bias (%); [e] intraday precision; [f] interday precision

Table 2: Determination of the validation parameters for each compound of interest. This table summarizes the range of calibration curves, the equation of the curves, the limit of detection (LOD), the limit of quantification (LOQ), the repeatability (intraday precision), the intermediate precision (interday precision), the accuracy and the linearity. **Range**: range of values of the drew calibration curves. **Equation**: equation of the linear regression line obtained for the different calibration curves. **Limit of detection (LOD) and quantification (LOQ)**: values in picomoles determined for each derivative. **Linearity**: slope and goodness of fit (R^2) of the linear regression line obtained when plotting the values (in femtomole) obtained for four different levels (quality control levels used in this case) with the theorical values used.

III.5 Method application

III.5.1 Assessment of the normalization procedure for caecal and fecal samples

As SCFA and BCFA are largely analysed in ceacal content and fecal samples, we wanted to determine an appropriate approach to assess the dry content of the samples. Indeed, if the water content of the samples is altered by an experimental condition, then this will have consequences on the reported SCFA (and other analytes) levels. Actually, some reports mentioned the importance of freeze-drying the samples (for caecal content and faeces analyses) as stool consistency (cfr. Bristol stool scale [347]) differs between pathological conditions and individuals [344, 450].

However, when analyzing volatile compounds, removing H_2O is not a trivial process. Thus, we examined here whether the use of freeze-drying as a sample preparation step could affect the quantification of the analytes of interest (most being volatile). For that, we compared samples analysed after the freeze-drying step and samples where the aqueous homogenate was split into two equivalent samples, one for the quantification and one for the lyophilization ensuring the consistency between both parts **(Figure 7)**.

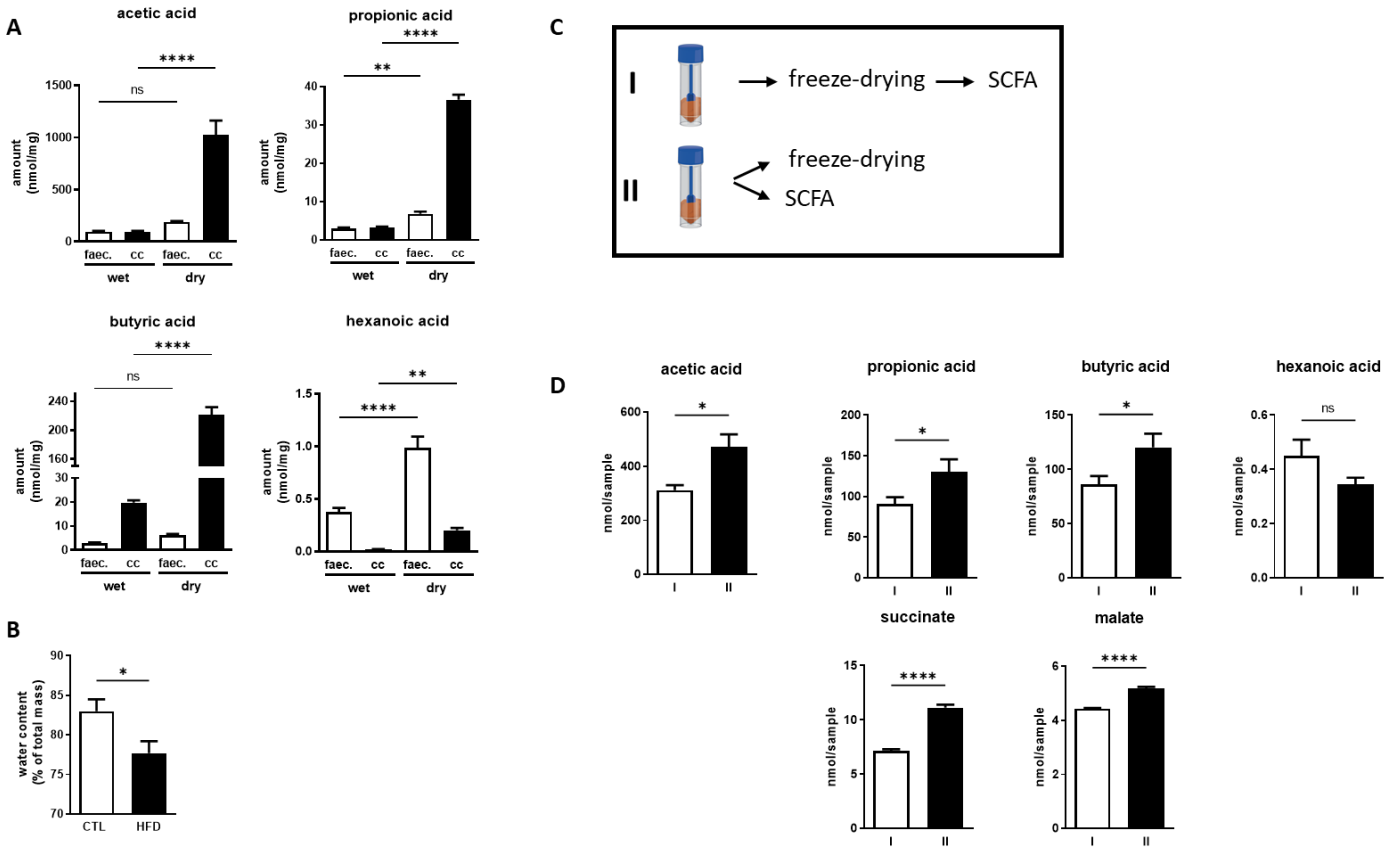
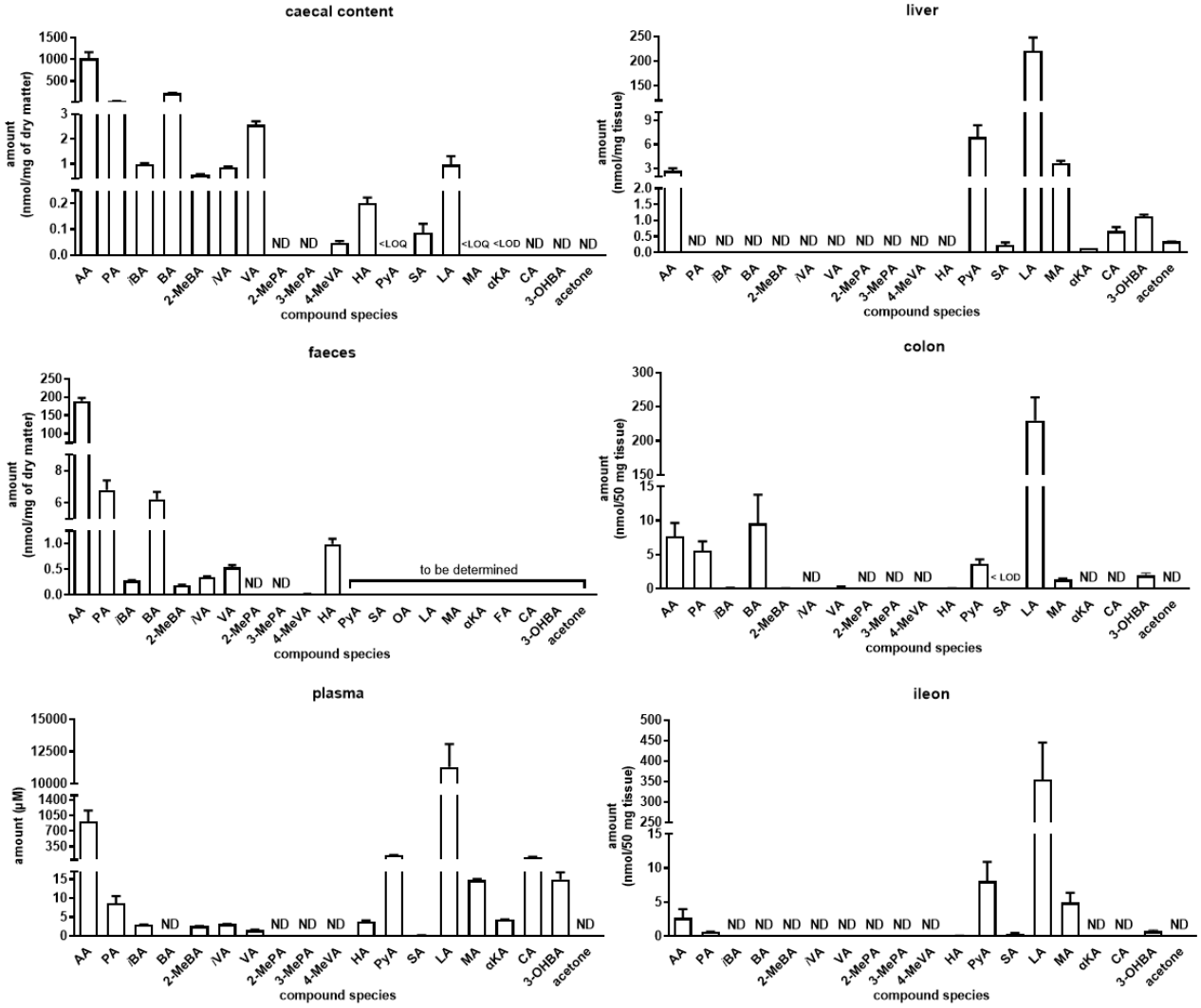


Figure 7: The application of a freeze-drying procedure separately to sample treatment allows to preserve analyte content. (**A**) The analyte amounts measured in caecal content (CC) and faecal (faec.) samples were normalized on the wet or dry mass of the samples. This shows the impact of the normalization on the reported levels. (**B**) Water content assessment in caecal contents of control and obese (high fat-fed) mice (10 mice/group). (**C**) Procedure description for the evaluation of the appropriate timing to apply the freeze-drying procedure. (**D**) Freeze-drying procedure was applied before or separately to the measurement of SCFA, BCFA, CCM and ketone bodies. Levels were normalized on the dry mass and expressed as nmol/sample. Values expressed in mean ± SEM. N=3 in triplicate. After normality assessment, student t-test was used when comparing two conditions and one-way Anova was used when comparing three or more conditions. ** p< 0,01; **** p< 0,0001 with Sidak's multiple comparison post-test.

As shown in **Figure 7C & D**, it is essential to determine the dry mass of the sample on another aliquot than the one used for the analyte quantification (process "B" in **Figure 7C**). Indeed, freeze-drying the sample to remove H_2O before analyzing the SCFA and CCM results in quite different results (process "A" in **Figure 7C**). Surprisingly, this effect is even more important in the case of CCM as gains in levels are between 20 % and 500 %. Those differences could be due to evaporation (i.e. for SCFA) or early degradation/oxidation (i.e. for malate).

III.5.2 Initial assessment of SCFA, BCFA and CCM in biological samples

Finally, we used our method to determine the basal endogenous amounts of SCFA, BCFA and CCM in various biological samples. To test our method, we selected several matrices that we thought are biologically relevant for the analytes of interest. These include GI tract tissue (colon and ileum) as well as caecal content and feces that are of particular interest for SCFA and BCFA. We selected the liver to have a control tissue for quantification of the CCM. Naturally the plasma is also an important matrix in the context of these analytes. In accordance with the literature, we observed important species- and matrices-dependent differences inside the families of compounds **(Figure 8A)**. As might be expected, SCFA and BCFA are particularly abundant in the caceal content and faeces while the CCM are relatively well detected in the tissues. However, it is important to keep in mind that while SCFA and BCFA are abundant in such matrices, a limited number of reports describe their levels in several tissues of the body. As for human samples, the easily accessible matrices during clinical studies are feces and plasma.


caecal content
amount (nmol/mg of dry matter)
compound species
AA PA iBA BA 2-MeBA iVA VA 2-MePA 3-MePA 4-MeVA HA PyA SA LA MA αKA CA 3-OHBA acetone
ND ND <LOQ <LOQ <LOD ND ND ND
liver
amount (nmol/mg tissue)
compound species
AA PA iBA BA 2-MeBA iVA VA 2-MePA 3-MePA 4-MeVA HA PyA SA LA MA αKA CA 3-OHBA acetone
ND ND ND ND ND ND ND ND ND ND
faeces
amount (nmol/mg of dry matter)
compound species
AA PA iBA BA 2-MeBA iVA VA 2-MePA 3-MePA 4-MeVA HA PyA SA OA LA MA αKA FA CA 3-OHBA acetone
ND ND
to be determined
colon
amount (nmol/50 mg tissue)
compound species
AA PA iBA BA 2-MeBA iVA VA 2-MePA 3-MePA 4-MeVA HA PyA SA LA MA αKA CA 3-OHBA acetone
ND ND ND ND < LOD ND ND ND
plasma
amount (μM)
compound species
AA PA iBA BA 2-MeBA iVA VA 2-MePA 3-MePA 4-MeVA HA PyA SA LA MA αKA CA 3-OHBA acetone
ND ND ND ND ND
ileon
amount (nmol/50 mg tissue)
compound species
AA PA iBA BA 2-MeBA iVA VA 2-MePA 3-MePA 4-MeVA HA PyA SA LA MA αKA CA 3-OHBA acetone
ND ND ND ND ND ND ND ND ND ND

B

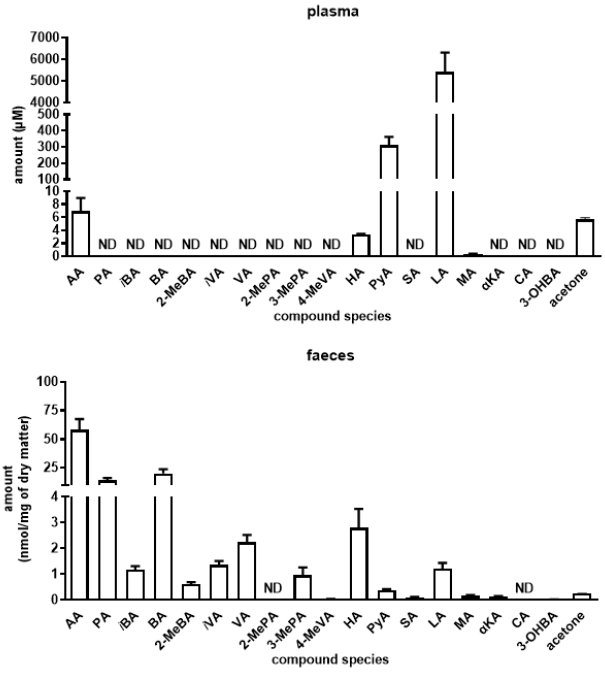

plasma
amount (μM)
compound species
AA PA iBA BA 2-MeBA iVA VA 2-MePA 3-MePA 4-MeVA HA PyA SA LA MA αKA CA 3-OHBA acetone
ND ND ND ND ND ND ND ND ND ND ND ND ND
faeces
amount (nmol/mg of dry matter)
compound species
AA PA iBA BA 2-MeBA iVA VA 2-MePA 3-MePA 4-MeVA HA PyA SA LA MA αKA CA 3-OHBA acetone
ND ND

Figure 8: Short chain and branched chain fatty acid, citrate cycle metabolites and ketone bodies in biologically relevant mouse and human matrices. Tissues (50 mg), faeces (10 mg), caecal content (20 mg) and plasma (75 µL for mice and 150 µL for human) were recovered and analysed using the complete procedure as described in the method section. (**A**) Detection observed in samples from naïve C57BL/6 mice. (**B**) Detection observed in samples obtained from (human) healthy subjects. AA: acetate; PA: propionate; *i*BA: isobutyrate; BA: butyrate; 2-MeBA: 2-methylbutyrate; *i*VA: isovalerate; VA: valerate; 2-MePA: 2-methylpentanoate; 3-MePA: 3-methylpentanoate; 4-MeVA: 4-methylvalerate; HA: hexanoate; PyA: pyruvate; SA: succinate; LA: lactate; MA: malate; αKA: alpha ketoglutarate; CA: citrate; 3-OHBA: 3-hydroxybutyrate. Data are expressed in mean ± SEM. Data expressed in nanomoles/ tissue amount.

However, we still need to apply the validated method in relevant pathological conditions. For instance, we plan to analyse feces from high-fat-diet-fed mice to understand whether the SCFA, BCFA, CCM and KB were affected by the onset of obesity. During a high-fat feeding experiment, faeces were recovered once a week from control and high fat diet mice during the 12 weeks of the HFD regimen. Samples still need to be analysed and will also allow us to determine the basal level of citrate cycle metabolites in mouse feces.

IV. CONCLUSION

We described here the development and characterization of a quantification method to analyse SCFA, BCFA as well as their synthesis metabolic derivatives known as CCM and ketone bodies. We also show the applicability of the method to a broad range of biological matrices. Thus, our method will ultimately allow us to quantify these interesting metabolites in several physio-pathological conditions. A description of the proposed method workflow is present at **figure 9**.

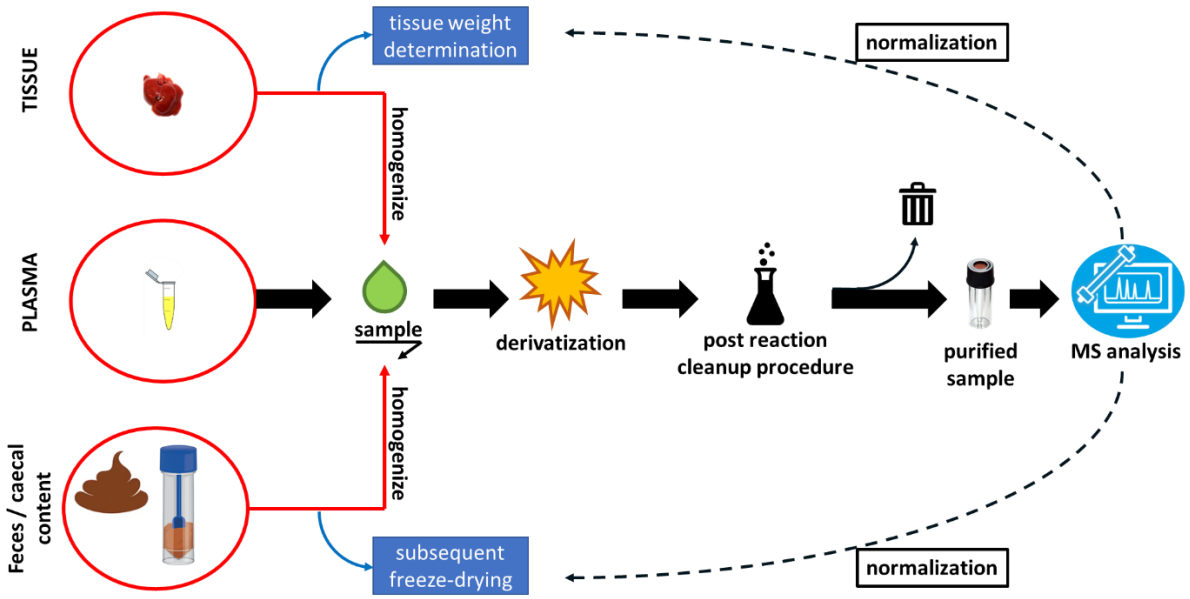


Figure 9: Proposed workflow for the analysis by LC-MS of short chain fatty acids, branched chain fatty acids, citrate cycle metabolites as well as ketone bodies.

We also emphasize in this manuscript the risk of sample alteration (e.g. contamination with SCFA from the environment). Our data clearly support the need to constantly take into consideration this potential issue when quantifying SCFA, BCFA and CCM.

In conclusion, we believe this method will lead to a better understanding of the implication of these metabolites in a physiological and pathophysiological context.

V. Acknowledgements

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VI. Authors contribution

J. Masquelier contributed with exploratory experiments involving the use of 3-NPH. A. Paquot and G. G. Muccioli designed the research; A. Paquot performed experiments and analysed the data; A. Paquot and G. G. Muccioli wrote the manuscript; G.G. Muccioli supervised the whole study.

VII. Conflicts of interest

The authors declare that they have no competing interests

GENERAL DISCUSSION

During my thesis I had the opportunity to analyse a large number of different compounds in very diversified matrices. In this thesis manuscript I choose to focus on two of these works as I think they nicely exemplify the challenges we face when we want to quantify endogenous compounds. Moreover, as reported in this manuscript, in these two works we opted for quite different approaches (i) a direct quantification by UPLC-MS/MS for the prostanoids and allied molecules and (ii) a quantification, post derivatization, by UHPLC-HRMS for the SCFA and allied molecules.

As described in the result section, for both methods we somehow increased the challenge by including molecules having quite diverse characteristics, but also close structure isomers, and a large variety in terms of endogenous levels. In addition, we wanted to develop methods allowing the analysis of a diversified set of matrices.

1. Quantification of the prostaglandins, prostaglandin-glycerol esters and prostaglandin-ethanolamides (and their respective precursors)

As described in the result section, we set up a complete procedure including extraction, purification and then analysis by LC-MS/MS. While our validated method was applied to the detection of prostaglandin derivatives in preclinicaly relevant tissues and models, its sensitivity might not be sufficient to obtain a complete characterisation of the compounds of interest.

I will discuss here several points that could still be explored in order to improve further the method.

1.1 Determining the best extraction procedure

At the beginning of the method development, the first question we asked ourselves was whether homogenization was an important parameter to assess in order to increase

metabolite extraction. Indeed, by testing several homogenization solvents we detected, using smaller quantities of matrices than usual, several PG-G and PG-EA that we never detected before in those amounts of tissue. Specifically, we replaced chloroform homogenization (which has the advantage of quenching protein activity and thus potential lipid metabolism) by water homogenization. Some detections were then observed but it was not possible to firmly establish if this was due to improved extraction or to some artefact due to ex vivo metabolism. Nevertheless, it will be interesting in future investigations to assess further the role of the homogenization solvents (or mixture of solvents). For instance, Araujo et. al. mentioned the interest of using acetonitrile as homogenization and precipitation solvent in the case of prostaglandin [451]. It seems that acetonitrile induces a flocculation process and increases extraction efficiency. The order of addition could also be assessed to favour protein precipitation without leading to induced turbidity in the extracted supernatant [451]. As prostaglandin derivatives are described to be cell-secreted mediators, it is possible that they bind to transport protein or are present in some vesicles. The selection of a solvent able to disrupt these structures could thus allow better extraction yield in the case of prostaglandin.

As for several methods used in the laboratory, we opted for a liquid / liquid extraction procedure. A first critic we can have regarding the protocol we applied is its potential toxicity. Indeed, we used chloroform as the organic solvent for lipid extraction as inspired by the Folch's procedure described in 1951 [91]. However, since the paper published by Folch the knowledge and awareness on chloroform toxicity largely increased [452-454]. The use of dichloromethane in replacement of chloroform is often promoted. For this reason, at the beginning of the method development, we tested whether the use of dichloromethane in place of chloroform could be considered an acceptable alternative. Even if the physicochemical properties of the two solvent are generally considered as similar, in our

hands dichloromethane was not a good option for prostaglandin derivatives extraction as only half the signal was recovered in comparison with what we observed with chloroform. Moreover, although new liquid / liquid extraction methods have been described over the years [92, 93, 107, 109, 110], Folch's procedure remains one of the most efficient methods for lipid extraction in complex biological matrices as nearly 100% of the targeted lipids were extracted from samples. Only some lipid species are not extracted using Folch's procedure (e.g. 25-hydroxycholesterol-3-sulfate or some bile acids). This extraction capacity is a key point in the case of low abundant compound such as prostaglandins. Based on these considerations, we continued to use chloroform for the liquid / liquid extraction.

An alternative to a liquid / liquid extraction in the case of prostaglandin derivatives is the so-called protein precipitation. This is usually followed by a direct injection on the LC-MS system (with or without concentration step) [232, 455]. Besides the fact that this strategy is less efficient in the case of tissue homogenate (due to the need of higher solvent volumes) the main issue is the absence of purification steps leading to high charge matrices. This increases the risk of decreased ionization for the compounds of interest in the ionization source resulting in decreased signal for low abundant compounds. Besides, the higher matrix charge decreases the life time of the analytical column and eventually of the instrument.

Even if in our developed method, extraction recoveries were good (based on the assessment made with deuterated standards), It could also be possible to think about approaches to increase extraction recovery to slightly increase prostaglandin extraction yield. For example, it is possible to use silanized glass vials to decrease the risk of lipid adsorption on the surface of the glass vials and tubes. This could be an interesting point to assess as the prostaglandin derivatives we quantified are less lipophilic than other lipids and thus might interact with the

glass. Others modifications consist in the use of salt exclusion processes to favour the extraction by the organic phase. Others pH modification than the one we assessed could also be possible even if the use of basic pH will potentially lead to the ionisation of the carboxylic group of prostaglandins and thus decreased extraction efficiency.

In conclusion, given the rather good extraction efficiency we obtained using the chloroform-based liquid / liquid extraction we did not explore further this point. However, if the throughput of the method needs to be improved then alternative approaches will have to be explored.

1.2 Improving chromatographic separation and analytes detection

When we started the setup of the analytical separation, we selected two different columns that were described in the literature for this type of compounds. Indeed, the number of different columns available is very large and it is not easy (if possible at all) to predict column efficiencies when developing a new method. Moreover, only limited information provided by suppliers is available on columns characteristics or applications. For these reasons the column choice is often based on already published papers or on the laboratory past experience. Having in the laboratory a “column collection” covering a wide variety of stationary phase characteristics of course helps in the process. We selected here a BEH column as isomeric forms were separated during the chromatographic run. However, achieving a reduction of peak width could increase peak intensity and thus sensitivity. Some suppliers allow to test several columns before selecting the best one but it remains an important issue in method development.

Another limitation we faced to increase method sensitivity was due to the set up of the autosampler. It is one of the main issues linked to the instrument that we faced, as the needle

of the autosampler was limited to a certain depth when withdrawing the samples from the vials. This forced us to dilute the sample more than desired in order to have a volume large enough to allow a consistent sampling. Knowing the injection volume required to obtain proper chromatographic peaks during separation on UPLC columns, we were limited to inject at maximum 5 out of 15µL of the sample extract. This issue could be solved by testing other vial inserts, or to try further to adapt the injection method.

Improvement of sensitivity could also be achieved using modification of the classical ionization sources provided by suppliers. Indeed, Lubin et al. described the addition of a stainless-steel rod inside the source to favour the nebulization [456]. This new ionization source, the Unispray®, seems to improve prostaglandin analysis leading to better detections in biofluids and several tissues. This type of device could be interesting to test in order to improve detection of PG-G and PG-EA in our settings.

As discussed in this manuscript, increasing the sensitivity was the main challenge during the method development as a not high enough sensitivity might have prevented us from detecting some of the compounds of interest. In this context, besides the bench part, improving the hardware (a.k.a the instrument) setup might also help improving the sensitivity.

1.3 Method development: to use or not to use a design of experiment approach?

Admittedly one of the points on which I could be challenged is the absence of design of experiment (DOE) during the set-up of the method. DOE consists in the statistical optimisation of parameters selection when developing analytical methods. The first step is to identify the parameters affecting the most the analytical signal and then modify the preselected parameters together to statistically determine the best compromise in terms of parameters.

This could sometimes improve detection capacities in comparison to the incremental strategy I used in this research.

While the approach is interesting, it is not devoid of limitations. The first one can be the need of a complete knowledge of the instrument and targeted compounds as it is crucial to know exactly on which parameters it is possible to play and which parameters could have some impact on detection. This requires extensive pretesting (sometimes equivalent to an incremental strategy). The second one is the difficulty to change any parameters once the DOE is completed. Finally, it is not easy to take into consideration the effect the matrix could have on the detection of standards during the DOE. By implementing it after the parameter selection, there is a risk to require further modifications to the DOE. These additional modifications can then lead to an increased time devoted to the method development.

1.4 Biological aspects that could explain the limited detection of PG-G and PG-EA

Besides the analytical aspects discussed above, detecting or not an endogenous compound can also depend on the choice of the biological settings.

As described in the literature, prostaglandins are ubiquitously present in the body, nonetheless, tissue abundance can differ depending to the type of tissue, the prostaglandin species, as well as the pathological environment (**see Appendix tables at the end of the manuscript**).

1.4.1 Does time mater for PG-G and PG-EA quantification?

One of the potential explanations for the absence of detection of several PG-G and PG-EA in our settings could be the kinetics of their production and degradation. All the prostaglandin derivatives share globally the same synthesis pathways. However, it is not well understood

yet if the activation of these pathways occurs at the same time or if they are depending to the inflammatory/pathological phases. Some reports suggest a competition between AA and 2-AG for COX-2 metabolic pathway [168]. This is an example of possible sequential activation of the pathways leading to different kinetics. We can then imagine arachidonic acid is first consumed by COX-2 (and others arachidonic inducible metabolizing enzymes) to generate prostaglandins, HETEs, leukotrienes in the early stage of inflammation and then, when there is a decrease in the excess of arachidonic acid, 2-AG is subsequently metabolized by COX-2 to generate PG-G. If this hypothesis is correct, then perhaps we would have detected higher levels of PG-G at later time points than those we assessed in vitro. Thus, it would be interesting to use our method to analyse over time the production of our metabolites of interest. Actually, when developing the in vitro model to achieve detection of prostaglandin derivatives, we compared 6 h and 24 h of LPS incubation (data not shown in result section I). By analysing both time-points we observed no, or limited, detection of PG-G and PG-EA after 6h of incubation when we detected all the derivatives after 24h. However, it should be kept in mind that keeping cells in culture (in the presence of LPS) for over 24 h could also have deleterious effect on the cells leading to artefactual data. Altogether these insights will be an interesting step in the comprehension of the inflammatory process occurring.

1.4.2 Does the model mater for PG-G and PG-EA quantification?

I discussed in the introduction that only a limited number of analytical methods have been described for the quantification of PG-G and PG-EA. Nonetheless, some research groups efficiently achieved their quantification [244, 246]. However, they often used large amounts of tissue and / or biologically “pushed” the system to favour the detection of prostaglandin derivatives. As example, Morgan et. al. used mouse overexpressing COX-2 together with a

monoacylglycerol lipase inhibitor (i.e. JZL184) to achieve PG-G detection [246]. In our hands, we also had difficulties to detect endogenous amounts of PG-G and PG-EA. However, thanks to our analytical method, we were able to detect pathophysiologically-produced PGE_2-G in a DSS-induced colitis model (i.e. without forcing the system to produce them) [457].

Still, we might have been more successful in quantifying PG-G and PG-EA if we had used another model instead of the DSS-induced colitis model. As the study of PG-G and PG-EA derivatives is a new research topic, there are, for now, only few descriptions of their presence in certain tissues depending of pathological models (**see appendix tables**). Thus, in contrast to the prostaglandin, we do not know if some of the family members are more abundant in given tissues or if some pathological conditions will preferentially activate one or the others pathways. We focused here on colitis and the assessment of relevant tissues linked to the pathophysiology of colitis. The choice was based on the previous detection of a PG-G in inflamed colon tissue [176]. In the study reported in this manuscript we only detected PGE_2-G. With the data available, it is not possible to know if the other PG-G or PG-EA are not produced in this context (i.e. DSS-induced acute colitis) or if they are produced at levels below the LOD. As for other tissues (e.g. the lung [458]), it is possible that the inflammation we induced activates differentially the pathways downstream of COX-2 resulting in increased levels of some prostaglandins (and PG-G, PG-EA) but decreased levels of other congeners. Actually, our data show a much stronger increase in PGE_2 levels, potentially suggesting a preferential activation of the mPGES pathway compared to the PGDS pathway (at least at that time point). This in turn could suggest a lower production of PGD_2-G compared to PGE_2-G in the colon during DSS-induced colitis. Of note, the opposite picture is observed with J774 cells activated by LPS. Besides, it is also known that depending on the pathological context the pathways leading to prostaglandins can be differently activated. In this context, using a

different model of colon inflammation (e.g. the oxazolone-induced colitis) could result in detection of another set of PG-G than what was detected here.

1.5 Can these lipids serve as biomarkers?

Inflammatory responses are one of the key components in a broad range of diseases. As bioactive inflammatory mediators, PG-G and PG-EA could play a broad range of activities. Increasing our understanding of the role of these lipids is certainly of interest. This requires additional pharmacological testing as it is performed for instance in our laboratory [176, 187]. However, I think that an efficient analytical method allowing to follow their levels would bring complementary insights to those obtained via pharmacological testing.

On the other hand, even if prostaglandin derivative levels increase during inflammatory events, it is complicated to point out this increase as a potential pathological marker. Indeed, as ubiquitously present in a broad range of tissue and responding easily to inflammatory insult, the specificity of prostaglandin for a unique disease is far from proven, and actually highly unlikely. Instead of serving as a specific marker, prostaglandin derivatives could more give an idea of the evolution of the inflammatory/pathological status of several diseases. Interestingly, differently from many others lipid mediators, the magnitude of variation in prostaglandin derivatives levels is often quite large, with modification going up to 10-fold compared to the basal level. This amplitude allows to quickly point out the pathway induction in pathological conditions and can then easily give an idea of the establishment of the inflammation.

1.6 Further developments

The method presented in this thesis could be considered as incomplete as we focused only on the prostaglandin derivatives. It is well described that, besides COX-2, other enzymes can have

their expression (or activity) increased in inflammatory settings [459, 460]. It is, for example, the case for the lipoxygenases 5, 12 and 15 or the leukotriene synthases. These other enzymes lead to the metabolization of arachidonic acid into 5-HETE, 12-HETE, 15-HETE or several leukotrienes. The activation of these other pathways is also an important component of the inflammatory environment in several diseases. Moreover, it is described that 2-AG and AEA can also be metabolized into HETE-G and HETE-EA derivatives respectively [169, 409, 461]. It could be also the case for others pathways not yet discovered. Thus, it could be interesting in the future to expand the actual method to, at least, the principal precursors in the several others pathways implicated in the pathological processes studied. As competition can occur between the three prostaglandin pathways, it could be the same with others activated pathways and to be sure to understand the related mechanism, it will be important to consider these others derivatives. Moreover, the lack of detection of PG-G and PG-EA could be explained, at least in part, by a preferential metabolization of 2-AG and AEA through lipoxygenases or others enzymes.

To conclude, the method developed here constitutes a piece of the puzzle but a lot of work remains before being able to characterize properly the prostaglandin pathways in health and diseases.

2. Set up of an UPLC-MS quantification method for short chain fatty acids, branched chain fatty acids, citrate cycle metabolites and ketone bodies

In the second part of this manuscript, I presented a method allowing the quantification of SCFA, BCFA, CCM as well as ketone bodies in various biological samples. The challenges linked to this method development were different compared to the ones observed faced for the PG-G and PG-EA quantification. Moreover, contrary to the development of a complete prostaglandin derivatives method, we were not pioneers in the domain as several methods

were already described in the literature. However, even if there are numerous methods published, the methodologies are diverse and no consensus exists on the best approach to quantify SCFA. Thus, one approach is to test several of the described procedures to compare them in terms of performance (e.g. sensitivity, specificity, sensitivity to contamination, …). Still, even if methods have been described several years ago, the scope is often limited to the principal mediators in the classical tissues (mainly caecal content, faeces and plasma). The challenge was to adapt the method to more complex matrices and to additional analytes (i.e. others carboxylic acid compounds but also several related isomers).

2.1 What can we do when the blank is not blank?

One of the main issues we faced in the developed method was the potential "blank contamination". Actually, the blank contamination reflects the contamination of the samples during the analytical process. As presented in the thesis, this contamination is sometimes quite high and sometimes variable between experiments. As it was complicated to predict the level of contamination, I opted to systematically perform several blank reactions (at least 4) in the numerous experiments performed over 5 years. It will be interesting in the future to better understand the sources of contaminants to favour a low and constant level of "background noise".

In the experiments I performed, I pointed out the importance of solvent selection in order to reduce the contamination, mainly for SCFA. Indeed, SCFA can be industrial solvent oxidation or degradation products leading to their presence in the final product. We could consider to conduct an additional purification step for the solvents we buy, such as distillation (knowing the higher volatility of organic solvent as compared with SCFA) to be able to clean up solvent before their use during experiments.

Besides acting on the solvents, using a controlled environment could help reduce the environmental contamination. For the method described above, a chemical hood was used for the majority of steps. However, the contamination by air could be additionally reduced using a laminar flow or more filtering hood rather than chemical hood in order to reduce as much as possible contamination from the environment. SCFA can logically be present in air due to their volatility. This is the case in most laboratories where acetic acid is often used. Besides, the commensal population of bacteria in the mouth could contribute to the presence of SCFA in the air [462, 463]. Finally, even if care was taken to minimize samples cross-contamination, it is important to keep in mind than highly concentrated samples could contaminate samples with lower abundances due to their volatilities. By acting on these several points, it might be possible to reduce further the contamination of the samples by exogenous SCFA.

I also presented in the method development (see result section II) the assessment of the reactional volume as strategy to minimize sample contamination. One of the critics of the work could be that we focused more on the contamination issue rather than on the reaction efficiency. In fact, as other researchers reported [366], a design of experiment could be interesting to apply in the case of the setup of the derivatization method. By comparing derivatization efficiency with blank levels during the design of experiment set up it could be a sounded strategy to select the best balance between efficiency and blank levels. Indeed, as shown by others, DOE is powerful to select the best proportion of the different reactants as changes in volume could affect derivatization. Unfortunately, it was too late in our method development to implement this additional step regarding the benefits it could add on the final method. However, this is something I would definitively consider in future works involving a derivatization step.

Another aspect that was difficult to assess during method development was the selection of sufficient excess of reagents. Of course, we calculated the molar ratio between derivatization reagent and endogenous levels of SCFA, BCFA, CCM and KB and add a sufficient security range during the method set up. But this point is impossible to predict as 3-NPH binds on the carboxylic or carbonyl moieties which are widely present in endogenous molecules (e.g. fatty acids, free amino acids and derivatives, eicosanoids, oxylipins, …). It is then possible that depending to the type of tissue as well as the pathological settings, we are not able to predict the endogenous carboxylic acid content leading to a default in derivatisation reagent. However, when developing the method, we selected as reagent concentration higher quantities than those described in the literature. Moreover, after having tested several concentrations of reagent focusing on the contamination, we increased the selected concentration as no differences in term of SFCA contamination was observed. In addition to those adjustments, we did not observe any saturation effect when drawing the calibration curves supporting a sufficient excess of the reagents in our conditions. Nonetheless, it will be interesting for future investigations to set up a method (e.g. colorimetric assay) to estimate the quantities of carboxylic acid or carbonyl functions in several endogenous tissues. To the best of my knowledge this has not been reported yet and it would be powerful to select the lower limit of concentration when developing a new method of derivatization. On the other hand, the large excess we choose, forced us to set up an additional clean up procedure which could be not necessary if we selected previously an appropriate excess.

2.2 How to ensure a complete lyophilisation of the samples? Can the freeze-drying procedure add variability to the results?

The easiest way to ensure that the freeze-drying procedure was complete is to weigh the tube containing the wet material before the procedure, then weigh the tube again after the freeze-drying step. By performing a second round of freeze-drying and reweighing the residual dry material, you can measure the efficiency of the freeze-drying procedure.

As mentioned in the results section II, it is crucial to consider the water content of the samples being analysed. However, it is also important to note that variation in the freeze-drying efficiency can introduce additional variability. Therefore, it is essential to ensure complete freeze-drying across various type of biological samples and with varying amount of matrix. This aspect may be lacking in the second part of the thesis. Nonetheless, the time of freeze-drying taken during the analyses exceeds the recommended duration for freeze-drying samples with similar water content. Moreover, there was minimal variability observed in the samples analysed during the numerous tests conducted for method set up and application.

2.3 How to ensure that we use sufficient excess of derivatizing reagents?

As for most of the methods involving a derivatization step, a potential issue is the amount of reagent used for the reaction that needs to be in sufficient excess. Even if we took higher amount of reagent than described in the literature and that one of the perspectives of this work is to evaluate the concentration of carboxylic functions in a particular matrix (and in various conditions applied to this matrix), the current proposed method did not exclude bias in term of saturation of the reaction. To have a better view of this sufficient excess (or not), we measured the level of internal standards (i.e. valproic acid and d_4-succinate) in the various

matrices we analysed to ensure we did not observe any decrease in their level within the several matrices (**Figure 1**).

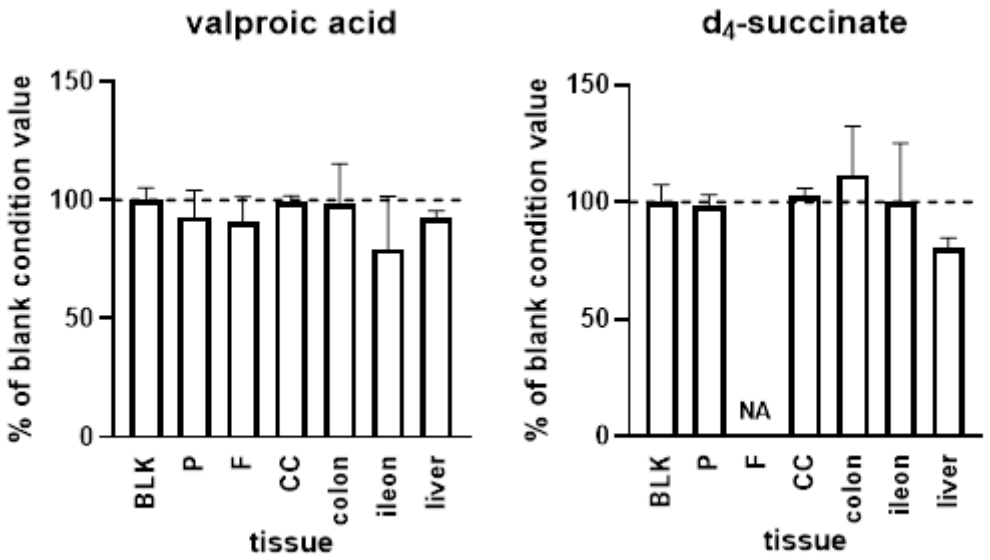

Figure 1: Comparison of the internal standard detection between blank samples and various matrices. Detected internal standards (in area under the curve) were normalized to the blank samples analyzed within the same experiments. Data are expressed in percentage on the detection observed in the blank sample. BLK: blank; P: plasma; F: Faeces; CC: caecal content

As shown on the figure, we did not observe significant decrease in the level of the internal standards within the different matrices. However, it is important to notice that matrix effect can also affect internal standard detections and that in the worst case, internal standards can undergo positive matrix effect from the tissue components and a saturation due to a lack of derivatization reagents. Additional work might have to be performed to better explore this point.

2.4 Comment on the internal standards selection

Another limitation related to this work is on the internal standard selection. In this work, valproic acid and d4-succinic acid were selected as internal standards. The selection of valproic

acid was done in the early stages of the method development for the SCFA and, unfortunately, we did not replace it with an internal standard of closer structure such as d4-butyric acid. The later would be a better option in the context of SCFA quantification. Another limitation could potentially be linked to the number of internal standards to cover the complete run time. An improvement of this validated method can be to select only deuterated internal standard and use an internal standard for each family of compounds. This selection of internal standard will be useful to assess better matrix effect as well as prevent saturation phenomenon as mentioned above.

2.5 Could we go further with this method in terms of analytes?

As discussed above, the described procedure allows to derivatize other types of carboxylic acid-containing compounds such as free amino acids. In fact, we know since the set up that the method of derivatization we used efficiently derivatises the free amino acid (tested on leucine, isoleucine and valine; data not shown). In fact, as branched chain fatty acids are generated through the metabolism of free branched chain amino acids, we wanted to add these molecules to the complete procedure when we started the set up. Moreover, it is also described that some others amino acid derivatives (i.e. indoles, …) are obtained from bacteria metabolism. Some of them also contain carboxylic acid moieties and could be important in the roles described for the microbiota. Unfortunately, in our hands, we achieved proper derivatization of the free amino acids but their extraction during the liquid-liquid extraction procedure we developed was not efficient enough. Thus, it could be interesting in the future to modify the extraction procedure in order to include them in the method. Indeed, their quantification would go in the direction of developing a more comprehensive method to characterize microbiota metabolome.

Finally, as suggested in the result section, SCFA are also considered as potential biomarkers of several diseases. In fact, there is a need to go further in this direction because, as mentioned, their levels are often associated to caecal content, faeces or plasma but it will be more interesting to focus on tissues where a microbiota-driven effect was shown. As they are mainly not related to an endogenous synthesis, the modulation of their levels in some tissues will be linked (positively or negatively) to precise conditions. They will then give an interesting output that could be used as biomarker for different pathologies. However, it remains to apply their quantification to several solid tissues together with the improvement of the analytical methods before going in this direction.

3. General comments

More globally, I think the development of analytical methods for endogenous compound characterization is a powerful tool to study health and diseases. It was demonstrated often that the variation of precise endogenous species could be related to pathological conditions and acting directly on these altered levels can allow to restore body homeostasis. Moreover, the use of non-targeted strategy could also provide new inputs to understand the aetiology of some illnesses. Nonetheless, as increasingly suggested, the use of more global “omics” strategy including compounds quantification as well as proteomics characterization of key proteins such as rate limiting enzymes would give a more complete picture of a pathology. It will be interesting, in the future, to apply omics methods to several well described diseases to validate a new model of characterization and applying it to others pathological settings to prospect to new potential targets. However, as mentioned in the introduction, despite methodological improvements, given the large variety of structures and the increasingly large number of lipid species (47718 lipids listed on the LipidMaps® database) it is still complex to have the complete lipidic fingerprint of a sample. Moreover, there is a need in the future to

fine-tune the high throughput technologies to develop quick and complete characterization of diseases. Mass spectrometry-related methods will take an important place in these settings as for now it is already one key player in sample characterization.

PERSPECTIVES

To go further in the reflexion about this thesis, I will expose below several improvement or perspective points that will help the research on prostaglandins, prostaglandin glycerol esters and prostamides on one hand and on SCFA, BCFA, CCM and KB on the other hand.

1. Short term perspectives

1.1 Finalize the short chain fatty acids method development

As mentioned in this thesis, some of the validation parameters required for the developed method still need to be assessed to finalize the development. Through them we will find intra- and interday precision, accuracy and bias. They were not included before submitting this thesis manuscript as those parameters are more linked to experimenter and instrument variation rather than an issue in the proper method development. However, as they are key in the validation process, it is important to add them as soon as possible.

1.2 Apply the validated short chain fatty acids method to pathological conditions

Once the method validation will be completed, we will then be able to apply the method to pathological conditions. Several relevant matrices (i.e. caecal content, faeces, plasma, colon, ileum, liver) were already analysed with the developed method showing its interest to be applied in biological samples. However, to confirm further the interest of this method, and to support that the metabolites that we selected for the method are important for a given disease, it will be necessary to apply the method in (models of) pathological conditions.

For that, a study on obesity was initiated simultaneously to the method development. The idea was to put forth the interconnection existing between the several SCFA synthetic pathways. For this reason, we recover once a week faeces from diet-induced obese mice during the course of obesity onset from the start of the diet until the end of the *in vivo* study.

Indeed, it was widely shown that obesity affects SCFA levels. With this study, we will bring additional inputs in the reflexion by putting forth which synthetic pathway was activated and which metabolites could be of more interest in the disease onset. Indeed, by analysing the metabolites at the earliest stages of obesity development we might be able to highlight changes that precede obesity and changes that accompany obesity.

2. Mid-term perspectives

2.1 Extend the prostaglandin method

As mentioned in the discussion section, besides the biochemical conversion of arachidonic acid, anandamide and 2-arachidnoylglycerol into the respective prostaglandin derivatives, these three precursors can also be metabolized by other enzymes leading to different compound families. Among them we find the hydroxyeicosatetraenoic acid (HETE) family of lipids. We can also think about leukotrienes or epoxyeicosatrienoic acid (EET). As mentioned in this thesis, it will be complex to include in a single analytical method all the metabolites involved in these metabolic pathways.

However, it will be possible to add to the validated method, some of the metabolites coming from the compound families generated from arachidonic acid (**Figure 1**). The idea will be to have a complete picture of the pathway activation during the study of pathophysiological conditions. Thus, one interesting perspective could be to add HETE, EET, leukotrienes but also the glycerol ester and ethanolamide derivatives (recently described [169]) to the previously validated method.

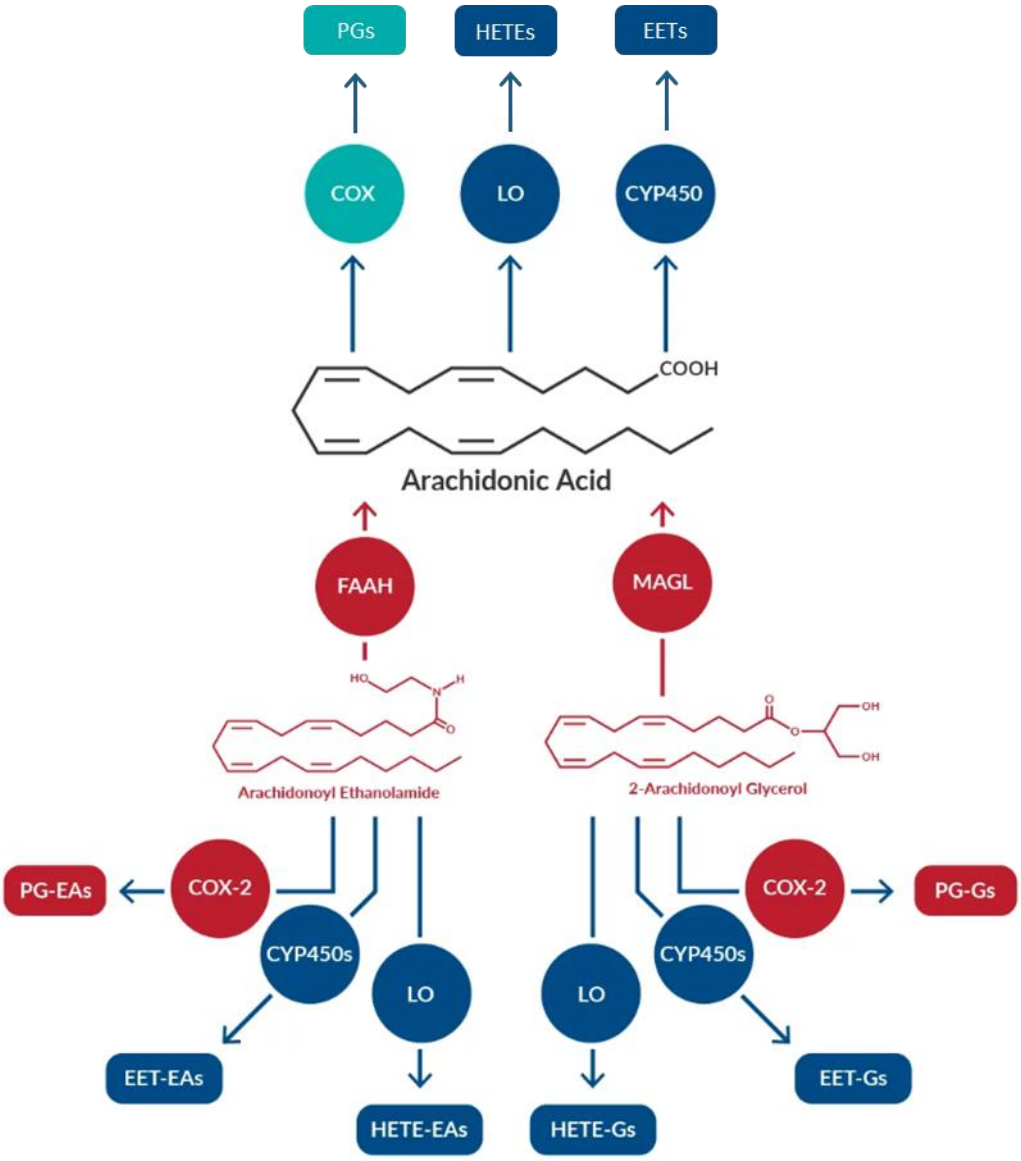

Figure 1: Metabolic families generated from the arachidonic, 2-arachidonoylglycerol, and arachidonoyl ethanolamide (anandamide) metabolization (adapted from "Interplay Between the AA Cascade & Endocannabinoids" [464])

It will not be an impossible task as I already observed satisfying detection of 5-HETE, 11-HETE, 12-HETE, 15-HETE, LTC4, LTD4, LTE4 in mice tissue [78]. One of the limitations is linked to the higher detection of some of those analytes in negative mode rather than the positive mode selected for the method described in chapter Results Part I. Of note, the XEVO-TQS instrument in theory allows to analyse simultaneously in positive and negative ionization mode.

2.2 Assess prostaglandins involvement in colitis

We showed in the chapter related to the prostaglandin quantification that colitis affects prostaglandin levels in several tissues. Our group previously showed that a treatment with prostaglandin D_2-glycerol ester but not with prostaglandin D_2 is able to prevent colitis in the DSS-induced colitis mice model [176]. Moreover, we also identified the DP1 receptor as key in this effect.

Nonetheless, as shown in Results Part I, one of the tissues where we observed the higher variations in prostaglandin derivative levels is the visceral adipose tissue. This tissue was not assessed during the previous study and might help to understand the observed effects for prostaglandin D_2-glycerol ester. The BPBL lab developed some skills in isolating adipose tissue (from mice and human) and separating stromal vascular fraction from the adipocytes (unpublished ongoing works). It will be of high interest to assess whether the increase of prostaglandin is linked to both fraction or not. Moreover, new pharmacological tests could be interesting directly in the adipose tissue to decipher the real implication of prostaglandin derivatives.

2.3 Assess the kinetic aspect of prostaglandins production

A large number of scientific publications underscore the importance of the kinetics in the inflammation process onset. Indeed, the inflammation process is divided in two parts with the acute phase and the resolution phase. Each phase induces the recruitment of different immune cells as well as the release of their own inflammatory mediators. Even if researchers suggest that prostaglandins are implicated in the acute phase, less is known on the implication of prostaglandin glycerol esters and prostamides in this context. As mentioned in the

discussion section, this difference of phase implication could be one of the reasons explaining the lack of detection for prostaglandin glycerol esters and prostamides.

It could then be interesting to perform a new colitis study and to sacrifice the mice at several timing after the last DSS administration in the way to point out the variation of prostaglandin levels in the onset of colitis in those mice. By analyzing mice tissues during the whole colitis experiment, we will be able to better understand the dynamics of inflammatory mediators production as well as if it exists some differences between prostaglandins and the others families of prostaglandin glycerol esters and prostamides.

2.4 Extend the SCFA method to others metabolites

As mentioned in this manuscript, free amino acids are also derivatized using the 3-NPH. As explained, the loss of free amino acids signal was due to the extraction procedure we decided to implement at the end of the protocol. Nonetheless, amino acids play key pivotal roles in the metabolic pathways linked to dietary fibres digestion by gut microbiota. Some of the effects provided by the gut bacteria are due to their capacity to generate amino acids.

Thus, it could be interesting to extend the proposed method to amino acids and perform additional experiment to include those small metabolites. This will require to find other conditions for the extraction procedure in order to extract the derivatized amino acids together with the other analytes of interest. In a second time it will be necessary to assess if the UPLC-MS parameters are also valid for the new compounds and add the validation steps required for new development.

2.5 Develop a more sensitive method for short chain fatty acids quantification

Even if SCFA (as mentioned in this work) are abundant lipids and that the sensitivities reached in the developed method were satisfactory for the method applicability, it could be interesting to improve the sensitivity further. Indeed, at the exception of faeces, caecal content and plasma, characterization of their levels was not applied in numerous other tissues. However, as suggested in the thesis, other tissues (e.g. digestive tissues or brain) could be relevant in the study of the SCFA.

The use of another type of mass spectrometer such as the tandem quadrupole, could help to improve method sensitivity. Moreover, one research group already efficiently developed a method allowing to analyse 3-NPH derivatives of citrate cycle metabolites on a tandem quadrupole mass spectrometer [375]. Interestingly, all the derivatives shared the same fragmentation pattern (consisting to the loss of the nitrophenyl hydrazine moiety) allowing to easily identify the compounds of interest.

We can then assess if the development of this type of analytical method could improve sensitivity. In first intention, we need to optimize the LC-MS parameters on the other instrument and then determine the new limit of detection. If reached values are better than for the Orbitrap method, the method could then be validated on the new instrument.

3. Long-term perspectives

3.1 Assess prostaglandin glycerol esters and prostamides involvement in the pathophysiology of other diseases

We limited our focus on colitis in this thesis. However, it is well known prostaglandins have several implications in the pathophysiology of lung related diseases as well as in several reproductive process [145, 146]. Even if there is a high number of research performed on

prostaglandins, few things are mentioned about the implication of prostaglandin glycerol esters and prostamides in these contexts.

It will then be interesting to apply the validated method on these types of conditions to improve our understanding of the pathway's activation. This could maybe bring some new features linked to the pathophysiology of lung diseases and to the reproductive process.

3.2 Development of a new strategy for the quantification of compounds low in abundance

The development of the prostaglandin, prostaglandin glycerol esters and prostamides method brought some reflexions about the limitation inherent to the low abundance of metabolites as well as the instrument limitations. As shown with the application of the method, even with an optimization until the limits of each parameter linked to the analysis of prostaglandin derivatives, we did not achieve a detection for all the metabolites. As we were able to pinpoint small variations in our analyse range, the issue with undetected metabolites is more due to the threshold of detection on the instrument rather than the capacity to detect small variations (if we consider that the several metabolites targeted in the described pathways are endogenously present).

The idea would then be to implement a new two-steps strategy to the detection of compounds low in abundance as prostaglandins. The first step will consist to develop and validate an original method allowing to quantify the compound of interest. It requires to optimize the sample treatment protocol and to assess the several validation parameters but not to ensure the applicability of the method on biological samples. The assessment of the matrix effect on the tissue of interest alone needs to be implemented at this point.

It is the beginning of the second step consisting to recover biological samples of interest. On those samples, we will spike each sample (control and pathological) with the value of the LOD

determined during the validation process. This to increase the detection threshold of each sample. As an instrument is limited to a certain threshold limit, as encountered in our case, we could then be able to artificially improve the threshold in all the samples.

The idea is not to perform an absolute determination of the endogenous compounds in both conditions but to pinpoint the variation between these two conditions (i.e. an increase or a decrease of the signal). For samples exhibiting high levels of compounds, the addition of the LOD values will be neglected as compared with the usual endogenous detection. For samples exhibiting usually too low levels to be seen, we will detect the artificial threshold plus the endogenous amount in the sample. This strategy will allow us to see variation of the several compounds of interest that was not seen with a conventional strategy.

Practically, this strategy first needs to be tested in known conditions. The use of J774 cells incubated in presence or not of LPS (i.e. as shown in the Result Part I section) will be a good starting point. We just need to spike the conditions with a mix of compounds at the LOD value and keep as comparison point the unspiked conditions. This test will reveal us if the strategy could be implemented in other biological matrices such as solid tissues.

3.3 Go into high throughput screening method development

With the help of my professional experience, a more long-term perspectives could be to try to develop both method in high throughput strategy. Indeed, my current job allow me to have access to high throughput methodologies allowing the detection and quantification of lipid derivatives within 1 to 2 min runs. The main limitation of this strategy is the importance to analyse very small amount of matrices to minimize the risk of matrix effect.

On the other hand, this strategy allows the analysis of a very large number of samples in extremely reduced times. In my case, it allows the drug screening of all the drug worldwide

on patient cells to be able to identify repurposing candidates that restore the defect caused by some rare diseases. This screening requires the analysis of thousands of samples (+4000) in a reduced time and can be permit by the extremely short run times.

Both the developed methods lead to run time of 20 min (i.e. prostaglandin method) to 45 min (i.e. SCFA method). The use of the strategy mentioned above could allow us to be able to perform different types of studies that require the analysis of many samples. For example, if the study is focusing on several tissues (as it was the case for the colitis study) or different timing (as it was proposed in another point of this section with the kinetic study).

Implementing this strategy opens the door to more complex studies that will be more and more required in the future to be able to assess the complexity of the human body. Of course, some challenges related to the analytical separation of the compounds need to be overpassed to achieve this goal.

3.4 Solve the stability issue linked to prostaglandin

As well-known from the literature, prostaglandin glycerol esters and prostamides have poor stability in vivo with half-life times in the order of the seconds to minutes for some of the derivatives [166]. We already shown at the BPBL's lab that the PGD_2-G have interesting properties in several inflammatory models [162, 176, 184]. However, as PGD_2-G metabolize quickly into PGD_2, we performed in parallel identical testing with PGD_2-G and PGD_2 to be sure that the effects observed with PGD_2-G was not due to its metabolization.

As Woodward et al. did it by generating a stable analogue of the prostamide F2α and used it as an anti-glaucoma drug [182], it will be interesting to generate stable analogues for the PGD_2-G and see if we are able to recapitulate its effects previously observed. This could allow

us to extend the characterization of its beneficial effects in other inflammatory conditions that the ones tested.

Practically, it will be necessary to generate a broad range of analogues, maybe by using similar strategy than the ones used for the generation of the bimatoprost and test it on cells activated with LPS as we did previously [162]. Once some of them will show similar effects than the ones observed with PGD_2-G, we could imagine to determine the PK/PD parameters *in vivo* and compare with those of the PGD_2-G. The newly developed and more stable analogue could then be tested in vivo in model already characterized to confirm its applicability in future projects.

Another strategy could be to develop a protective formulation that allows to improve the stability of the compound. However, it is a complex task, already initiated with the development of a formulation based on lipid nanocapsules [185], that still need to be improved if we want to reach better stability of the compound in vivo.

OTHER WORKS AND CONTRIBUTIONS

While the result section of this manuscript reports the development of two analytical methods, during my PhD thesis I had the chance to participate to several other studies that resulted in published papers.

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APPENDIXES

Appendix Table 1: Endogenous amount and biological distribution of prostaglandin derivatives in animals (control condition).

prostaglandin derivative	**tissue** (Mouse (M); Rat (R); others (O))	**amount**	**amount (pmol/g or mL)**	**method**	**Ref.**
arachidonic acid	plasma (M)	1.5 µg/mL	4934 pmol/mL	GC-MS	[465]
	plasma (M)	1180 fmol/µL	1180 pmol/mL	LC-MS/MS	[466]
	plasma (R)	500 µg/mL	1644000 pmol/mL	LC-MS/MS	[467]
	plasma (O-cows)	251.5 nmol/mL	251.5 pmol/mL	LC-MS/MS	[468]
	plasma (O-cows)	581.2 pmol/mL	581.2 pmol/mL	LC-MS/MS	[469]
	serum (M)	65 ng/mL	213.8 pmol/mL	ELISA	[470]
	serum (R)	820.9 ± 125.7 µg/mL	2700000 pmol/mL	GC-MS	[471]
	lung (M)	800 pmol/mg of lung protein	800 pmol/mg of lung protein	LC-MS/MS	[472]
	lung (M)	15 µmol/g	15000000 pmol/g	LC-MS/MS	[473]
	liver (M)	2.7 ± 0.4 pmol/g	2.7 ± 0.4 pmol/g	LC-MS/MS	[250]
	liver (M)	13.5 ± 2.2 µmol/g	13500000 ± 2200000 pmol/g	LC-MS/MS	[474]
	liver (O-cows)	6.9 pmol/mg	6900 pmol/g	LC-MS/MS	[469]
	colon (M)	86405.26 ± 40368.8 ng/g	284228 ± 132792 pmol/g	LC-MS/MS	[475]
	brain (M)	603.5 ± 150.8 nmol/g	603500 ± 150800 pmol/g	LC-MS/MS	[476]
	cortex (M)	350 nmol/g	350000 pmol/g	LC-MS/MS	[477]
	cortex (M)	15500 ± 1860 pmol/g	15500 ± 1860 pmol/g	LC-MS/MS	[478]
	cortex (M)	460 pmol/mg	460000 pmol/g	LC-MS	[479]
	periaqueductal gray (M)	150 nmol/g	150000 pmol/g	LC-MS/MS	[477]
	amygdala (M)	20000 nmol/g protein	20000 pmol/mg protein	LC-MS/MS	[480]
	amygdala (M)	200 nmol/g	200000 pmol/g	LC-MS/MS	[477]
	striatum (M)	23800 ± 5170 pmol/g	23800 ± 5170 pmol/g	LC-MS/MS	[478]
	hippocampus (M)	28000 nmol/g protein	28000 pmol/mg protein	LC-MS/MS	[480]
	hippocampus (M)	33700 ± 6290 pmol/g	33700 ± 6290 pmol/g	LC-MS/MS	[478]
	cerebellum (M)	16100 ± 1460 pmol/g	16100 ± 1460 pmol/g	LC-MS/MS	[478]
	thalamus (M)	20900 ± 3080 pmol/g	20900 ± 3080 pmol/g	LC-MS/MS	[478]
	hippothalamus (M)	13300 ± 2040 pmol/g	13300 ± 2040 pmol/g	LC-MS/MS	[478]
	midbrain (M)	22900 ± 2670 pmol/g	22900 ± 2670 pmol/g	LC-MS/MS	[478]
	brainstem (M)	13500 ± 1900 pmol/g	13500 ± 1900 pmol/g	LC-MS/MS	[478]
	spinal cord (M)	45000 pmol/g	45000 pmol/g	LC-MS/MS	[481]

	sciatic nerve (M)	40 ng/mg	131600 pmol/g	LC-MS/MS	[482]
	paw (M)	5000 pmol/g	5000 pmol/g	LC-MS/MS	[481]
	retina (M)	8.38 ± 1.02 pmol/retina	8.38 ± 1.02 pmol/retina	GC-MS	[483]
	muscle (M)	1400 pg/mg	4605 pmol/g	LC-MS/MS	[484]
	adipose tissue (O-cows)	1097.0 fmol/mg	1097 pmol/g	LC-MS/MS	[468]
	adipose tissue (O-cows)	1.3 pmol/mg	1300 pmol/g	LC-MS/MS	[469]
	bone (M)	8 pmol/mg	8000 pmol/g	LC-MS/MS	[485]
prostaglandin D_2	plasma (M)	118.6 ± 19.9 pg/µL	336.7 ± 56.5 pmol/mL	LC-MS/MS	[486]
	plasma (M)	0.13 ± 0.044 ng/mL	0.38 ± 0.13 pmol/g	LC-MS/MS	[368]
	plasma (M)	0.07 ± 0.01 ng/mL	0.2 ± 0.03 pmol/mL	LC-MS/MS	[487]
	plasma (R)	4.04 ± 1.5 ng/mL	11.5 ± 4.3 pmol/mL	LC-MS/MS	[487]
	serum (M)	130 pg/mL	0.37 pmol/mL	ELISA	[488]
	lung (M)	80.74 pmol/g	80.74 pmol/g	LC-MS/MS	[489]
	lung (M)	11.24 ± 2.16 pg/mg	31.9 ± 6.13 pmol/g	LC-MS/MS	[490]
	lung (M)	76.5 ± 88.9 pg/mg	217.2 ± 252.4 pmol/g	LC-MS/MS	[486]
	lung (M)	10 pmol/mg of lung protein	10 pmol/mg of lung protein	LC-MS/MS	[472]
	BALF (M)	0.5 ng/mL	1.4 pmol/mL	ELISA	[491]
	liver (M)	1.85 pg/mg	5.25 pmol/g	LC-MS/MS	[490]
	liver (M)	1.2 ± 0.7 pg/mg	3.41 ± 1.9 pmol/g	LC-MS/MS	[486]
	liver (M)	13.6 ± 15.8 ng/g	38.6 ± 44.8 pmol/g	LC-MS/MS	[368]
	colon (M)	1169.16 ± 279.4 ng/g	3319.6 ± 793.3 pmol/g	LC-MS/MS	[475]
	brain (M)	29.4 ± 6.2 pg/mg	83.5 ± 17.6 pmol/g	LC-MS/MS	[486]
	brain (M)	0.76 ± 0.29 nmol/g	760 ± 290 pmol/g	LC-MS/MS	[476]
	cortex (M)	0.15 pmol/mg	150 pmol/g	LC-MS	[479]
	hippocampus (M)	2.5 pmol/mg	2500 pmol/g	LC-MS/MS	[492]
	spinal cord (M)	45.4 ± 5.0 pg/mg	128.9 ± 14.2 pmol/g	LC-MS/MS	[486]
	spinal cord (R)	16.2 ± 1.5 pg/mg	45.9 ± 4.3 pmol/g	LC-MS/MS	[493]
	kidney (M)	0.9 ± 0.7 pg/mg	2.56 ± 1.99 pmol/g	LC-MS/MS	[486]
	bladder (M)	0.2 ng/mg	567.8 pmol/g	LC-MS/MS	[494]
	spleen (M)	13.9 ± 8.5 pg/mg	39.5 ± 24.2 pmol/g	LC-MS/MS	[486]
	sciatic nerve (M)	20.4 ± 14.8 pg/mg	57.9 ± 42 pmol/g	LC-MS/MS	[486]
	brown adipose tissue (M)	0.001 ng/mg	2.8 pmol/g	LC-MS/MS	[495]
	aorta (O-rabbit)	1.5 pg/mg	4.2 pmol/g	ELISA	[496]
	muscle (M)	15.33 ± 0.73 pmol/g	15.33 ± 0.73 pmol/g	LC-MS/MS	[497]

	uterus (R)	0.10 ± 0.044 ng/mg	283.9 ± 124.9 pmol/g	LC-MS/MS	[498]
	corporea lutea (O-cow)	2.15 ± 0.80 pmol/g	2.15 ± 0.80 pmol/g	LC-MS/MS	[499]
prostaglandin E_2	plasma (M)	5.7 nM	5.7 pmol/mL	LC-MS/MS	[500]
	plasma (M)	0.13 ± 0.06 ng/mL	0.36 ± 0.17 pmol/mL	LC-MS/MS	[368]
	plasma (M)	0.61 ± 0.12 pmol/mL	0.61 ± 0.12 pmol/mL	LC-MS/MS	[501]
	plasma (M)	1.1 pg/mL	0.003 pmol/mL	ELISA	[502]
	plasma (R)	100 pg/mL	0.28 pmol/mL	ELISA	[503]
	plasma (R)	750 pmol/L	0.75 pmol/mL	ELISA	[504]
	plasma (R)	496 ± 27 pg/mL	1.41 ± 0.08 pmol/mL	ELISA	[505]
	plasma (R)	7.86 ± 0.96 ng/mL	22.1 ± 2.7 pmol/mL	LC-MS/MS	[487]
	plasma (O-rabbit)	75 ng/mL	212.9 pmol/mL	ELISA	[506]
	serum (M)	20 pg/mL	0.57 pmol/mL	ELISA	[507]
	serum (R)	6.43 ± 0.25 ng/mL	18.3 ± 0.7 pmol/mL	ELISA	[508]
	serum (R)	19 pg/mL	0.05 pmol/mL	ELISA	[509]
	serum (R)	250 pg/mL	0.71 pmol/mL	ELISA	[510]
	blood (R)	7.35 ng/mL	20.9 pmol/mL	ELISA	[511]
	cerebrospinal fluid (M)	1.34 pg/mL	0.004 pmol/mL	ELISA	[502]
	cerebrospinal fluid (R)	45 pg/100 µL	1.28 pmol/mL	ELISA	[512]
	cerebrospinal fluid (R)	500 pg/mL	1.4 pmol/mL	ELISA	[513]
	cerebrospinal fluid (R)	0.0027 ± 0.0027 pg/µL	0.0077 ± 0.007 pmol/mL	LC-MS/MS	[493]
	cerebrospinal fluid (R)	400 pg/mL	1.1 pmol/mL	ELISA	[514]
	lung (M)	4091.98 pmol/g	4091.98 pmol/g	LC-MS/MS	[489]
	lung (M)	23.8 ± 11.8 pg/mg	67.6 ± 33.5 pmol/g	LC-MS/MS	[490]
	lung (M)	633.3 ± 667.5 pg/mg	1798.1 ± 1895.2 pmol/g	LC-MS/MS	[486]
	lung (M)	2.5 pg/mg	7.1 pmol/g	LC-MS/MS	[515]
	lung (M)	50 pmol/mg of lung protein	50 pmol/mg of lung protein	LC-MS/MS	[472]
	BAL (M)	30 pg (total BAL fluid)	0.085 pmol (total BAL fluid)	LC-MS/MS	[515]
	BAL (M)	1250 pg/mL	3.5 pmol/mL	ELISA	[516]
	pleural exudate (M)	2 ng/mL	5.7 pmol/mL	ELISA	[517]
	liver (M)	0.25 pg/mg	0.71 pmol/g	LC-MS/MS	[490]
	liver (M)	0.1 ± 0.0 pg/mg	0.28 ± 0 pmol/g	LC-MS/MS	[486]
	liver (M)	3.9 ± 6.4 ng/g	11.3 ± 18.1 pmol/g	LC-MS/MS	[368]
	colon (M)	5403.9 ± 126.4 ng/g	15343 ± 358.9 pmol/g	LC-MS/MS	[475]

	intestine (R)	75 pg/mg protein	0.21 pmol/mg protein	ELISA	[518]
	stomach (R)	45.02 ± 1.6 pg/g	0.13 pmol/g	ELISA	[519]
	stomach (R)	7.5 pg/mg	21.3 pmol/g	ELISA	[520]
	brain (M)	6 nmol/g	6000 pmol/g	LC-MS/MS	[500]
	brain (M)	3.7 ± 0.9 pg/mg	10.5 ± 2.55 pmol/g	LC-MS/MS	[486]
	brain (M)	0.84 ± 0.30 nmol/g	840 ± 300 pmol/g	LC-MS/MS	[476]
	brain (M)	15 pg/mg	42.6 pmol/g	ELISA	[521]
	cortex (M)	2980 ± 560 pmol/g	2980 ± 560 pmol/g	LC-MS/MS	[478]
	cortex (M)	0.012 pmol/mg	12 pmol/g	LC-MS	[479]
	cortex (R)	7.5 pg/mg protein	0.02 pmol/mg protein	ELISA	[522]
	striatum (M)	580 ± 145 pmol/g	580 ± 145 pmol/g	LC-MS/MS	[478]
	striatum (M)	170 ± 42.1 pmol/g	170 ± 42.1 pmol/g	LC-MS/MS	[523]
	hippocampus (M)	1620 ± 174 pmol/g	1620 ± 174 pmol/g	LC-MS/MS	[478]
	hippocampal homogenate (R)	500 pg/mL	1.4 pmol/mL	ELISA	[524]
	cerebellum (M)	553 ± 84.1 pmol/g	553 ± 84.1 pmol/g	LC-MS/MS	[478]
	thalamus (M)	704 ± 166 pmol/g	704 ± 166 pmol/g	LC-MS/MS	[478]
	hippothalamus (M)	301 ± 27.3 pmol/g	301 ± 27.3 pmol/g	LC-MS/MS	[478]
	midbrain (M)	681 ± 196 pmol/g	681 ± 196 pmol/g	LC-MS/MS	[478]
	brainstem (M)	568 ± 190 pmol/g	568 ± 190 pmol/g	LC-MS/MS	[478]
	spinal cord (M)	20 pmol/g	20 pmol/g	LC-MS/MS	[481]
	spinal cord (M)	10.2 ± 3.3 pg/mg	28.9 ± 9.4 pmol/g	LC-MS/MS	[486]
	spinal cord (R)	8.8 ± 0.7 pg/mg	24.9 ± 1.9 pmol/g	LC-MS/MS	[493]
	spinal cord (R)	3 pg/mg	8.5 pmol/g	LC-MS/MS	[525]
	spleen (M)	10.88 ± 3.74 pg/mg	30.8 ± 10.6 pmol/g	LC-MS/MS	[490]
	spleen (M)	3.0 ± 3.4 pg/mg	8.52 ± 9.7 pmol/g	LC-MS/MS	[486]
	kidney (M)	1.5 ± 0.5 pg/mg	4.26 ± 1.4 pmol/g	LC-MS/MS	[486]
	kidney (R)	2.7 ng/mg protein	7.7 pmol/mg protein	ELISA	[526]
	kidney (R)	20 pg/g	0.057 pmol/g	ELISA	[527]
	bladder (M)	1.7 ng/mg	4826.8 pmol/g	LC-MS/MS	[494]
	sciatic nerve (M)	22.6 ± 15.3 pg/mg	64.2 ± 43.5 pmol/g	LC-MS/MS	[486]
	paw (M)	50 pmol/g	50 pmol/g	LC-MS/MS	[481]
	paw (R)	1400 pg/mg protein	3.98 pmol/mg protein	ELISA	[528]
	paw inflammatory exudate (R)	5 pg/mL	0.014 pmol/mL	ELISA	[529]
	brown adipose tissue (M)	0.0025 ng/mg	7.1 pmol/g	LC-MS/MS	[495]
	aorta (M)	53.7 ± 2.0 pg/mg	152.5 ± 5.7 pmol/g	ELISA	[530]
	aorta (R)	75 pg/mg	212.9 pmol/g	ELISA	[531]

	aorta (O-rabbit)	7.5 pg/mg	21.3 pmol/g	ELISA	[496]
	muscle (M)	53.74 ± 4.05 pmol/g	53.74 ± 4.05 pmol/g	LC-MS/MS	[497]
	muscle (O-salmon)	16.2 ± 3.0 ng/g	45.9 ± 8.5 pmol/g	shotgun lipidomic	[229]
	muscle (O-cat)	100 pg/mg protein	0.28 pmol/mg protein	ELISA	[532]
	uterus (R)	0.034 ± 0.018 ng/mg	96.5 ± 51.1 pmol/g	LC-MS/MS	[498]
	gingival tissue (R)	174.33 ± 49.5 pg/mg protein	0.49 ± 0.14 pmol/mg protein	ELISA	[533]
prostaglandin $F_{2\alpha}$	plasma (M)	0.9 ± 0.3 pM	900 ± 300 pmol/mL	LC-MS/MS	[250]
	plasma (M)	0.19 ± 0.13 ng/mL	0.53 ± 39.9 pmol/g	LC-MS/MS	[368]
	plasma (R)	6000 pmol/L	6 pmol/mL	ELISA	[504]
	plasma (R)	0.55 ± 0.11 ng/mL	0.15 ± 0.03 pmol/mL	LC-MS/MS	[487]
	lung (M)	1848.58 pmol/g	1848.58 pmol/g	LC-MS/MS	[489]
	lung (M)	14.13 ± 2.21 pg/mg	39.8 ± 6.2 pmol/g	LC-MS/MS	[490]
	lung (M)	83.7 ± 89.4 pg/mg	235.5 ± 251.6 pmol/g	LC-MS/MS	[486]
	lung (M)	1 pmol/mg of lung protein	1 pmol/mg of lung protein	LC-MS/MS	[472]
	liver (M)	0.79 pg/mg	2.23 pmol/g	LC-MS/MS	[490]
	liver (M)	0.6 ± 0.3 pg/mg	1.7 ± 8.4 pmol/g	LC-MS/MS	[486]
	liver (M)	11.9 ± 14.2 ng/g	33.5 ± 39.9 pmol/g	LC-MS/MS	[368]
	liver (M)	20.6 ± 19.1 pmol/g	20.6 ± 19.1 pmol/g	LC-MS/MS	[250]
	colon (M)	1573.86 ± 147.5 ng/g	4429.7 ± 415.1 pmol/g	LC-MS/MS	[475]
	brain (M)	8.4 ± 1.7 pg/mg	23.6 ± 4.8 pmol/g	LC-MS/MS	[486]
	brain (M)	0.7 ± 0.2 nmol/g	700 ± 200 pmol/g	LC-MS/MS	[476]
	cortex (M)	406 ± 57.6 pmol/g	406 ± 57.6 pmol/g	LC-MS/MS	[478]
	striatum (M)	374 ± 50.9 pmol/g	374 ± 50.9 pmol/g	LC-MS/MS	[478]
	striatum (M)	192 ± 24.1 pmol/g	192 ± 24.1 pmol/g	LC-MS/MS	[523]
	hippocampus (M)	1.5 pmol/mg	1500 pmol/g	LC-MS/MS	[492]
	hippocampus (M)	575 ± 46.5 pmol/g	575 ± 46.5 pmol/g	LC-MS/MS	[478]
	cerebellum (M)	245 ± 33.5 pmol/g	245 ± 33.5 pmol/g	LC-MS/MS	[478]
	thalamus (M)	364 ± 41.8 pmol/g	364 ± 41.8 pmol/g	LC-MS/MS	[478]
	hippothalamus (M)	256 ± 31.8 pmol/g	256 ± 31.8 pmol/g	LC-MS/MS	[478]
	midbrain (M)	364 ± 55.6 pmol/g	364 ± 55.6 pmol/g	LC-MS/MS	[478]
	brainstem (M)	300 ± 43.9 pmol/g	300 ± 43.9 pmol/g	LC-MS/MS	[478]
	spinal cord (M)	10 pmol/g	10 pmol/g	LC-MS/MS	[481]
	spinal cord (M)	11.2 ± 6.7 pg/mg	31.5 ± 18.8 pmol/g	LC-MS/MS	[486]
	spinal cord (R)	25.5 ± 1 pg/mg	71.8 ± 2.8 pmol/g	LC-MS/MS	[493]
	spleen (M)	1.5 ± 1.5 pg/mg	4.2 ± 4.2 pmol/g	LC-MS/MS	[486]
	kidney (M)	1.3 ± 0.7 pg/mg	3.7 ± 1.9 pmol/g	LC-MS/MS	[486]

	bladder (M)	0.3 ng/mg	844.3 pmol/g	LC-MS/MS	[494]
	brown adipose tissue (M)	0.004 ng/mg	11.2 pmol/g	LC-MS/MS	[495]
	sciatic nerve (M)	5.4 ± 3.4 pg/mg	15.2 ± 9.6 pmol/g	LC-MS/MS	[486]
	aorta (M)	80.7 ± 7.3 pg/mg	227.1 ± 20.5 pmol/g	ELISA	[530]
	aorta (R)	50 pg/mg	140.7 pmol/g	ELISA	[531]
	paw (M)	25 pmol/g	25 pmol/g	LC-MS/MS	[481]
	muscle (M)	35.31 ± 2.51 pmol/g	35.31 ± 2.51 pmol/g	LC-MS/MS	[497]
	uterus (R)	0.038 ± 0.018 ng/mg	106.9 ± 50.6 pmol/g	LC-MS/MS	[498]
	gingival tissue (R)	1405.56 ± 558.9 pg/mg protein	3.96 ± 1.6 pmol/mg protein	ELISA	[533]
15deoxy$\delta^{12,14}$prostaglandin J_2	plasma (M)	0.13 ± 0.1 pM	130 ± 100 pmol/mL	LC-MS/MS	[250]
	lung (M)	95.56 pmol/g	95.56 pmol/g	LC-MS/MS	[489]
	liver (M)	0.15 ± 0.03 pmol/g	0.15 ± 0.03 pmol/g	LC-MS/MS	[250]
	colon (M)	474.2 ± 144.3 ng/g	1495.9 ± 454.9 pmol/g	LC-MS/MS	[475]
15-keto PGE_2	colon (M)	0.2 pg/mg	0.57 pmol/g	LC-MS/MS	[207]
	colon (M)	1500.58 ± 259.15 ng/g	4281.2 ± 739.4 pmol/g	LC-MS/MS	[475]
	muscle (M)	3.77 ± 0.52 pmol/g	3.77 ± 0.52 pmol/g	LC-MS/MS	[497]
6-keto prostaglandin $F_{1\alpha}$	plasma (M)	1067.7 ± 534.9 pg/µL	2881.8 ± 1443.7 pmol/g	LC-MS/MS	[486]
	plasma (M)	0.072 ± 0.022 ng/mL	0.2 ± 0.06 pmol/mL	LC-MS/MS	[368]
	plasma (M)	0.06 ± 0.01 ng/mL	0.2 ± 0.02 pmol/mL	LC-MS/MS	[487]
	plasma (R)	175 pg/mL	0.47 pmol/mL	ELISA	[503]
	plasma (R)	0.19 ± 0.05 ng/mL	0.5 ± 0.1 pmol/mL	LC-MS/MS	[487]
	lung (M)	1063.4 ± 1004.9 pg/mg	2870.2 ± 2712.3 pmol/g	LC-MS/MS	[486]
	pleural exudate (M)	40 ng/mL	107.9 pmol/mL	ELISA	[517]
	liver (M)	7.6 ± 7.5 ng/g	20.5 ± 20.2 pmol/g	LC-MS/MS	[368]
	liver (M)	4.4 ± 2.2 pg/mg	11.9 ± 5.9 pmol/g	LC-MS/MS	[486]
	liver (M)	11 ± 7.36 pmol/g	11 ± 7.36 pmol/g	LC-MS/MS	[250]
	colon (M)	70 pg/mg	188.9 pmol/g	LC-MS/MS	[207]
	colon (M)	11156.4 ± 1458.9 ng/g	30111.7 ± 3937.6 pmol/g	LC-MS/MS	[475]
	brain (M)	11.3 ± 1.3 pg/mg	30.5 ± 3.5 pmol/g	LC-MS/MS	[486]
	brain (M)	0.23 ± 0.04 nmol/g	230 ± 40 pmol/g	LC-MS/MS	[476]
	spinal cord (M)	67.5 ± 26.8 pg/mg	182.2 ± 72.3 pmol/g	LC-MS/MS	[486]
	spinal cord (R)	4.78 ± 0.7 pg/mg	12.9 ± 1.9 pmol/g	LC-MS/MS	[493]
	cerebrospinal fluid (R)	0.054 ± 0.04 pg/µL	0.14 ± 0.1 pmol/mL	LC-MS/MS	[493]

	spleen (M)	32.8 ± 33.7 pg/mg	88.5 ± 90.9 pmol/g	LC-MS/MS	[486]
	kidney (M)	7.0 ± 6.9 pg/mg	18.9 ± 18.6 pmol/g	LC-MS/MS	[486]
	bladder (M)	0.7 ng/mg	1889.3 pmol/g	LC-MS/MS	[494]
	sciatic nerve (M)	32.7 ± 26.6 pg/mg	88.3 ± 71.8 pmol/g	LC-MS/MS	[486]
	muscle (M)	7.34 ± 0.86 pmol/g	7.34 ± 0.86 pmol/g	LC-MS/MS	[497]
	brown adipose tissue (M)	0.008 ng/mg	21.6 pmol/g	LC-MS/MS	[495]
	uterus (R)	0.39 ± 0.10 ng/mg	1052.6 ± 269.9 pmol/g	LC-MS/MS	[498]
	aorta (R)	200 pg/mg	539.8 pmol/g	ELISA	[531]
	aorta (R)	4000 pg/mg protein	10.8 pmol/mg protein	ELISA	[534]
	mesenteric artery (R)	1750 pg/mg protein	4.7 pmol/mg protein	ELISA	[534]
13,14-dihydro-15-keto prostaglandin F1α	colon (M)	1.5 pg/mg	4.99 pmol/g	LC-MS/MS	[207]
8-iso prostaglandin $F_{2\alpha}$	plasma (M)	130 pg/mL	0.37 pmol/mL	ELISA	[530]
	plasma (M)	5 nM	5 pmol/mL	LC-MS/MS	[474]
	serum (R)	130 pg/mL	0.37 pmol/mL	ELISA	[535]
	lung (M)	73.60 pmol/g	73.60 pmol/g	LC-MS/MS	[489]
	lung (M)	0.12 pg/mg	0.34 pmol/g	LC-MS/MS	[490]
	BALF (M)	50 pg/mL	0.14 pmol/mL	ELISA	[536]
	liver (M)	0.03 pmol/mg	30 pmol/g	GC-MS	[537]
	colon (M)	93.03 ± 7.3 ng/g	262.4 ± 20.59 pmol/g	LC-MS/MS	[475]
2-arachidonoylglycerol	plasma (M)	16.43 ± 3.01 ng/mL	43.4 ± 7.95 pmol/mL	GC-MS	[538]
	plasma (M)	90 ± 2.9 pmol/mL	90 ± 2.9 pmol/mL	LC-MS/MS	[501]
	plasma (M)	1.25 ng/mL	3.3 pmol/mL	ELISA	[502]
	plasma (M)	50 pmol/mL	50 pmol/mL	LC-MS/MS	[539]
	plasma (M)	3.3 fmol/µL	3.3 pmol/mL	LC-MS/MS	[466]
	plasma (R)	12.5 pmol/mL	12.5 pmol/mL	LC-MS/MS	[540]
	plasma (R)	48.88 ± 6.15 pmol/ml	48.88 ± 6.15 pmol/ml	LC-MS	[541]
	plasma (O-cows)	16.8 nmol/mL	16800 pmol/mL	LC-MS/MS	[468]
	plasma (O-cows)	10.5 fmol/mL	0.0105 pmol/mL	LC-MS/MS	[469]
	cerebrospinal fluid (M)	0.69 ng/mL	1.8 pmol/mL	ELISA	[502]
	lung (M)	400 nmol/g	400000 pmol/g	LC-MS/MS	[473]
	lung (M)	0.14 ng/mg	370.1 pmol/g	LC-MS/MS	[542]
	liver (M)	3 nmol/g	3000 pmol/g	LC-MS/MS	[162]
	liver (M)	782.8 ± 1290.9 pmol/g	782.8 ± 1290.9 pmol/g	LC-MS/MS	[250]
	liver (R)	10 ng/mg	26400 pmol/g	LC-MS/MS	[543]
	liver (O-cows)	287.6 fmol/mg	287.6 pmol/g	LC-MS/MS	[469]
	ileum (M)	6000 ng/mg	15860000 pmol/g	LC-MS	[544]

	jejunum (M)	14 nmol/g	14000 pmol/g	LC-MS/MS	[545]
	colon (M)	24.08 ± 2.03 pmol/g	24.08 ± 2.03 pmol/g	LC-MS/MS	[546]
	gut (O-shrew)	4 nmol/g	4000 pmol/g	LC-MS	[547]
	cortex (M)	17 nmol/g	17000 pmol/g	LC-MS/MS	[548]
	cortex (M)	12 pmol/mg	12000 pmol/g	LC-MS	[549]
	cortex (M)	200 nmol/g	200000 pmol/g	LC-MS/MS	[477]
	cortex (M)	23.0 nmol/g	23000 pmol/g	LC-MS/MS	[550]
	cortex (M)	544 ± 85.9 pmol/g	544 ± 85.9 pmol/g	LC-MS/MS	[478]
	cortex (M)	6 pmol/mg	6000 pmol/g	LC-MS	[479]
	cortex (R)	11.2 ± 0.7 nmol/g	11200 ± 700 pmol/g	LC-MS/MS	[551]
	cortex (R)	11 nmol/g	11000 pmol/g	LC-MS/MS	[552]
	cortex (R)	1.75 nmol/g	1750 pmol/g	LC-MS/MS	[553]
	cortex (R)	1.5 pmol/mg	1500 pmol/g	LC-MS	[554]
	amygdala (M)	18.72 ± 4.6 nmol/g	18720 ± 4600 pmol/g	LC-MS/MS	[555]
	amygdala (M)	900 nmol/g protein	900 pmol/mg protein	LC-MS/MS	[480]
	amygdala (M)	27 pmol/mg	27000 pmol/g	LC-MS	[549]
	amygdala (M)	100 nmol/g	100000 pmol/g	LC-MS/MS	[477]
	amygdala (R)	10 nmol/g	10000 pmol/g	LC-MS/MS	[552]
	basolateral amygdala (R)	2 pmol/mg	2000 pmol/g	LC-MS/MS	[556]
	substance nigra (M)	55 pg/mg	145.4 pmol/g	LC-MS/MS	[557]
	periaqueductal gray (M)	160 nmol/g	160000 pmol/g	LC-MS/MS	[477]
	periaqueductal gray (R)	100 nmol/g	100000 pmol/g	LC-MS/MS	[558]
	striatum (M)	36 nmol/g	36000 pmol/g	LC-MS/MS	[548]
	striatum (M)	1100 ± 239 pmol/g	1100 ± 239 pmol/g	LC-MS/MS	[523]
	striatum (M)	1400 ± 328 pmol/g	1400 ± 328 pmol/g	LC-MS/MS	[478]
	hippocampus (M)	9.11 ± 1.45 nmol/g	9110 ± 1450 pmol/g	LC-MS/MS	[555]
	hippocampus (M)	450 nmol/g protein	450 pmol/mg protein	LC-MS/MS	[480]
	hippocampus (M)	40 pmol/mg	40000 pmol/g	LC-MS	[549]
	hippocampus (M)	1100 ± 354 pmol/g	1100 ± 354 pmol/g	LC-MS/MS	[478]
	hippocampus (R)	20 nmol/g	20000 pmol/g	LC-MS/MS	[552]
	hippocampus (R)	7.5 pmol/mg	7500 pmol/g	LC-MS	[554]
	cerebellum (M)	40 nmol/g	40000 pmol/g	LC-MS/MS	[548]
	cerebellum (M)	1840 ± 286 pmol/g	1840 ± 286 pmol/g	LC-MS/MS	[478]
	thalamus (M)	35 nmol/g	35000 pmol/g	LC-MS/MS	[548]
	thalamus (M)	2150 ± 266 pmol/g	2150 ± 266 pmol/g	LC-MS/MS	[478]
	midbrain (M)	25 nmol/g	25000 pmol/g	LC-MS/MS	[548]

	midbrain (M)	2390 ± 760 pmol/g	2390 ± 760 pmol/g	LC-MS/MS	[478]
	brain medulla (R)	25 pmol/mg	25000 pmol/g	LC-MS	[559]
	brainstem (M)	2070 ± 526 pmol/g	2070 ± 526 pmol/g	LC-MS/MS	[478]
	brain (R)	2 pmol/mg	2000 pmol/g	LC-MS	[560]
	brain (O-shrew)	1.75 nmol/g	1750 pmol/g	LC-MS	[547]
	hypothalamus (M)	1200 ± 548 pmol/g	1200 ± 548 pmol/g	LC-MS/MS	[478]
	hypothalamus (M)	49.9 nmol/g	49900 pmol/g	LC-MS/MS	[550]
	hypothalamus (R)	32 pmol/mg	32000 pmol/g	LC-MS/MS	[561]
	spinal cord (M)	3 µg/g	7930 pmol/g	LC-MS/MS	[562]
	spinal cord (M)	50000 pmol/g	50000 pmol/g	LC-MS/MS	[481]
	spinal cord (R)	18 pmol/mg	18000 pmol/g	LC-MS	[559]
	spinal cord (R)	120 pg/mg	317.2 pmol/g	ELISA	[563]
	spinal cord (R)	5 µg/mg protein	13217 pmol/mg protein	ELISA	[564]
	trigeminal ganglia (R)	5 pmol/mg	5000 pmol/g	LC-MS	[559]
	dorsal root ganglion (R)	15 µh/mg protein	39651 pmol/mg protein	ELISA	[564]
	sciatic nerve (M)	2 ng/mg	5286.8 pmol/g	LC-MS/MS	[482]
	subcutaneous adipose tissue (R)	7.74 ± 2.47 pmol/mg	7740 ± 2470 pmol/g	LC-MS	[541]
	adipose tissue (O-cows)	199.7 nmol/mg	199.7 pmol/g	LC-MS/MS	[468]
	adipose tissue (O-cows)	97 fmol/mg	97 pmol/g	LC-MS/MS	[469]
	paw (M)	2000 pmol/g	2000 pmol/g	LC-MS/MS	[481]
	hair (M)	225.9 ± 29.8 pg/mg	597.1 ± 78.8 pmol/g	LC-MS/MS	[565]
	ovary (M)	40 ng/g	105.7 pmol/g	LC-MS/MS	[566]
	muscle (M)	70 pg/mg	185.1 pmol/g	LC-MS/MS	[484]
	muscle (R)	0.60 ± 0.3 pmol/mg	600 ± 300 pmol/mg	LC-MS	[541]
	bone (M)	6 fmol/mg	6 pmol/g	LC-MS/MS	[485]
	heart (R)	5.5 nmol/g	5500 pmol/g	LC-MS/MS	[540]
prostaglandin D_2 - glycerol ester			**No amount reported in publications**		
prostaglandin E_2 - glycerol ester	dorsal root ganglion (M)	90 fmol/g of lipids	0.09 pmol/g of lipids	LC-MS/MS	[421]
	plasma (M)	0.17 ± 0.08 fmol/mL	0.00017 ± 0.00008 pmol/mL	LC-MS/MS	[501]
prostaglandin $F_{2\alpha}$ - glycerol ester			**No amount reported in publications**		
15deoxy$\delta^{12,14}$prostaglandin J_2 - glycerol ester			**No amount reported in publications**		
anandamide	plasma (M)	646.56 ± 48.68 pg/mL	1.86 ± 0.14 pmol/mL	GC-MS	[538]
	plasma (M)	2.43 ± 0.3 pM	2430 ± 300 pmol/mL	LC-MS/MS	[250]
	plasma (M)	0.67 ± 0.08 pmol/mL	0.67 ± 0.08 pmol/mL	LC-MS/MS	[501]
	plasma (M)	1.24 ng/mL	3.6 pmol/mL	ELISA	[502]

	plasma (M)	0.71 pmol/mL	0.71 pmol/mL	LC-MS/MS	[539]
	plasma (M)	0.75 fmol/µL	0.75 pmol/mL	LC-MS/MS	[466]
	plasma (R)	2.8 pmol/mL	2.8 pmol/mL	LC-MS/MS	[540]
	plasma (R)	0.7 ng/mL	2 pmol/mL	LC-MS/MS	[467]
	plasma (R)	1.94 ± 0.21 pmol/mL	1.94 ± 0.21 pmol/mL	LC-MS	[541]
	plasma (O-hamster)	0.8 ± 0.2 ng/mL	2.3 ± 0.6 pmol/mL	LC-MS/MS	[567]
	plasma (O-cows)	308.6 fmol/mL	0.31 pmol/mL	LC-MS/MS	[468]
	plasma (O-cows)	232.2 fmol/mL	0.23 pmol/mL	LC-MS/MS	[469]
	cerebrospinal fluid (M)	0.84 ng/mL	2.4 pmol/mL	ELISA	[502]
	lung (M)	0.9 nmol/g	900 pmol/g	LC-MS/MS	[473]
	lung (M)	0.1 nmol/g protein	0.1 pmol/mg protein	LC-MS/MS	[568]
	lung (O-guinea pigs)	0.002 ng/mg	5.7 pmol/g	LC-MS/MS	[542]
	liver (M)	1.09 ± 0.2 pmol/g	1.09 ± 0.2 pmol/g	LC-MS/MS	[250]
	liver (M)	0.01 nmol/g protein	0.01 pmol/mg protein	LC-MS/MS	[568]
	liver (M)	3 pmol/g	3 pmol/g	LC-MS/MS	[569]
	liver (R)	0.5 ng/mg	1435.5 pmol/g	LC-MS/MS	[543]
	liver (O-hamster)	2.4 ± 0.2 ng/mg	6892.5 ± 574.4 pmol/g	LC-MS/MS	[567]
	liver (O-cows)	0.7 fmol/mg	0.7 pmol/g	LC-MS/MS	[469]
	colon (M)	10.04 ± 1.9 pmol/g	10.04 ± 1.9 pmol/g	LC-MS/MS	[546]
	intestine (O-hamster)	8.8 ± 2.9 ng/mg	25.3 ± 8.3 pmol/g	LC-MS/MS	[567]
	gut (O-shrew)	105 nmol/g	105000 pmol/g	LC-MS	[547]
	cortex (M)	15 pmol/g	15 pmol/g	LC-MS/MS	[548]
	cortex (M)	3 pmol/g	3 pmol/g	LC-MS/MS	[477]
	cortex (M)	13.3 pmol/g	13.3 pmol/g	LC-MS/MS	[550]
	cortex (M)	32.7 ± 6.8 pmol/g	32.7 ± 6.8 pmol/g	LC-MS/MS	[478]
	cortex (M)	0.008 pmol/mg	8 pmol/g	LC-MS	[479]
	cortex (R)	10 pmol/g	10 pmol/g	LC-MS/MS	[552]
	cortex (R)	33 pmol/g	33 pmol/g	LC-MS/MS	[553]
	cortex (R)	750 pmol/g	750 pmol/g	LC-MS	[554]
	periaqueductal gray (M)	3 pmol/g	3 pmol/g	LC-MS/MS	[477]
	periaqueductal gray (R)	55 pmol/g	55 pmol/g	LC-MS/MS	[558]
	striatum (M)	8 pmol/g	8 pmol/g	LC-MS/MS	[548]
	striatum (M)	26 ± 5.9 pmol/g	26 ± 5.9 pmol/g	LC-MS/MS	[523]
	striatum (M)	41.8 ± 8.9 pmol/g	41.8 ± 8.9 pmol/g	LC-MS/MS	[478]
	amygdala (M)	6.89 ± 1.04 pmol/g	6.89 ± 1.04 pmol/g	LC-MS/MS	[555]
	amygdala (M)	400 pmol/g protein	0.4 pmol/mg protein	LC-MS/MS	[480]
	amygdala (M)	2.5 pmol/g	2.5 pmol/g	LC-MS/MS	[477]
	amygdala (R)	25 nmol/g	25000 pmol/g	LC-MS/MS	[570]

	amygdala (R)	10 pmol/g	10 pmol/g	LC-MS/MS	[552]
	hippocampus (M)	8.30 ± 0.8 pmol/g	8.30 ± 0.8 pmol/g	LC-MS/MS	[555]
	hippocampus (M)	0.1 pmol/mg	100 pmol/g	LC-MS/MS	[492]
	hippocampus (M)	450 pmol/g protein	0.45 pmol/mg protein	LC-MS/MS	[480]
	hippocampus (M)	175 ng/mg protein	502.4 pmol/mg protein	ELISA	[571]
	hippocampus (M)	79.1 ± 15.7 pmol/g	79.1 ± 15.7 pmol/g	LC-MS/MS	[478]
	hippocampus (R)	22 pmol/g	22 pmol/g	LC-MS/MS	[552]
	hippocampus (R)	4000 pmol/g	4000 pmol/g	LC-MS	[554]
	cerebellum (M)	20 pmol/g	20 pmol/g	LC-MS/MS	[548]
	cerebellum (M)	34.7 ± 8.2 pmol/g	34.7 ± 8.2 pmol/g	LC-MS/MS	[478]
	thalamus (M)	20 pmol/g	20 pmol/g	LC-MS/MS	[548]
	thalamus (M)	49.5 ± 11 pmol/g	49.5 ± 11 pmol/g	LC-MS/MS	[478]
	hypothalamus (M)	18.7 ± 3.8 pmol/g	18.7 ± 3.8 pmol/g	LC-MS/MS	[478]
	hypothalamus (R)	0.025 pmol/mg	25 pmol/g	LC-MS/MS	[561]
	midbrain (M)	11 pmol/g	15-1 pmol/g	LC-MS/MS	[548]
	midbrain (M)	39.1 ± 16.6 pmol/g	39.1 ± 16.6 pmol/g	LC-MS/MS	[478]
	brainstem (M)	15.6 ± 3.4 pmol/g	15.6 ± 3.4 pmol/g	LC-MS/MS	[478]
	brain (M)	0.13 nmol/g protein	0.13 pmol/mg protein	LC-MS/MS	[568]
	brain (R)	6 pmol/g	6 pmol/g	LC-MS	[560]
	brain medulla (R)	0.015 pmol/mg	15 pmol/g	LC-MS	[559]
	brain medulla (R)	0.01 pmol/mg	10 pmol/g	LC-MS/MS	[572]
	brain (O-shrew)	65 nmol/g	65000 pmol/g	LC-MS	[547]
	spinal cord (M)	5 ng/g	14.4 pmol/g	LC-MS/MS	[562]
	spinal cord (M)	10 pmol/g	10 pmol/g	LC-MS/MS	[481]
	spinal cord (R)	0.02 pmol/mg	20 pmol/g	LC-MS	[559]
	spinal cord (R)	50 pg/mg	143.6 pmol/g	ELISA	[563]
	spinal cord (R)	30 pg/mg	86.1 pmol/g	LC-MS/MS	[525]
	spinal cord (R)	0.012 pmol/mg	12 pmol/g	LC-MS/MS	[572]
	spinal cord (R)	20 ng/mg protein	57.4 pmol/mg protein	ELISA	[564]
	dorsal root ganglion (R)	4 pmol/g	4 pmol/g	????	[573]
	dorsal root ganglion (R)	25 ng/mg protein	71.8 pmol/mg potein	ELISA	[564]
	trigeminal ganglia (R)	0.025 pmol/mg	25 pmol/g	LC-MS	[559]
	trigeminal ganglia (R)	0.02 pmol/mg	20 pmol/g	LC-MS/MS	[572]
	spleen (M)	0.02 nmol/g protein	0.02 pmol/mg protein	LC-MS/MS	[568]
	kidney (M)	0.05 nmol/g protein	0.05 pmol/mg protein	LC-MS/MS	[568]
	paw (M)	1 pmol/g	1 pmol/g	LC-MS/MS	[481]
	hair (M)	159.1 ± 27.0 pg/mg	456.9 ± 77.5 pmol/g	LC-MS/MS	[565]

	ovary (M)	3.5 ng/g	10 pmol/g	LC-MS/MS	[566]
	muscle (M)	1 pg/mg	2.9 pmol/g	LC-MS/MS	[484]
	muscle (R)	4.86 ± 0.8 pmol/g	4.86 ± 0.8 pmol/g	LC-MS	[541]
	skin (M)	0.07 nmol/g protein	0.07 pmol/mg protein	LC-MS/MS	[568]
	bone (M)	4 fmol/mg	4 pmol/g	LC-MS/MS	[485]
	subcutaneous adipose tissue (R)	36.22 ± 2.7 pmol/g	36.22 ± 2.7 pmol/g	LC-MS	[541]
	adipose tissue (O-cows)	1.6 fmol/mg	1.6 pmol/g	LC-MS/MS	[468]
	adipose tissue (O-cows)	0.7 fmol/mg	0.7 pmol/g	LC-MS/MS	[469]
	heart (M)	0.025 nmol/g protein	0.025 pmol/mg protein	LC-MS/MS	[568]
	heart (R)	60 pmol/g	60 pmol/g	LC-MS/MS	[540]
	basolateral amygdala (R)	8 pmol/g	8 pmol/g	LC-MS/MS	[556]
prostaglandin D_2 - ethanolamide			**No amount reported in publications**		
prostaglandin E_2 - ethanolamide			**No amount reported in publications**		
prostaglandin $F_{2\alpha}$ - ethanolamide	liver (M)	0.127 ± 0.1 pmol/g	0.127 ± 0.1 pmol/g	LC-MS/MS	[250]

Appendix Table 2: Endogenous amount and biological distribution of prostaglandin derivatives in human (control condition).

prostaglandin derivative	**tissue** (Human (H))	**reported amount**	**amount (pmol/g or mL)**	**method**	**Ref.**
arachidonic acid	plasma (H)	5060 ± 2610 pmol/mL	5060 ± 2610 pmol/mL	LC-MS/MS	[420]
	plasma (H)	306.3 ± 127.5 ng/mL	1006.9 ± 419.13 pmol/mL	LC-MS/MS	[574]
	plasma (H)	279.4 ng/mL	918.4 pmol/mL	LC-MS/MS	[575]
	plasma (H)	3000 pmol/mL	3000 pmol/mL	LC-MS/MS	[576]
	plasma (H)	1000 pmol/mL	1000 pmol/mL	LC-MS/MS	[577]
	serum (H)	2235.09 ng/mL	7352.2 pmol/mL	LC-MS/MS	[578]
	serum (H)	0.85 ng/mL	2.79 pmol/mL	LC-MS/MS	[446]
	serum (H)	439.2 ± 102.3 ng/mL	1443.7 ± 336.3 pmol/mL	LC-MS/MS	[574]
	saliva (H)	7659.1 ± 1876.1 fmol/mg protein	7.7 ± 1.9 pmol/mg protein	LC-MS/MS	[579]
	saliva (H)	2327.15 ± 2539.3 fmol/mg protein	2.3 ± 2.5 pmol/mg protein	LC-MS/MS	[580]
	cerebrospinal fluid (H)	15,62 ± 7.49 pmol/mL	15,62 ± 7.49 pmol/mL	LC-MS/MS	[581]
	breast milk (H)	0.49 mg/mL	1611000 pmol/mL	GC-FID	[582]
	breast milk (H)	4712.24 ± 1698.3 ng/mL	15500.8 ± 5586.5 pmol/mL	LC-MS/MS	[583]
prostaglandin D_2	plasma (H)	9.6 ± 2.2 pg/mL	0.027 ± 0.006 pmol/mL	LC-MS/MS	[203]
	plasma (H)	44.6 ± 109.1 nM	44.6 ± 109.1 pmol/mL	LC-MS/MS	[584]
	plasma (H)	0.094 ± 0.05 nM	0.094 ± 0.05 pmol/mL		[585]
	serum (H)	0.57 ng/mL	1.62 pmol/mL	LC-MS/MS	[578]
	serum (H)	315.9 ± 297.3 pg/mL	0.89 ± 0.84 pmol/mL	LC-MS/MS	[574]
	BAL (H)	5.6 pM	5600 pmol/mL	LC-MS/MS	[586]
prostaglandin E_2	plasma (H)	2.5 pmol/mL	2.5 pmol/mL	LC-MS/MS	[587]
	plasma (H)	300 pg/mL	0.85 pmol/mL	ELISA	[588]
	plasma (H)	4.5 ± 4.2 pg/mL	0.013 ± 0.012 pmol/mL	LC-MS/MS	[203]
	plasma (H)	0.026 ± 0.028 ng/mL	0.074 ± 0.079 pmol/mL	LC-MS/MS	[589]
	plasma (H)	0.26 ± 0.19 nM	0.26 ± 0.19 pmol/mL	LC-MS/MS	[584]
	plasma (H)	10.2 ± 10.2 pg/mL	0.03 ± 0.03 pmol/mL	LC-MS/MS	[574]
	plasma (H)	3.6 ng/mL	10.2 pmol/mL	LC-UV	[590]
	serum (H)	0.34 ng/mL	0.97 pmol/mL	LC-MS/MS	[578]
	serum (H)	0.49 ng/mL	1.39 pmol/mL	LC-MS/MS	[446]
	serum (H)	554.8 ± 103.9 **?? No units reported**		ELISA	[591]

	serum (H)	19947.8 ± 10190.5 pg/mL	56.6 ± 28.9 pmol/mL	LC-MS/MS	[574]
	serum (H)	400 pg/mL	1.14 pmol/mL	ELISA	[592]
	serum (H)	49.23 ± 18.7 pg/mL	0.14 pmol/mL	ELISA	[593]
	urine (H)	10.0 ng/mmol creatinine	28.3 pmol/mmol creatinine	LC-MS/MS	[594]
	BAL (H)	1.6 pM	1600 pmol/mL	LC-MS/MS	[586]
	menstrual fluid (H)	369.4 ± 80.6 ng/mL	1048.9 ± 228.9 pmol/mL	ELISA	[595]
	gingival cervicular fluid (H)	500 pg/µL	1419.6 pmol/mL	ELISA	[596]
	esophagus (H)	200 pg/mg protein	0.57 pmol/mg protein	ELISA	[597]
prostaglandin $F_{2\alpha}$	plasma (H)	5.25 ± 1.2 ng/mL	14.8 ± 3.4 pmol/mL	ELISA	[595]
	plasma (H)	0.8 ± 0.82 ng/mL	2.2 ± 2.3 pmol/mL	LC-MS/MS	[589]
	plasma (H)	0.14 ± 0.37 nM	0.14 ± 0.37 pmol/mL	LC-MS/MS	[584]
	serum (H)	0.81 ng/mL	2.29 pmol/mL	LC-MS/MS	[578]
	serum (H)	4470.1 ± 1347.9 pg/mL	12.6 ± 3.4 pmol/mL	LC-MS/MS	[574]
	urine (H)	97 ng/mmol creatinine	273.1 pmol/mmol creatinine	LC-MS/MS	[594]
	BAL (H)	13 pM	13000 pmol/mL	LC-MS/MS	[586]
	brochial wash (H)	21 pM	21000 pmol/mL	LC-MS/MS	[586]
	menstrual fluid (H)	1955.1 ± 194.8 ng/mL	5502.7 ± 548.3 pmol/mL	ELISA	[595]
15deoxy$\delta^{12,14}$prostaglandin J_2	plasma (H)	2.5-349.6 pg/mL	0.0079 - 1.1 pmol/mL	LC-MS/MS	[598]
	plasma (H)	20 pmol/mL	20 pmol/mL	LC-MS/MS	[587]
15-keto PGE_2	serum (H)	0.79 ng/mL	2.25 pmol/mL	LC-MS/MS	[578]
6-keto prostaglandin $F_{1\alpha}$	plasma (H)	580 pg/mL	1.6 pmol/mL	ELISA	[588]
	plasma (H)	6.8 ± 2.9 pg/mL	0.018 ± 0.0077 pmol/mL	LC-MS/MS	[203]
	plasma (H)	0.036 ± 0.016 ng/mL	0.097 ± 0.04 pmol/mL	LC-MS/MS	[589]
	plasma (H)	0.48 ± 0.06 ng/mL	1.3 ± 0.1 pmol/mL	LC-MS/MS	[487]
	plasma (H)	0.18 ± 0.08 nM	0.18 ± 0.08 pmol/mL	LC-MS/MS	[585]
	serum (H)	1.09 ng/mL	2.94 pmol/mL	LC-MS/MS	[578]
	brochial wash (H)	4.1 pM	4100 pmol/mL	LC-MS/MS	[586]
13,14-dihydro-15-keto prostaglandin F1α	plasma (H)	49 ± 41 pg/mL	0.16 ± 0.13 pmol/mL	LC-MS/MS	[599]
8-iso prostaglandin $F_{2\alpha}$	plasma (H)	8.26 ± 2.98 pg/mL	0.023 pmol/mL	LC-MS/MS	[600]
	serum (H)	0.48 ng/mL	1.35 pmol/mL	LC-MS/MS	[578]
	urine (H)	0.34 ± 0.02 ng/mL	0.96 pmol/mL	LC-MS/MS	[601]
	urine (H)	0.13 ng/mL	0.37 pmol/mL	LC-MS/MS	[430]
	urine (H)	0.35 ng/mg creatinin	0.99 pmol/mg creatinin	LC-MS/MS	[602]

	urine (H)	23 ng/mmol creatinine	64.9 pmol/mmol creatinine	LC-MS/MS	[594]
2-arachidonoylglycerol	plasma (H)	1.85 ± 0.79 ng/mL	4.89 ± 2.09 pmol/mL	GC-MS	[538]
	plasma (H)	16.5 ± 5.3 pmol/mL	16.5 ± 5.3 pmol/mL	LC-MS/MS	[420]
	plasma (H)	2.5 pmol/mL	2.5 pmol/mL	LC-MS	[603]
	plasma (H)	0.74 pmol/mL	0.74 pmol/mL	LC-MS/MS	opsycho.2022.108281
	plasma (H)	0.2 ng/mL	0.53 pmol/mL	LC-MS/MS	[604]
	plasma (H)	25 pmol/mL	25 pmol/mL	LC-MS/MS	[605]
	plasma (H)	8.86 ± 2.43 ng/mL	23.4 pmol/mL	ELISA	[606]
	plasma (H)	1.5 pmol/mL	1.5 pmol/mL	LC-MS/MS	[576]
	plasma (H)	15 pmol/mL	15 pmol/mL	LC-MS/MS	[577]
	plasma (H)	15.8 ± 5.96 nM	15.8 ± 5.96 pmol/mL	LC-MS/MS	[607]
	plasma (H)	4 pmol/mL	4 pmol/mL	LC-MS/MS	[587]
	plasma (H)	0.27 ng/mL	0.71 pmol/mL	LC-MS/MS	[608]
	plasma (H)	4 pmol/mL	4 pmol/mL	LC-MS/MS	[609]
	serum (H)	5 pmol/mL	5 pmol/mL	LC-MS/MS	[610]
	saliva (H)	41.3 ± 40.6 fmol/mg protein	0.04 ± 0.04 pmol/mg protein	LC-MS/MS	[579]
	saliva (H)	54.71 ± 36.5 fmol/mg protein	0.054 ± 0.04 pmol/mg protein	LC-MS/MS	[580]
	cerebrospinal fluid (H)	0.076 ± 0.04 pmol/mL	0.076 ± 0.04 pmol/mL	LC-MS/MS	[581]
	breast milk (H)	233.3 ± 60.2 ng/mL	616.7 ± 159.1 pmol/mL	LC-MS/MS	[583]
	hair (H)	33.21 ± 13.5 pg/mg	95.3 ± 38.7 pmol/g	LC-MS/MS	[611]
	hair (H)	67.4 ± 40.2 pg/mg	178.2 ± 110 pmol/g	LC-MS/MS	[612]
	hair (H)	113 pg/mg	298.7 pmol/g	LC-MS/MS	[613]
	hair (H)	9.28 ± 3.25 pg/mg	24.5 ± 8.6 pmol/g	LC-MS/MS	[614]
	nails (H-children)	39.4 pg/mg	104.2 pmol/g	LC-MS/MS	[615]
	nails (H-adults)	25.1 pg/mg	66.3 pmol/g	LC-MS/MS	[615]
prostaglandin D_2 - glycerol ester	**No amount reported in publications**				
prostaglandin E_2 - glycerol ester	**No amount reported in publications**				
prostaglandin $F_{2\alpha}$ - glycerol ester	**No amount reported in publications**				
15deoxy$\delta^{12,14}$prostaglandin J_2 - glycerol ester	**No amount reported in publications**				
anandamide	plasma (H)	290.6 ± 72.0 pg/mL	0.83 ± 0.2 pmol/mL	LC-MS/MS	[203]
	plasma (H)	0.9 ± 0.2 ng/mL	2.6 ± 0.6 pmol/mL	LC-MS/MS	[567]
	plasma (H)	1.7 ± 0.4 pmol/mL	1.7 ± 0.4 pmol/mL	LC-MS/MS	[420]

	plasma (H)	0.47 pmol/mL	0.47 pmol/mL	LC-MS/MS	opsycho.2022.108281
	plasma (H)	0.5 ng/mL	1.4 pmol/mL	LC-MS/MS	[604]
	plasma (H)	2 pmol/mL	2 pmol/mL	LC-MS/MS	[605]
	plasma (H)	6.12 ± 1.6 ng/mL	17.6 ± 4.6 pmol/mL	ELISA	[606]
	plasma (H)	0.25 pmol/mL	0.25 pmol/mL	LC-MS/MS	[576]
	plasma (H)	350 fmol/mL	0.35 pmol/mL	LC-MS/MS	[577]
	plasma (H)	0.77 ± 0.47 nM	0.77 ± 0.47 pmol/mL	LC-MS/MS	[607]
	plasma (H)	2.5 pmol/mL	2.5 pmol/mL	LC-MS/MS	[587]
	plasma (H)	0.2 ng/mL	0.57 pmol/mL	LC-MS/MS	[608]
	plasma (H)	1.59 ± 0.3 nM	1.59 ± 0.3 pmol/mL	LC-MS/MS	[585]
	plasma (H)	0.75 pmol/mL	0.75 pmol/mL	LC-MS/MS	[609]
	serum (H)	2 pmol/mL	2 pmol/mL	LC-MS/MS	[610]
	urine (H)	0.01 ng/mL	0.029 pmol/mL	LC-MS/MS	[616]
	saliva (H)	0.17 ± 0.3 fmol/mg protein	0.00017 ± 0.0003 pmol/mg protein	LC-MS/MS	[579]
	saliva (H)	0.17 ± 0.18 fmol/mg protein	0.00017 ± 0.0002 pmol/mg protein	LC-MS/MS	[580]
	cerebrospinal fluid (H)	1.51 ± 1.08 fmol/mL	0.0015 ± 0.001 pmol/mL	LC-MS/MS	[581]
	breast milk (H)	0.11 ± 0.02 ng/mL	0.32 ± 0.6 pmol/mL	LC-MS/MS	[583]
	colon (H)	0.7 pmol/mg	700 pmol/g	LC-MS/MS	[617]
	hair (H)	0.75 ± 0.75 pg/mg	2.2 ± 2.2 pmol/g	LC-MS/MS	[611]
	hair (H)	1.6 ± 2.0 pg/mg	4.6 ± 5.8 pmol/g	LC-MS/MS	[612]
	hair (H)	1.9 pg/mg	5.5 pmol/g	LC-MS/MS	[613]
	hair (H)	0.76 ± 0.65 pg/mg	2.2 ± 1.9 pmol/g	LC-MS/MS	[614]
	nails (H-children)	1.8 pg/mg	5.2 pmol/g	LC-MS/MS	[615]
	nails (H-adults)	0.95 pg/mg	2.7 pmol/g	LC-MS/MS	[615]
prostaglandin D_2-ethanolamide	**No amount reported in publications**				
prostaglandin E_2-ethanolamide	**No amount reported in publications**				
prostaglandin $F_{2\alpha}$-ethanolamide	**No amount reported in publications**				

Appendix Table 3: Endogenous amount and biological distribution of short chain fatty acids and branched chain fatty acids in animals (control condition).

prostaglandin derivative	**tissue** Mouse (M)	**amount**	**amount (µmol/g or mL)**	**Method (D: dried; W: wet)**	**Ref.**
Acetate	Plasma (M)	0.5 mM	0.5 µmol/mL	Elisa	[618]
	Serum (M)	115 µg/mL	1.9 µmol/mL	LC-MS/MS	[619]

	Faeces (M)	1700 ng/mg	28 µmol/g	LC-MS (W)	[620]
	Faeces (M)	28 nmol/mg	28 µmol/g	LC-MS (W)	[621]
	Faeces (M)	750 µg/g	12.5 µmol/g	GC-MS (W)*	[622]
	Faeces (M)	11 mg/g	183 µmol/g	GC-MS (W)	[623]
	Faeces (M)	8.04 mM/g	?	GC-FID (W)	[624]
	Faeces (M)	6 µg/mg	1000 µmol/g	GC-MS (W)	[625]
	Faeces (M)	15 µmol/g	15 µmol/g	GC-MS (W)	[626]
	Colon (M)	43 mmol/kg	43 µmol/g	GC-MS	[627]
	Caecum (M)	120 mmol/kg	120 µmol/g	GC-MS	[627]
Propionate	Serum (M)	70 µg/mL	0.9 µmol/mL	LC-MS/MS	[619]
	Faeces (M)	250 ng/mg	3.4 µmol/g	LC-MS (W)	[620]
	Faeces (M)	7 nmol/mg	7 µmol/g	LC-MS (W)	[621]
	Faeces (M)	175 µg/g	2.3 µmol/g	GC-MS (W)*	[622]
	Faeces (M)	1.25 mg/g	16.9 µmol/g	GC-MS (W)	[623]
	Faeces (M)	4.79 mM/g	?	GC-FID (W)	[624]
	Faeces (M)	1 µg/mg	13.5 µmol/g	GC-MS (W)	[625]
	Faeces (M)	3 µmol/g	3 µmol/g	GC-MS (W)	[626]
	Colon (M)	13 mmol/kg	13 µmol/g	GC-MS	[627]
	Caecum (M)	45 mmol/kg	45 µmol/g	GC-MS	[627]
Isobutyrate	Faeces (M)	21 µg/g	0.2 µmol/g	GC-MS (W)*	[622]
	Faeces (M)	0.001 mg/g	0.01 µmol/g	GC-MS (W)	[623]
	Colon (M)	2 mmol/kg	2 µmol/g	GC-MS	[627]
	Caecum (M)	8 mmol/kg	8 µmol/g	GC-MS	[627]
Butyrate	Plasma (M)	7.5 µg/g	0.09 µmol/mL	GC-MS	[628]
	Serum (M)	15 µg/mL	0.17 µmol/mL	LC-MS/MS	[619]
	Faeces (M)	8 nmol/mg	8 µmol/g	LC-MS (W)	[621]
	Faeces (M)	115 µg/g	1.3 µmol/g	GC-MS (W)*	[622]
	Faeces (M)	400 µg/g	4.5 µmol/g	GC-MS (W)	[628]
	Faeces (M)	7.5 mg/g	85.2 µmol/g	GC-MS (W)	[623]
	Faeces (M)	2.08 mM/g	?	GC-FID (W)	[624]
	Faeces (M)	0.75 µg/mg	8.5 µmol/g	GC-MS (W)	[625]
	Faeces (M)	5 µmol/g	5 µmol/g	GC-MS (W)	[626]
	Colon (M)	8 mmol/kg	8 µmol/g	GC-MS	[627]
	Colon (M)	13 µg/g	0.15 µmol/g	GC-MS	[628]
	Caecum (M)	30 mmol/kg	30 µmol/g	GC-MS	[627]
2-methylbutyrate	**No amount reported in publications**				
Isovalerate	Faeces (M)	17.5 ng/mg	0.17 µmol/g	LC-MS (W)	[620]
	Faeces (M)	24 µg/g	0.2 µmol/g	GC-MS (W)*	[622]
	Faeces (M)	0.013 mg/g	0.13 µmol/g	GC-MS (W)	[623]
	Colon (M)	2 mmol/kg	2 µmol/g	GC-MS	[627]
	Caecum (M)	5 mmol/kg	5 µmol/g	GC-MS	[627]
Valerate	Faeces (M)	15 ng/mg	0.15 µmol/g	LC-MS (W)	[620]
	Faeces (M)	21 µg/g	0.2 µmol/g	GC-MS (W)*	[622]
	Faeces (M)	0.07 mg/g	0.69 µmol/g	GC-MS (W)	[623]
	Colon (M)	1 mmol/kg	1 µmol/g	GC-MS	[627]
	Caecum (M)	5 mmol/kg	5 µmol/g	GC-MS	[627]

2-methylpentanoate	**No amount reported in publications**				
3-methylpentanoate	**No amount reported in publications**				
4-methylvalerate	**No amount reported in publications**				
Hexanoate	Faeces (M)	5.5 ng/mg	0.05 µmol/g	LC-MS (W)	[620]
	Faeces (M)	0.018 mg/g	0.16 µmol/g	GC-MS (W)	[623]

* use of 4-methylvalerate as internal standard whereas it is an endogenous compound

Appendix Table 4: Endogenous amount and biological distribution of short chain fatty acids and branched chain fatty acids in human (control condition).

prostaglandin derivative	**tissue** Human (H)	**amount**	**amount (nmol/g or mL)**	**Method (D: dried; W: wet)**	**Ref.**
Acetate	Faeces (H)	1.5 mg/g	25 µmol/g	GC-? (W)**	[629]
	Faeces (H)	32.1 µmol/g	32.1 µmol/g	GC-? (D)**	[630]
	Faeces (H)	1200 mg/kg	20 µmol/g	Ion-Chrom. (W)	[631]
	Faeces (H)	75 µmol/g	75 µmol/g	GC-MS (W)	[632]
	Faeces (H)	2200 µg/g	36.7 µmol/g	GC-MS (W)	[633]
Propionate	Faeces (H)	2 mg/g	27 µmol/g	GC-? (W)**	[629]
	Faeces (H)	9.9 µmol/g	9.9 µmol/g	GC-? (D)**	[630]
	Faeces (H)	160 mg/kg	2 µmol/g	Ion-Chrom. (W)	[631]
	Faeces (H)	25 µmol/g	25 µmol/g	GC-MS (W)	[632]
	Faeces (H)	600 µg/g	8.1 µmol/mL	GC-MS (W)	[633]
Isobutyrate	Faeces (H)	1.2 µmol/g	1.2 µmol/g	GC-? (D)**	[630]
Butyrate	Faeces (H)	0.8 mg/g	9 µmol/g	GC-? (W)**	[629]
	Faeces (H)	18.2 µmol/g	18.2 µmol/g	GC-? (D)**	[630]
	Faeces (H)	100 mg/kg	1 µmol/g	Ion-Chrom. (W)	[631]
	Faeces (H)	10 µmol/g	10 µmol/g	GC-MS (W)	[632]
	Faeces (H)	700 µg/g	7.9 µmol/g	GC-MS (W)	[633]
2-methylbutyrate	**No amount reported in publications**				
Isovalerate	Faeces (H)	1.1 µmol/g	1.1 µmol/g	GC-? (D)**	[630]
Valerate	Faeces (H)	0.9 µmol/g	0.9 µmol/g	GC-? (D)**	[630]
	Faeces (H)	5 mg/kg	0.049 µmol/g	Ion-Chrom. (W)	[631]
	Faeces (H)	125 µg/g	1.2 µmol/g	GC-MS (W)	[633]
2-methylpentanoate	Faeces (H)	0.0 µmol/g	0.0 µmol/g	GC-? (D)**	[630]
3-methylpentanoate	**No amount reported in publications**				
4-methylvalerate	**No amount reported in publications**				
Hexanoate	Faeces (H)	0.2 µmol/g	0.2 µmol/g	GC-? (D)**	[630]

** nature of the detector not mentioned in the publication

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