Efficient methodology for the extraction and analysis of lipids from porcine pulmonary artery by supercritical fluid chromatography coupled to mass spectrometry

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Abstract

Pulmonary artery grafts are needed as cardiovascular bioprosthetics. For successful tissue recellularization after transplantation, lipids have to be removed from the donor artery. Developing a selective process to remove lipids without damaging the extracellular matrix greatly depends on knowing the amount and type of lipid compounds in the specific tissue. Here we present an efficient methodology for the study of lipids present in porcine pulmonary arteries. The performance of six extraction methods to recover lipids from artery was evaluated. For this purpose, a supercritical fluid chromatography method coupled to quadrupole time-of-flight mass spectrometry detection (UHPSFC/QTOF-MS) was adapted. The method enabled separation of lipids of a wide range of polarity according to lipid class in less than 7 minutes. One dichloromethane-based extraction method was shown to be the most efficient one for the recovery of lipids from pulmonary artery. However, one MTBE-based extraction method was able to show the highest fatty acid extraction yields (to the expense of longer extraction times). Lipids were relative quantified according to class, and the major species within each class were identified. Triacylglycerols and glycerophospholipids were the most abundant classes, followed by sphingomyelins, monoacylglycerols and fatty acyls. The matrix effect exerted no interference on the analytical method, except for some few combinations of extraction method and lipid class. These results are of relevance for lipidomic studies from solid tissue, in particular for studies on pulmonary and cardiovascular diseases. Finally, our work sets the basis for the further development of a selective processes to remove lipids from pulmonary artery without damaging the tissue prior to transplantation.

Keywords: pulmonary arteries; lipids; extraction; supercritical fluid chromatography; mass spectrometry.

1. Introduction

Cardiovascular disease is the number one cause of death globally [1]. Several hundred thousands of cardiovascular prosthetics are needed to replace failed cardiac valves and blood vessels every year [2]. In addition, pulmonary artery grafts are needed for children born with congenital heart defects [3, 4].

An ideal approach to overcome immune reactions and provide more durable bioprosthetics is to develop a process that removes cellular components but keeps the structure and function of the extracellular matrix [5, 6]. Lipids are one component that is crucial to remove in order to ensure tissue recellularization with repopulating cells after transplantation. The amount and composition of lipid compounds are tissue dependent; developing a process to successfully remove the lipids without damaging the extracellular matrix greatly depends on knowing the amounts and types of lipid compounds present in the specific tissue.

Lipid analysis from biological material requires reliable and efficient extraction and analytical methodologies due to their enormous structural diversity. For a successful lipid recovery, extraction solvents must be able to penetrate the sample matrix and dissolve both free lipids and those attached to the matrix, which will depend on matching polarities between lipid and solvent, and accessibility of the lipids within the matrix. Therefore, while several methods for lipid extraction from soft tissue have been developed, an optimal extraction method depends on the tissue of interest. An efficient extraction method results in high extraction yields of lipids with the smallest amount of solvent and in the shortest time. Other factors like how easy to handle is the method, may also be relevant. To date, methods using chlorinated solvents have been widely used for lipid extraction of both dried and wet biological samples like liver, brain and blood [7-10]. The use of $CHCl_3$:MeOH mixtures was originally reported by Folch et al. [11] and was later improved by Blight et al. [12], have been adapted to specific biological matrices, with the objective to reach higher efficiencies [7, 13]. Carcinogenicity of chloroform and the fact that lipids had to be recovered from the lower layer of separated phases were the main drawbacks of this method. Later, less toxic and noncarcinogenic solvents were demonstrated to be as effective as $CHCl_3$ i.e. methyl-*tert*-butyl ether (MTBE) [14-18]. MTBE gained importance since it is a non-corrosive and non-reactive solvent that avoids lipid degradation during storage [16]. In addition, it enables easy handling since it floats over MeOH, which minimizes contamination when the extract is collected. Butanol was also proposed as a safe candidate but longer times are needed for its complete evaporation [19]. Extraction methods based on lipid partitioning through a sequential process were considered ideal for the separation of the most polar and non-polar lipids

from complex matrices [7, 20, 21]. The mentioned extraction methods have been applied to a great variety of biological samples however, to date, no method has been adapted to pulmonary arteries.

Chromatography coupled to mass spectrometry (MS) is a technique that allows for comprehensive lipid analysis [22]. Reverse phase [23], normal phase [24] or hydrophilic high performance liquid chromatography [25] have been used for the separation of lipids based on dissimilar carbon number and polarity. Shorter times of analysis, less solvent consumption and higher sensitivity have been achieved by ultrahigh performance liquid chromatography (UHPLC) methods [26, 27]. However, Ultrahigh performance supercritical fluid chromatography (UHPSFC) is becoming popular for lipidomic studies. UHPSFC allows even more efficient separations, with one-third to one-fifth lower run times and greater throughputs than UHPLC (with the same column size). In addition, lipids with a wide range of polarities can be simultaneously separated with high-resolution, higher peak capacity and excellent reproducibility [22, 28], which makes UHPSFC the preeminent technique for the analysis of the whole lipid content from complex matrices such as animal tissue.

In this work, we present an efficient methodology for the extraction and analysis of lipids present in porcine pulmonary arteries. A screening of six extraction methods, adapted from those most commonly used in lipidomics, is performed. The methods are evaluated in terms of relative amount and type of lipids extracted as well as repeatability. For this purpose, a chromatographic method based on UHPSFC/quadrupole time-of-flight (QTOF)-MS/MS is presented that facilitates the identification and quantification of lipids cover a wide range of polarities, by enabling their separation according to lipid class. This work sets the basis for developing selective processes to remove lipids from pulmonary artery without damaging the extracellular matrix, which may preserve mechanical properties and increase the lifetimes of cardiovascular grafts by facilitating recellularization after transplantation.

2. Materials and Methods

2.1. Biological material

Lung tissue was obtained from pigs used for transplantation studies, approved by the regional Research Ethical Committee (2015-174). The lungs were flushed with Krebs solution to remove blood. Pulmonary arteries were then dissected and isolated from the lung tissue ensuring removal of remaining blood and connective tissue. Cleaned fresh and freeze-dried pieces of pulmonary arteries were stored at -80°C until further use.

2.2. Chemicals and standards

Ultrapure water (18 Ω/cm) was dispensed by Milli-Q devices from Merck Millipore (Darmstadt Germany). Methanol (LC-MS grade), dichloromethane (stabilized with about 0.002% of methyl-2-butene) and chloroform (stabilized by 0.6% ethanol) were purchased from VWR Chemicals (Fontenay-sous-bois, France). Methyl-tert-butyl ether (MTBE, ≥99.5 % purity), ammonium formate (≥99% purity) were from Sigma-Aldrich (St. Louis, MO, USA). Deuterated triacylglyceride (15:0-18:0-d7-15:0 TG), monoacylglicerol (18:1-d7 MG), phosphatidylcholine (15:0-18:1-d7 PC), sphingomyelin (18:1-d9 SM) and fatty acid (d11-arachidonic FA), used as internal standards, were purchased from Avanti polar lipids (Alabama, USA). Hydrochloric acid (37% solution in water) was from Acros Organics (Geel, Belgium). Liquid CO_2 (99.9993% purity) was provided by AGA Gas AB (Lidingö, Sweden).

2.3. Lipid extraction

Six methods, widely used in lipidomics and already described for other biological matrices i.e. brain, liver or blood [11, 16, 17, 29-36], were adapted to extract lipids from porcine pulmonary arteries. Table 1 shows an overview of the methods (MA-MF) to facilitate comparisons.

Table 1. Comparison of extraction methods used for the recovery of lipids from porcine pulmonary arteries.

		Extraction parameters				
Method		**Total time (min)±**	**Position of the recovered phase**	**Solvent system (v/v)**	**Environmentally friendly process**	**Equipment necessary**
MA		45	Lower	CH_2Cl_2:MeOH (2:1)	No (CH_2Cl_2)	- Refrigerated micro-centrifuge - Vortex
MB		45	Lower	CH_2Cl_2:0.1 M HCl in MeOH (2:1)	No (CH_2Cl_2)	- Refrigerated micro-centrifuge -Vortex
MC		110	Upper x2	MTBE:MeOH (10:3)	Yes	-Refrigerated centrifuge -Rotary shaker
MD		110	Upper x2	MTBE: 75% MeOH (10:3)	Yes	-Refrigerated centrifuge -Rotary shaker
ME		1490	Upper x2	MTBE:MeOH (10:3)	Yes	-Refrigerated centrifuge -Rotary shaker
MF	Polar fraction	20	Upper	MeOH:H_2O (1:1)	Yes	- Refrigerated micro-centrifuge -Vortex
	Nonpolar fraction	40	Upper	CH_2Cl_2:MeOH (3:1)	No (CH_2Cl_2)	

Differences between methods involve incubation times, solvent composition, extraction cycles and equipment needed. A more detailed description of the methods can be found in the Appendix. Preliminary studies showed that fresh arteries gave inconsistent extraction results. Therefore, samples were freeze dried

before extraction. Sample-to-solvent ratio was set to 1:120. This ratio was similar to what has been reported for human brain (1:130) [37] or liver from rats (1:100) [38]. Independent extractions (n=5 or 6) were carried out per method in two batches (interday experiments), following a randomized order. Samples were kept on ice whenever possible. All the obtained extracts were gently dried under N_2 stream and kept at -80°C in a N_2 atmosphere until further use. Extraction blanks were performed following similar procedures described below but in the absence of biological material. It was not possible to homogeneously introduce the internal standard in such solid sample, tissue. For this reason, losses during handling may not affect internal standard and analytes proportionally. Therefore, the sample was intentionally not spiked with an internal standard prior to extraction

2.4. Supercritical fluid chromatography and mass spectrometry conditions

The chromatographic and spectrometric method were adopted from our previous study [39] with some modifications. In this study, different stationary phases, temperature and pressure settings were non-systematically tested on lipid standards for the best separation and signal intensity. Modifier composition (concentration of ammounium formate in methanol) and the percentage of modifier in the mobile phase were adjusted to obtain separation of lipid standards (i.e. deuterated standards) in the shortest time possible. Note that the final aim is not to achieve chromatographic separation of each individual lipid from the extracts, but rather elute lipids from the same class within a given time range to facilitate quantification. The composition of the make-up solvent was also adjusted to ensure good ionization (i.e. as high intensity as possible). Mass spectrometry parameters such as cone gas flow, gas desolvation temperature and sampling cone voltage were adapted to obtain the highest possible peak resolution for the standards. The best results were obtained under the conditions as shown below.

For untargeted analysis, fractions obtained after each extraction process were suspended in 200 µL of $CHCl_3$:MeOH (1:1, v/v). Afterwards, for the relative quantification study, those samples were spiked with a IS stock solution, to a final concentration of 250 ppm in $CHCl_3$:MeOH (1:1, v/v) per IS-lipid class. In order to obtain reliable and high quality data acquisition, IS stock solution was also used as quality controls throughout the analysis [40].

Afterwards, the samples were analysed on an Ultra Performance Convergence Chromatography system (a SFC system) (ACQUITY UPC^2, Waters, Milford, MA, USA) with an ACQUITY UPC^2 Torus DIOL column (130Å, 1.7 µm, 3 mm x 100 mm, Waters, MA, USA) coupled to a column guard Torus DIOL (130Å, 1.7 µm, 2.1 mm x 5 mm, Waters, MA, USA). The temperature of the column and the active back

pressure regulator (ABPR) were set at 50°C and 110 bar respectively. The injection volume was 1 µL and the CO_2 flow rate was set at 1.6 mL/min. Methanol containing 10 mM of ammonium formate was used as modifier in the following proportions, 0 min, 2%; 2 min, 2%; 6 min, 27%; 7 min, 27%; 7.5 min, 45%; 10 min 2%. The injector needle was washed after each injection with the same mixture used to dissolve the samples, $CHCl_3$:MeOH (1:1, v/v).

The SFC system was coupled via a flow splitter (ACQUITY UPC^2 splitter, Waters) to a Xevo-G2 quadrupole time-of-flight MS (QTOF-MS; Waters, MA, USA). Two T-pieces (Waters, MA, USA) were placed for backpressure control and an infusion of MeOH with 10 mM ammonium formate (0.25 mL/min) was used as makeup liquid. The approximate split ratio was estimated to 1:100 [41]. The capillary voltage was set at 3.0 kV and 2.5 kV for positive and negative modes respectively, the sampling cone voltage at 40 V, the source temperature at 120 °C, the drying temperature at 200 °C, the cone gas flow at 100 L/h and the drying gas flow at 800 L/h. A collision energy ramp of 15 to 45 eV in ESI+ and ESI- with a scanning range of *m/z* 150−1000. The resolution of the MS was tracked during the analysis and found greater than 20000 for the mass range between *m/z* 500 and 900. All analyses were performed with leucine-enkephalin (1500 ng/mL, 5 µL/min) in the lockspray, monitored at 0.1 Hz and averaging over 10 scans. Data was acquired in MS^E mode and processed using MassLynx v4.1 (Waters, MA, USA).

Extraction blanks and samples were randomly injected in one batch. Quality controls were strategically inserted throughout the sample list.

2.5. Sample analysis

2.5.1. Gravimetric analysis of lipid extracts

The initial dry tissue (prior to extraction) and the dry extracts were weighed in a 5-decimal place balance. Extraction yields were expressed as µg of lipid extract per mg of initial weight. Results from gravimetric analysis were used to study the repeatability of the six extraction methods.

2.5.2. Identification of lipids

Chromatograms, MS and MS/MS spectra of the analytes were used for the identification of lipid classes and their species. The confidence in metabolite identification corresponded to level 1, following the annotation recommended by Viant, et al. (2017) [42], because two orthogonal analytical techniques were applied to both standards and analytes under interest, i.e. accurate MS data with retention time as well as accurate MS data and fragmentation pattern.

Lipid compounds were classified in a specific lipid class based on retention times of the lipid standards followed by their accurate mass (*m/z*) signals by formula recognition. Lipid species were identified by MS spectra and MS/MS fragment ion recognition and proposed analytes compare with published mass data.

Lipid nomenclature in this work is based on the work published by Liebisch et al. [43], which is in line with the recommendation of the Lipid MAP consortium.

2.5.3. Relative quantification of lipids by targeted analysis of the extracts

Deuterated triacylglycerides and phosphatidylcholines were selected as internal standards for relative quantification, as they are expected to be found in human and animal samples. Glycerophospholipids are major components in cell membranes (plasma and organelle) and enable the communication between the exterior and interior spaces by forming a semi-permeable barrier [44]. These compounds, together with cholesterol, maintain the integrity of the membrane. Cholesterol also gives fluidity to the membrane; it is positioned between glycerophospholipids and avoids extreme situations such as too firm or too fluid bilayers [45]. On the contrary, triacylglycerols are not forming part of the cellular membrane but they can be present as part of the lipoproteins or as lipid droplets surrounded by glycerophospholipids in the cytoplasm [46] as well as in the extracellular matrix of the sub-endothelial layer of the cell wall (intima layer) [47]. Moreover, other deuterated internal standards, i.e. monoacylglicerols, sphingomyelins and fatty acids, were chosen for relative quantification of their respective lipid classes. Monoisotopic ions for deuterated triacylglicerol, monoacylglicerol, phosphatidylcholine, sphingomyelin and fatty acid were respectively, *m/z* 829.7991 ($[M+NH_4]^+$), *m/z* 363.5830 ($[M+Na]^+$), *m/z* 753.6105 ($[M+H]^+$), *m/z* 738.6528 ($[M+H]^+$) and *m/z* 314.5239 ($[M-H]^-$).

To allow a reliable comparison between extraction methods, the area obtained from the monoisotopic ion per identified lipid specie was normalized according to the area of their respective IS monoisotopic ion. Therefore, the areas of identified triacylglicerides, monoacylglicerides, phosphatidylcholines, sphingomyelins and fatty acids species were firstly normalized using their respective IS-species areas, resulting in "normalized area" (NA). Afterwards, NAs were corrected following the exact initial weight per sample after tissue drying, since tissue pieces were unique in terms of sample weight. Lipid content was expressed as normalized lipid specie area per mg of dried pulmonary artery (NA/mg tissue).

Moreover, possible matrix effects resulting from having performed extractions using different procedures were also evaluated. Matrix effect could result decisive, when comparing several extraction methods, to

select the most suitable method for lipid extraction from pulmonary arteries. Matrix effect was calculated as follow (n=6):

$$Matrix\ effect = 100 \times \frac{Area\ of\ the\ IS\ per\ lipid\ class\ in\ samples}{Area\ of\ the\ IS\ per\ lipid\ class\ in\ CHCl_3{:}MeOH\ (1{:}1, v/v)}$$

2.5.4. Statistical analysis

Dixon´s Q test and one-way ANOVA test (at 95% of confidence level) were respectively used for identification of outliers and variance analysis. Both tests were performed at several phases of the experimental procedure. Moreover, one-way ANOVA with post-hoc Tukey HSD (Honestly-significant-difference) test (α=0.05) was used for the study of the relative quantification of lipid classes and species. Statistical analyses were performed using the GraphPad Quick Calculations software (La Jolla, CA, USA).

3. Results and Discussion

The six extraction methods MA-MF (methods specification showed in Table 1 and Appendix section) were evaluated based on gravimetric results as well as on the identification and relative quantification of lipid classes by UHPSFC/QTOF-MS/MS. Time-consumption, repeatability, type of solvents used (chlorinated vs non-chlorinated) were also considered in order to propose an optimal methodology for a reliable and efficient extraction of lipids from porcine pulmonary arteries.

3.1. Evaluation of extraction methods based on gravimetric analysis

The extraction methods MA-C, E and F showed an acceptable repeatability since no statistical differences ($p<0.05$, 95% of confidence level) were observed between repetitions. MD, however, showed much lower interday precision (Fig. 1). For this reason, it was considered unsuitable for the lipid extraction from porcine pulmonary artery and MD extracts were discarded from further analysis.

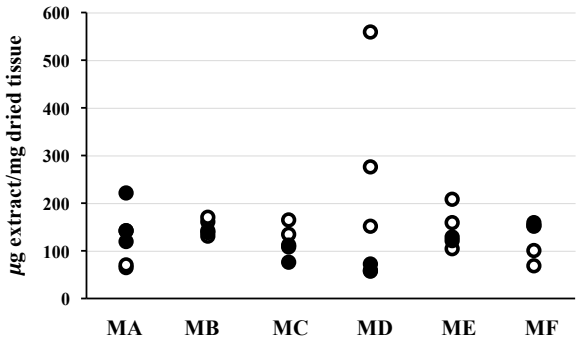

Fig. 1. Extraction yields (μg/mg dried tissue) obtained from each extraction method in the 1st and 2nd batch, solid and empty circles respectively (number of replicates, n=6). MA-MF correspond to the six different

methods used for lipid extraction from porcine pulmonary arteries; specifications are described in the Appendix section and summarized in Table 1.

One-way ANOVA analysis (α= 0.05) showed no significant differences ($p>0.07$) between the gravimetric results obtained with MA-C, ME and MF. Average values varied from 119.11 to 148.16 µg extract/mg dried tissue corresponding to MC and MB respectively. Although there is no clear effect of the extraction method on the total lipid recovery, each method may result in a particular extract composition due to different lipid partitioning into the extraction solvents used [35]. Therefore, lipid identification and relative quantification of the MA-C, ME and MF extracts were performed by UHPSFC/QTOF-MS/MS.

3.2. Evaluation of extraction methods based on results from UHPSFC/QTOF-MS/MS

Extraction methods MA-C, ME and MF were evaluated in terms of class and relative amount of lipids present in the extracts.

3.2.1. Identification and separation of lipid classes

Based on their biosynthesis and, in consequence, their chemical nature, long-chain lipids can be grouped in four lipid classes i.e. free FAs, glycerolipids (including monoacylglycerols, MGs, and triacylglycerols, TGs), glycerophospholipids and sphingolipids (sphingomyelins, SMs) [48]. The adapted chromatographic method was able to separate lipids according to class and polarity, from less polar lipids such as TGs to more polar lipids such as SMs. As previously detailed, the identification of analytes corresponded to the first level of confidence [42]; since orthogonal data from lipid standards as well as unknown analytes was studied. Lipids specie were identified by their retention times, accurate MS spectra, fragmentation pattern by MS/MS spectra and comparison with the reported lipids specie commonly found in biological samples. In most extracts, the mass measurement error for all the identified lipids was lower than 10 mDa when comparing with the corresponding theoretical m/z ions. The same lipid classes were found in the extracts from all tested extraction methods. Most lipid classes were identified in positive mode, i.e. TGs ($[M+NH_4]^+$), MGs ([M+Na]+), PCs ([M+H]+) and SMs ([M+NH4]+), as well as cholesterol [M-H2O+H]+. However, FAs ([M-H]-) were identified in negative mode. Lipids were separated according to class, as can be observed in Fig. 2.

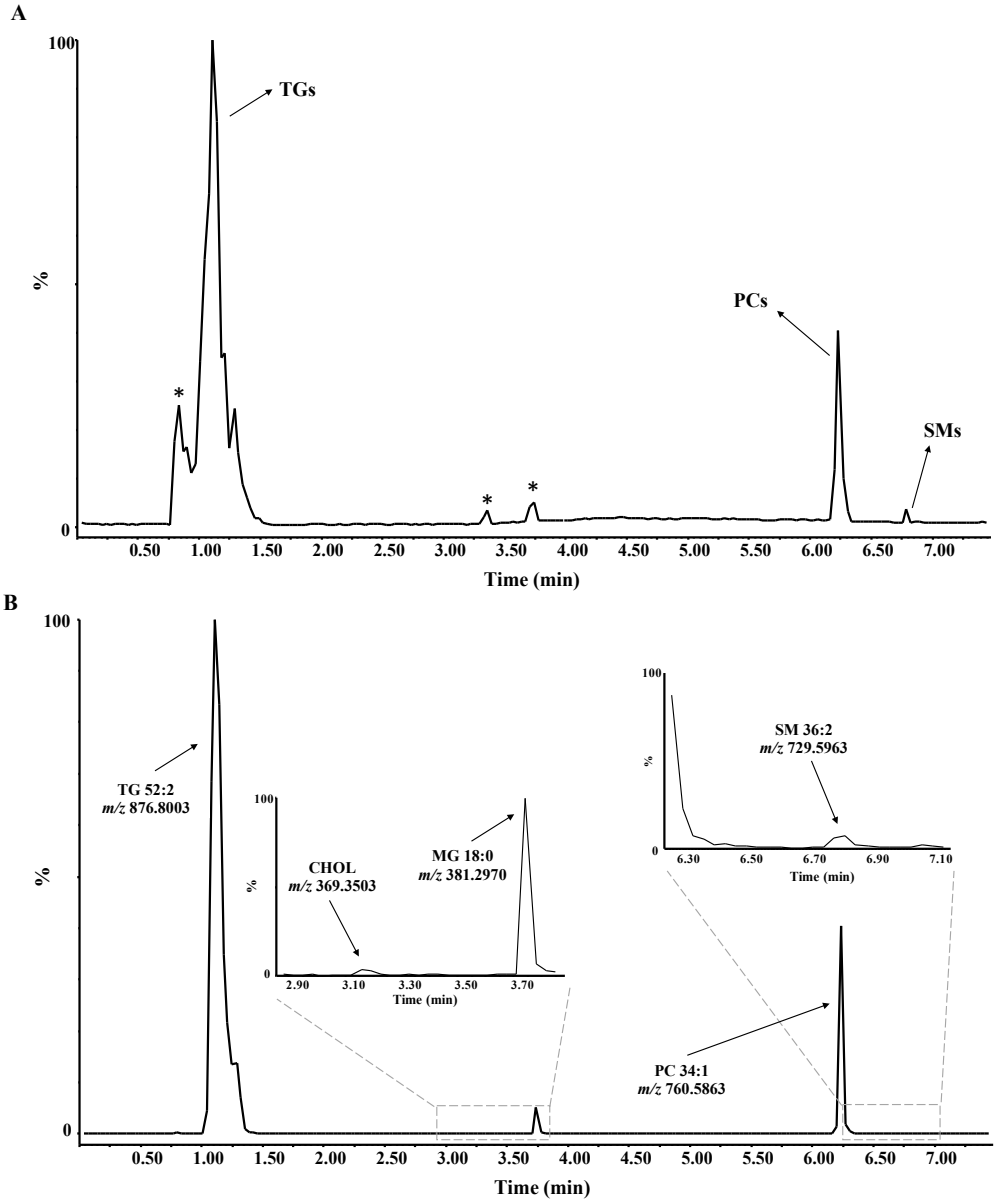

Fig. 2. Example of a base peak intensity UHPSFC/QTOF-MS chromatogram (ESI+) (A) and selected representative ions of the lipid classes (B) extracted from porcine pulmonary arteries with method A.*Peaks corresponding to unidentified compounds present in extraction controls.

Retention times of identified compounds are shown in Table 2. Time values displayed are an average of the results from all extraction methods. TGs eluted from 0.80-1.42 min followed by cholesterol and MGs with retention times of 3.16 and 3.71-3.78 min respectively. Then, PCs and SMs eluted at 6.16-6.31 min and 6.75-6.79 min respectively. For free FAs, a wider range of retention times was observed, from 1.66 to 3.18 min. As expected, retention times of the compounds within a lipid class were influenced by the fatty acyl chain length and the saturation grade. Longer retention times were observed for longer fatty acyl chains

and higher number of double bonds in TGs, free FAs and MGs, with the exception of TG 50:3. On the contrary, for more polar lipid classes such as PCs and SMs, retention times decreased with the number of carbons of the fatty acyl chains, while increased with number of double bonds (as for lipids of lower polarity). In addition, it was observed that the number of double bonds in the PC class affected the retention time more than the number of carbons. It was possible to separate a saturated from an unsaturated PC with the same number of carbons (i.e. PC 34:0 vs 34:2), but not to reach a successful separation of PCs with two carbons of difference and same number of double bonds (i.e. PC 36:2 vs 34:2) (SI Fig. S1). However, in the case of TGs, it seemed that both structural features positively contributed to the chromatographic separation.

MGs were subjected to an in-source fragmentation during the analytical process. The phenomenon was revealed by the presence of FA chains at the same retention time as the MGs (3.71-3.78 min) but in the negative mode spectra (SI Fig. S2). MG 16:0, MG 18:0 and MG 18:1 were fragmented into FA 16:0, FA 18:0 and FA 18:1 respectively with a neutral loss of 74.0368 mDa in all cases. This smaller fragment may be attributed to the glycerol molecule ($C_3H_6O_2$) according to calculations performed by the Thermo Scientific Xcalibur software (Thermo Scientific, Massachusetts, USA), with an accuracy of +0.02 mDa. An example of such phenomenon was described by Murphy et al. [48], who observed the appearance of FA signals from the glycerolipid class when an electron ionization source was used. Also, Gao et al. [49] reported that MGs and diacylglycerides yield a very low abundance due to their fragmentation in the ion source when cone voltages higher than 100 or 120 V respectively were used. In our work, the molecular integrity of MGs was affected by the collision energy ramp (15-45 eV) set for data acquisition in MS^E mode. This is a relevant phenomenon to consider for the relative quantification of MGs.

The same lipid species per class were found for all extracts. Therefore, no relevant differences were found in the extraction capability of each method in qualitative terms.

Table 2. Identified lipids in porcine pulmonary artery using UHPSFC/QTOF-MS. Experimental and theoretical data for $^{1}[M+NH_4]^+$, $^{2}[M-H]^-$, $^{3}[M-H_2O+H]^+$, $^{4}[M+Na]^+$ and $^{5}[M+H]^+$ are shown. *Most intense peaks inside lipid class.

Retention time (min)	Lipid	*m/z* experimental	*m/z* theoretical
Triacylglycerols (TGs)[1]			
0.80	TG 48:0	824.7692	824.7702
0.83	TG 48:1	822.7558	822.7545
0.87	TG 48:2	820.7424	820.7389
0.9	*TG 50:1	850.7871	850.7858
1.04	TG 50:2	848.7720	848.7702
1.04	TG 52:0	880.8281	880.8328
1.04	*TG 52:1	878.8182	878.813
1.11	TG 50:3	846.7593	846.7545
1.11	*TG 52:2	876.8036	876.8015
1.21	TG 52:3	874.7880	874.7806
1.25	TG 54:2	904.8335	904.8297
1.29	*TG 54:3	902.8193	902.8171
1.39	TG 54:4	900.8039	900.8015
1.42	TG 54:5	898.7832	898.7858
Fatty acids (FAs)[2]			
1.66	FA 14:0	227.1994	227.1980
1.69	FA 15:0	241.2180	241.2192
1.79	*FA 16:0	255.2311	255.2324
1.90	*FA 16:1	253.2176	253.2192
1.96	*FA 18:0	286.2624	283.2605
2.06	*FA 18:1	281.2467	281.2494
2.20	FA 18:2	279.2313	279.2307
2.20	FA 20:1	309.2799	309.2813
2.37	FA 18:3	277.2197	277.2194
2.37	FA 20:2	307.2632	307.2638
2.47	FA 24:0	367.3584	367.3604
2.50	FA 20:3	305.2471	305.2454
2.88	FA 22:4	331.2650	331.2644
3.01	FA 22:5	329.2470	329.2477
3.18	FA 22:6	327.2293	327.2266
Free cholesterol[3]			
3.16	5-cholesten-3ß-ol	369.3515	369.3503

3.2.2. *Extraction efficiency by relative quantification of lipids*

While all extraction methods (except MD) gave similar results based on gravimetric analysis, differences between MA-C, ME and MF were found based on the relative quantification of TGs, MGs, PCs, SMs and FAs analysis.

As expected, a poor ionization efficiency of cholesterol was achieved using ESI [50], so this compound was not considered in the relative quantification study. Moreover, because of the observed in-source fragmentation, the relative quantification for MGs was approached through the study of their respective FA areas ($[M-H]^-$), after normalizing it to the FA area obtained from MG-IS fragmentation (*m/z* 288.2909 ($[M-H]^-$)).

Regardless the extraction method, TGs showed the most intense signals from those lipids found in pulmonary arteries (Fig. 2A). MA resulted in the highest values for almost all TGs species, being MF the less efficient method for such lipid class (Fig. 3).

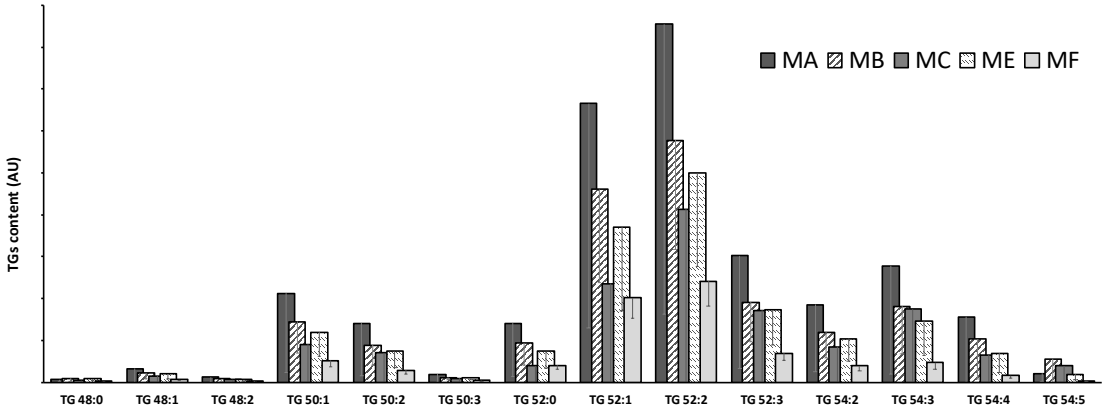

Fig. 3. Relative TGs content per lipid specie extracted by MA-C, ME and MF expressed as NA (normalized area)/mg dried porcine pulmonary arteries. Data are expressed as means ± SD; n=6 for MA-C and n=5 for ME-F.

No statistical differences were observed between extraction methods ($p>0.05$, $\alpha= 0.95$), most probably because of the biological variability. Moreover, a notably high contribution to the lipid class was observed by TG 52:1 and TG 52:2 for all methods. Such contributions varied from 21-6.6% for TG 52:1 and 28-7.9% for TG 52:2 of the total TGs extracted (considering MA-C, ME and MF). These dissimilar proportions obtained for the two most abundant TGs per extraction method, suggested a varied selectivity in the extraction of each TG specie. Therefore, MA seemed to be the most suitable method, showing the highest total content of TG with the highest representation of the most abundant TG species (TG 52:1 and TG 52:2). However, because of the lack of statistical differences, none of the methods was discarded based on TGs results.

Similar tendency was observed for PCs compared to TGs, i.e. highest PCs content for most of the PCs species was observed in MA. No statistical differences were found between MA and the other methods, except for MF in which significantly lower content of PCs was observed ($p<0.05$, $\alpha= 0.95$). Similar results were found between MC and MF. The most abundant species corresponded to PC 34:1, followed by PC32:0, PC 36:2 and PC 38:4, with a cumulative contribution of 73.0-78.0% with respect to the total PCs identified per method (Fig. 4). Unlike with the TGs results, all methods showed similar selectivity for all PCs species.

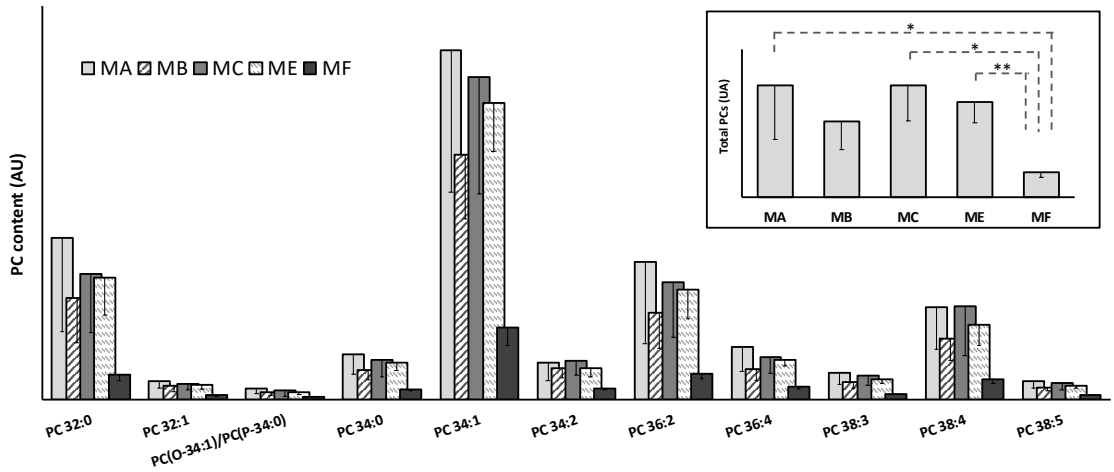

Fig. 4. Relative PCs content per lipid specie extracted by MA-C, ME and MF expressed as NA (normalized area)/mg dried porcine pulmonary arteries. Total PCs content extracted per method (framed histogram) was also detailed. * $p<0.05$ between the corresponding data pairs obtained from the analysis of the variance (ANOVA), followed by Tukey´s test. * * $p<0.05$ between the corresponding data pairs for the most representative PC specie (PC 34:1) obtained from the analysis of the variance (ANOVA), followed by Tukey´s test. Data are expressed as means ± SD; n=6 for MA-C and n=5 for ME-F.

According to PCs results, MF was discarded as a candidate for the most suitable method. The main difference between MF and MA is the lower polarity of the solvent mixture used in MF, that is (CH_2Cl_2:MeOH, 3:1) vs CH_2Cl_2:MeOH (2:1) in MA. Significant differences with MC and ME can be the result of different solvent mixtures, longer extraction times and a two-cycle extraction.

Since most abundant lipid classes, TGs and PCs did not enable a reliable discrimination between extraction methods MA, MB, MC and ME, SMs and MGs were also analyzed. Within the three identified SMs species, SM 34:1 was the most abundant, resulting in a 67% of total SMs content for all methods. Extraction methods seemed to show similar selectivity for the three identified SMs species. MC yielded

the highest content for all SMs species although no statistically differences were observed ($p>0.05$, $\alpha= 0.95$) when comparing with MA, MB and ME (SI Fig. S3). However, the total amount of MGs in MA extracts, indirectly analyzed as FAs derivatives from MGs because of the in-source fragmentation, was significantly higher than in MB and MC ($p<0.05$, $\alpha= 0.95$), as it is shown in Fig. 5. These results suggested that the acidic conditions of MB led to lower extraction efficiency of MGs from porcine pulmonary arteries. Therefore, MB was discarded as a method candidate due to the notably lower content of total MGs compared to MA (Fig. 5). The non-chlorinated extraction method MC also resulted in a lower content of MGs compared to MA ($p<0.05$, $\alpha= 0.95$) so, MC was also excluded for further analysis. Moreover, the MGs statistical results found in MC and MF compared to MA, could point to extraction time as the responsible parameter for an efficient extraction of MGs in MTBE-based methods (see Table 1).

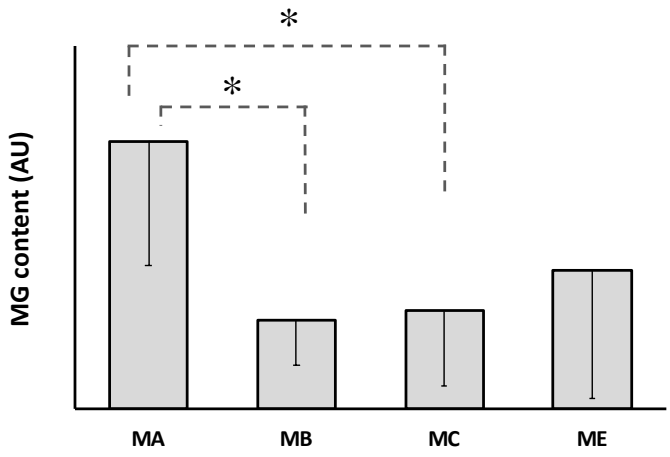

Fig. 5. Content of FAs species from MGs in-source fragmentation in MA, MB and ME expressed as relative area per mg of dried tissue. * $p<0.05$ between the corresponding data pairs obtained from the analysis of the variance (ANOVA), followed by Tukey´s test. Data are expressed as means ± SD; n=6 for MA-C and n=5 for ME.

According to the results from the lipid classes identified in positive mode, MA showed the best results in quantitative terms. ME showed no statistically differences compared to MA however, a lower content trend for any of the analyzed lipid classes was observed respect to MA. Therefore, the analysis of lipid classes in negative mode was performed to evaluate the efficiency of MA and ME to extract fatty acids (FAs).

The total content of FAs present in ME extracts was notably higher ($p<0.01$, $\alpha= 0.95$) than with MA, mostly due to the significantly higher amounts ($p<0.01$, $\alpha= 0.95$) of two saturated FAs extracted with ME, FA 16:0 and FA 18:0 (Fig. 6, Table 3).

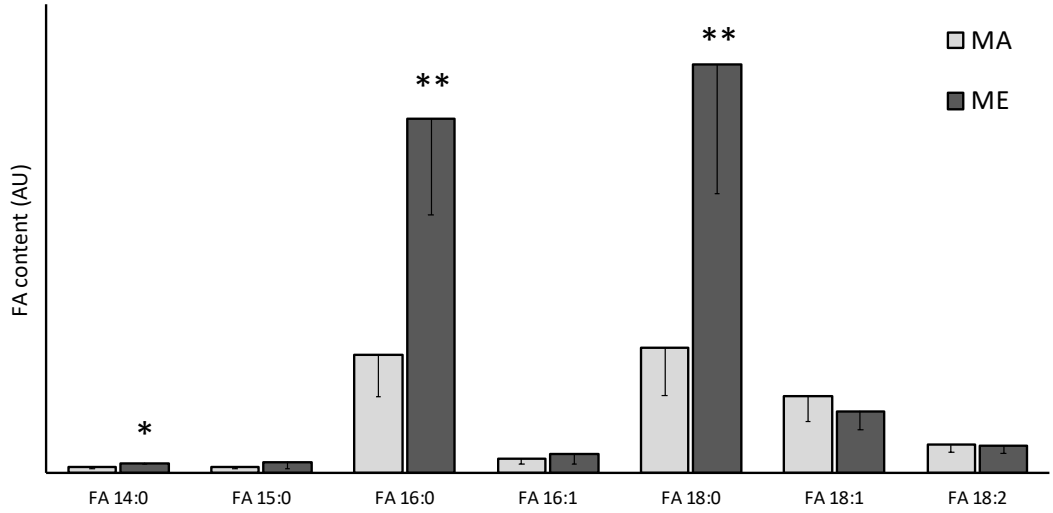

Fig. 6. Relative quantification of the FAs species showing a contribution to the total FAs content higher than 1% in MA and ME expressed as normalized area (NA) per mg of dried tissue. Data are expressed as means ± SD; n=6 for MA and n=5 for ME. * $p<0.05$, ** $p<0.01$ between the corresponding methods obtained from the analysis of the variance (ANOVA), followed by Tukey´s test.

Table 3. Content of FAs species expressed as normalized area/mg dried tissue and their contribution to the total FAs content (%) extracted from porcine pulmonary arteries by MA and ME. Showed values represent the averaged of the replicates per method. *Data statistically different, $p<0.01$ $\alpha=0.95$, in comparison with those from the same row.

Class of FA	**NA/mg dried tissue**		**Contribution to total FAs (%)**	
	MA	**ME**	**MA**	**ME**
Saturated	99.30	302.46*	63.54	85.58
Monounsaturated	36.11	32.02	23.10	9.06
Polyunsaturated	20.85	18.91	13.34	5.35
Total	156.27	353.40*	100	100

The cumulative percentage of FA 16:0 and FA 18:0 was 83% and 60% for ME and MA respectively, followed by FA18:1 (6.7% ME, 18% MA), FA 18:2 (3% ME, 7% MA), FA 16:1(2% ME, 3% MA) FA

15:0 and FA 14:0 (both less than 2% for MA and ME). The rest of the identified FAs contributed to the total FAs content in less than 1%.

The contribution of the FAs class based on the acyl chain saturation to the total FAs content was evaluated, and results suggested that the saturated FAs could be responsible of the higher total FAs content in ME samples, compared to MA ($p<0.01$, $\alpha= 0.95$) (Table 3). Therefore, MTBE seemed to facilitate the extraction of saturated FAs from pulmonary artery more than CH_2Cl_2, which contrasts with the case of lipid extraction from adipose tissue [51], probably due to the dissimilar biological material used.

In order to check the higher FAs extraction rates reported by Jensen et al. [34] due to acidic media (MB), FAs content in MB was also analyzed. Results showed no differences between the FAs total content in MB and MA (121.5 ± 26.6 and 156.2 ± 49.0 NA/mg of dried tissue respectively). Therefore, acid-methanolic environment (MB) had no positive effect on the extraction efficiency for FAs from porcine pulmonary arteries, which contradicts previous published data [34], probably due to the dissimilar biological material used.

3.3. Study of the matrix effect

As an additional information, the effects on the analytical method caused by other potential components in lipid extracts were evaluated by the matrix effect approach. It was calculated as a percentage of the ratio IS in sample vs IS in $CHCl_3$:MeOH (1:1, v/v) for each lipid class. No significant differences between ISs signals were found for most of the methods and lipid classes. Matrix effect values close to 100% were obtained, except for a significant suppression of TGs in MA, SMs in MF and FAs in MC ($p<0.05$, $\alpha= 0.95$) (SI Table S1).

These results suggested that, whenever ISs are not available, a standard addition method would be needed for the absolute quantification of those mentioned lipid classes extracted with some of the methods above described. In example, the TGs content in MA could be underestimated if other method for quantitative analysis is selected.

3.4. Summary of the evaluation of extraction methods

All the methods that performed with acceptable repeatability in the gravimetric analysis showed similar lipid profiles. However, statistically differences in the total lipid class and lipid species content were found between the extraction method. Along the study, less efficient extraction methods in terms of sample lipid content were discarded according to their respective statistic results. Finally, two methods,

MA and ME, were found suitable for the extraction of lipids from pulmonary artery. A tendency for a higher extracted lipid contents was noticed for TGs, PCs and MGs in MA compared to ME however, no significant differences were observed. ME led to better extraction yields of total FAs (around a 50% more), with a high contribution of saturated FAs species. Other aspects may be considered in order to choose between MA and ME. In example, the dichloromethane/methanol/water-based method (MA) needed lower extraction time than the MTBE-based method (ME), 45 min versus 1490 min respectively (see Table 1). Time consumption becomes especially relevant for studies based on biological samples, since a high number of replicates are needed, and experimental total time increase notably. Moreover, large sample batches could negatively affect to the quality of the results, because of the operator fatigue in long times of sample preparation. However, it is also important to consider the fact that the recovered phase in ME is on top of the mixture, while at the bottom in MA, which makes it easier to handle and avoid loses. In addition, MA uses chlorinated solvents which are absent in the ME method, making of ME a more environmentally adapted methodology.

4. Conclusion

In this paper, the optimal methodology for the extraction and analysis of lipid classes from porcine pulmonary arteries has been discussed. The chlorinated- and MTBE-based methods evaluated for the extraction of lipids in this work resulted in extracts with similar lipid chromatographic profiles. The elution order of lipids was achieved according to polarity of lipid class. Lipid classes and lipid species were relatively quantified by UHPSFC/QTOF-MS and –MS/MS after their signal normalization using one deuterated internal standard per lipid class. Triacyclglycerols and phosphatidylcholines were the lipid classes that showed the most intense mass spectrometry signals, followed by sphingomyelins and monoacylglycerols in positive mode. Cholesterol was discarded for relative quantification because of its trivial contribution to the total lipid fractions, most probably due to the bad ionization using ESI. In addition, the extraction method affected the fragmentation of monoacylglycerols in the ion source, which was relevant for their relative quantification. The results obtained from the relative quantification of lipids showed that the MTBE-based method (ME) with the longest extraction time (24 h of incubation) gave one of the highest yields and was the most reliable method for lipid extraction from porcine pulmonary arteries. However, a dichloromethane-based extraction method (MA) was the most efficient one, the one that consumes the least time as well as the more suitable for a global analysis of lipids from pulmonary

arteries. No matrix effect was noticed for ME but a suppression in triacyclglycerols signal was detected in MA, which gives useful information for further absolute quantification studies.

New knowledge about lipid composition of porcine pulmonary arteries has been acquired. An extraction method has been proposed that is of relevance for further lipidomic studies in blood vessels. These results are crucial for the development of selective extraction processes aimed to delipidate arterial tissue without damaging the extracellular matrix. Such a process is the focus of our current research with the ambition to improve tissue quality for transplantations.

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Appendix

Detailed description of extraction methods

1. *Method A (MA)*

Method A corresponded to a dichloromethane/methanol/water system based on a combination of Folch et al. [11] and Cífková et al. [29] methods with some modifications, i.e. the substitution of chloroform by dichloromethane. CH_2Cl_2 was suggested a healthier and more secure solvent in comparison with $CHCl_3$ [30], and it was pointed out as equally effective for total lipid extraction and even more effective for the extraction of FAs [30-32]. Moreover, the higher polarity of CH_2Cl_2 with respect to $CHCl_3$ could enhance the extraction of polar lipids like glycerophospholipids.

Briefly, 5 mg of dried tissue was mixed with 600 µl of CH_2Cl_2:MeOH (2:1, v/v) and homogenized for 10 min (2 min per cycle) at 25 Hz in a Qiagen TissueLyser (Qiagen GmbH, Hilden, Germany) in presence of stainless steel beads. Then, the samples were vigorously shaken for 15 min at room temperature in a VX-2500 Multi-tube Vortexer (VWR, Fontenay-sous-bois, France) and immediately centrifuged for 10 min at 12.000 rpm and 4 °C in a 5424R Eppendorf centrifuge (Hørsholm, Denmark). Supernatant was collected and submitted again to centrifugation for 5 min at 13.000 rpm and 4 °C to remove undesirable particles in

suspension, before adding 120 µl of 0.9% wt NaCl aqueous solution. Then, the mixture was vigorously shaken for 10 seconds at room temperature (RT) and centrifuged once more at RT and 3000 rpm for 5 min, to compact the interfacial layer and enhance phase separation. The chlorinated fraction (lower phase) containing the lipids was recovered for further analysis.

2. *Method B (MB)*

MB differs from MA in that pure MeOH was replaced by 0.1 M HCl in MeOH. Acidic conditions were expected to enhance the partitioning of acid lipids i.e. glycerophospholipids into the chlorinated phase, by suppressing ionization of polyphosphoinositide phosphate groups [33]. The recovery of total FAs and polyunsaturated FAs could also be enhanced under such conditions [34].

3. *Method C (MC)*

The extraction method C was a combination of those reported by Abbott et al. [17] and Matyash et al. [16]. Briefly, 5 mg of dried tissue were mixed with 600 µl of pure MeOH and homogenized for 10 min (2 min per cycle) at 25 Hz in a Qiagen TissueLyser (Qiagen GmbH, Hilden, Germany). Then, additional 324 µl of MeOH and 3.08 ml of methyl-*tert*-butyl ether (final ratio MTBE/MeOH 10:3, v/v) were added to the extraction tubes and gently shaken for 1h at 4 °C using a Roto-Shake Genie from Scientific Industries, Inc (Bohemia, NY, USA). After the incubation time, 760 µl of 0.15 M ammonium acetate was incorporated to the mixture before a 10 min of rotary agitation at RT. Afterwards, the mixture was centrifuged for 10 min at 3000 rpm and 4 °C in a 4K15-centrifuge from Sigma laboratory (Santa Fe, NM, USA). Once the upper phase was recovered, the lower fraction was re-extracted by adding 1.233 ml of MTBE:MeOH:0.15 M ammonium acetate (20:6:5, v/v/v) mixture, followed by the above described agitation and centrifugation processes. Finally, both upper phases were pooled together for further analysis.

4. *Method D (MD)*

This method resulted from a slightly-modified MC in which pure MeOH was replaced by 75% MeOH in water, following the procedure described by Chen et al. [35]. It is expected that higher amounts of water in the solvent may lead to a more efficient extraction of glycerophospholipids.

5. *Method E (ME)*

Method E was similar to MC except for the incubation time. In ME, the tissues were in contact with the extraction solvents for longer times, 24 h instead 1 h. Method MC was developed for brain tissue but the structural proteins in arteries may slow down desorption and diffusion of lipids from the biological matrix

into the bulk solvent. An increased time was therefore aimed to evaluate whether there was a kinetic limitation in the extraction of lipids from arteries.

6. *Method F (MF)*

MF was an adaptation of the method previously reported by Want et al. [36]. It was based in a two-step process allowing the partitioning of more and less polar compounds in two individual fractions.

Summarizing, dried tissue (5 mg approximately) was mixed with 600 µl of MeOH:H_2O (1:1, v/v) and submitted to tissue disruption for 10 min (2 min per cycle) at 25 Hz in a TissueLyser from Qiagen GmbH (Hilden, Germany), followed by centrifugation at 10.000 rpm and 4 °C for 10 min in a 5424R Eppendorf centrifuge (Hørsholm, Denmark). The supernatant was recovered, N_2 dried and kept at -80 °C until use (it was considered as MF-polar fraction). Then, 640 µl of cold CH_2Cl_2:MeOH (3:1, v/v) was mixed with the solid precipitate and submitted again to the homogenization process (same conditions described above). The supernatant obtained after centrifugation (at 11.000 rpm and 4 °C for 10 min) was also recovered for analysis (it was considered as MF-nonpolar fraction).

Bibliography

[1] World Health Organization, Cardiovascular diseases (CVDs) Fact sheets for the http://www.who.int/mediacentre/factsheets/fs317/en/, 2017 (accessed 09 October 2017).

[2] P. Lancellotti, P. Pibarot, J. Chambers, T. Edvardsen, V. Delgado, R. Dulgheru, M. Pepi, B. Cosyns, M.R. Dweck, M. Garbi, J. Magne, K. Nieman, R. Rosenhek, A. Bernard, J. Lowenstein, M.L.C. Vieira, A. Rabischoffsky, R.H. Vyhmeister, X. Zhou, Y. Zhang, J.-L. Zamorano, G. Habib, Recommendations for the imaging assessment of prosthetic heart valves: a report from the European Association of Cardiovascular Imaging endorsed by the Chinese Society of Echocardiography, the Inter-American Society of Echocardiography, and the Brazilian Department of Cardiovascular Imaging†, Eur. Heart J. - Cardiovascular Imaging, 17 (2016) 589-590.

[3] J.M. Forbess, Conduit selection for right ventricular outflow tract reconstruction: contemporary options and outcomes, Semin. Thorac. Cardiovasc. Surg. Pediatr. Card. Surg. Annu., 7 (2004) 115-124.

[4] O. Reinhartz, V.M. Reddy, E. Petrossian, M. MacDonald, J.J. Lamberti, S.J. Roth, G.E. Wright, S.B. Perry, S. Suleman, F.L. Hanley, Homograft valved right ventricle to pulmonary artery conduit as a modification of the norwood procedure, Circulation, 114 (2006) I-594-I-599.

[5] J.L. Balestrini, L.E. Niklason, Extracellular matrix as a driver for lung regeneration, Ann. Biomed. Eng., 43 (2015) 568-576.

[6] L.F. Tapias, H.C. Ott, Decellularized scaffolds as a platform for bioengineered organs, Curr. Opin. Organ Tran., 19 (2014) 145-152.

[7] P. Masson, A.C. Alves, T.M.D. Ebbels, J.K. Nicholson, E.J. Want, Optimization and evaluation of metabolite extraction protocols for untargeted metabolic profiling of liver samples by UPLC-MS, Anal. Chem., 82 (2010) 7779-7786.

[8] S. Meier, S.A. Mjøs, H. Joensen, O. Grahl-Nielsen, Validation of a one-step extraction/methylation method for determination of fatty acids and cholesterol in marine tissues, J. Chromatogr. A, 1104 (2006) 291-298.

[9] A. De Tonnac, S. Karim-Luisset, J. Mourot, Effect of different dietary linseed sources on fatty acid composition in pig tissues, Livest. Sci., 203 (2017) 124-131.

[10] K.i. Ichihara, K. Yoneda, A. Takahashi, N. Hoshino, M. Matsuda, Improved methods for the fatty acid analysis of blood lipid classes, Lipids, 46 (2011) 297-306.

[11] J. Folch, M. Lees, G.H.S. Stanley, A simple method for the isolation and purification of total lipides from animal tissues, J. Biol. Chem., 226 (1957) 497-509.

[12] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Phys., 37 (1959) 911-917.

[13] W. Zou, Total Lipid Extraction, in, Nature Publishing Group, 2011.

[14] M. Kosicek, S. Kirsch, R. Bene, Z. Trkanjec, M. Titlic, L. Bindila, J. Peter-Katalinic, S. Hecimovic, Nano-HPLC–MS analysis of phospholipids in cerebrospinal fluid of Alzheimer's disease patients—a pilot study, Anal. Bioanal. Chem., 398 (2010) 2929-2937.

[15] M. Kosicek, H. Zetterberg, N. Andreasen, J. Peter-Katalinic, S. Hecimovic, Elevated cerebrospinal fluid sphingomyelin levels in prodromal Alzheimer's disease, Neurosci. Lett., 516 (2012) 302-305.

[16] V. Matyash, G. Liebisch, T.V. Kurzchalia, A. Shevchenko, D. Schwudke, Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics, J. Lipid Res., 49 (2008) 1137-1146.

[17] S.K. Abbott, A.M. Jenner, T.W. Mitchell, S.H.J. Brown, G.M. Halliday, B. Garner, An improved high-throughput lipid extraction method for the analysis of human brain lipids, Lipids, 48 (2013) 307-318.

[18] J. Graessler, D. Schwudke, P.E.H. Schwarz, R. Herzog, A. Shevchenko, S.R. Bornstein, Top-down lipidomics reveals ether lipid deficiency in blood plasma of hypertensive patients, Plos One, 4 (2009) e6261.

[19] M. Murph, T. Tanaka, J. Pang, E. Felix, S. Liu, R. Trost, A.K. Godwin, R. Newman, G. Mills, Liquid chromatography mass spectrometry for quantifying plasma lysophospholipids: potential biomarkers for cancer diagnosis, in: Methods in Enzymology, Academic Press, 2007, pp. 1-25.

[20] Z. Zhao, Y. Xu, An extremely simple method for extraction of lysophospholipids and phospholipids from blood samples, J. Lipid Res., 51 (2010) 652-659.

[21] A. Reis, A. Rudnitskaya, G.J. Blackburn, N.M. Fauzi, A.R. Pitt, C.M. Spickett, A comparison of five lipid extraction solvent systems for lipidomic studies of human LDL, J. Lipid Res., 54 (2013) 1812-1824.

[22] M. Lísa, M. Holčapek, High-throughput and comprehensive lipidomic analysis using ultrahigh-performance supercritical fluid chromatography–mass spectrometry, Anal. Chem., 87 (2015) 7187-7195.

[23] Y. Sato, I. Suzuki, T. Nakamura, F. Bernier, K. Aoshima, Y. Oda, Identification of a new plasma biomarker of Alzheimer's disease using metabolomics technology, J. Lipid Res., 53 (2012) 567-576.

[24] P. Olsson, J. Holmbäck, B. Herslöf, Separation of lipid classes by HPLC on a cyanopropyl column, Lipids, 47 (2012) 93-99.

[25] E. Cífková, M. Holčapek, M. Lísa, Nontargeted lipidomic characterization of porcine organs using hydrophilic interaction liquid chromatography and off-line two-dimensional liquid chromatography–electrospray ionization mass spectrometry, Lipids, 48 (2013) 915-928.

[26] K. Sterz, G. Scherer, J. Ecker, A simple and robust UPLC-SRM/MS method to quantify urinary eicosanoids, J. Lipid Res., 53 (2012) 1026-1036.

[27] O.L. Knittelfelder, B.P. Weberhofer, T.O. Eichmann, S.D. Kohlwein, G.N. Rechberger, A versatile ultra-high performance LC-MS method for lipid profiling, J. Chromatogr. B, 951–952 (2014) 119-128.

[28] T. Yamada, K. Taguchi, T. Bamba, Chapter 14 - Separation of lipids A2 - Poole, Colin F, in: Supercritical Fluid Chromatography, Elsevier, 2017, pp. 419-438.

[29] E. Cífková, M. Holčapek, M. Lísa, M. Ovčačíková, A. Lyčka, F. Lynen, P. Sandra, Nontargeted quantitation of lipid classes using hydrophilic interaction liquid chromatography–electrospray

ionization mass spectrometry with single internal standard and response factor approach, Anal. Chem., 84 (2012) 10064-10070.

[30] E. Cequier-Sánchez, C. Rodríguez, Á.G. Ravelo, R. Zárate, Dichloromethane as a solvent for lipid extraction and assessment of lipid classes and fatty acids from samples of different natures, J. Agr. Food Chem., 56 (2008) 4297-4303.

[31] L. Carlson, Extraction of lipids from human whole serum and lipoproteins and from rat liver tissue with methylene chloride-methanol: a comparison with extraction with chloroform-methanol, Clin. Chim. Acta, 149 (1985) 89-93.

[32] Y. Li, F. Ghasemi Naghdi, S. Garg, T.C. Adarme-Vega, K.J. Thurecht, W.A. Ghafor, S. Tannock, P.M. Schenk, A comparative study: the impact of different lipid extraction methods on current microalgal lipid research, Microb. Cell Fact., 13 (2014) 14.

[33] A. Kielkowska, I. Niewczas, K.E. Anderson, T.N. Durrant, J. Clark, L.R. Stephens, P.T. Hawkins, A new approach to measuring phosphoinositides in cells by mass spectrometry, Adv. Biol. Regul., 54 (2014) 131-141.

[34] S.K. Jensen, Improved bligh and dyer extraction procedure, Lipid Tech., 20 (2008) 280-281.

[35] S. Chen, M. Hoene, J. Li, Y. Li, X. Zhao, H.-U. Häring, E.D. Schleicher, C. Weigert, G. Xu, R. Lehmann, Simultaneous extraction of metabolome and lipidome with methyl tert-butyl ether from a single small tissue sample for ultra-high performance liquid chromatography/mass spectrometry, J. Chromatogr. A, 1298 (2013) 9-16.

[36] E.J. Want, P. Masson, F. Michopoulos, I.D. Wilson, G. Theodoridis, R.S. Plumb, J. Shockcor, N. Loftus, E. Holmes, J.K. Nicholson, Global metabolic profiling of animal and human tissues via UPLC-MS, Nat. Protocols, 8 (2013) 17-32.

[37] A. Demel, M. Wolf, C.F. Poets, A.R. Franz, Effect of different assumptions for brain water content on absolute measures of cerebral oxygenation determined by frequency-domain near-infrared spectroscopy in preterm infants: an observational study, BMC Pediatr., 14 (2014) 206.

[38] R.F. Reinoso, B.A. Telfer, M. Rowland, Tissue water content in rats measured by desiccation, J. Pharmacol. Tox. Met., 38 (1997) 87-92.

[39] Hamimi S Al, Sandahl M, Armeni M, Turner C, Spégel P, Screening of stationary phase selectivities for global lipid profiling by ultrahigh performance supercritical fluid chromatography, J Chromatogr A, 1548 (2018) 76–82.

[40] Broadhurst D, Goodacre R, Reinke SN, Kuligowski J, Wilson ID, Lewis MR, Dunn WB, Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies, Metabolomics, 14 (2018) 72.

[41] Jumaah F, Larsson S, Essén S, Cunico L, Holm C, Turner C, Sandahl M, A rapid method for the separation of vitamin D and its metabolites by ultra-high performance supercritical fluid chromatography-mass spectrometry, J Chromatogr A, 1440 (2016) 191-200.

[42] Viant MR, Kurland IJ, Jones MR, Dunn WB, How close are we to complete annotation of metabolomes? Curr Opin Chem Biol., 36 (2017) 64–9.

[43] G. Liebisch, J. A. Vizcaíno , H. Köfeler , M. Trötzmüller , W. J. Griffiths , G. Schmitz , F. Spener, M. J. O. Wakelam, Shorthand notation for lipid structures derived from mass spectrometry, J. Lipid Res., 54 (2013) 1523-1530.

[44] T.H.M. Da Costa, M.K. Ito, PHOSPHOLIPIDS | Physiology A2 - Caballero, Benjamin, in: Encyclopedia of Food Sciences and Nutrition (Second Edition), Academic Press, Oxford, 2003, pp. 4523-4531.

[45] R.A. Cooper, Influence of increased membrane cholesterol on membrane fluidity and cell function in human red blood cells, J. Supramol. Str., 8 (1978) 413-430.

[46] T.C. Walther, R.V. Farese, Lipid droplets and cellular lipid metabolism, Ann. Rev. Biochem., 81 (2012) 687-714.

[47] K. Öörni, M.O. Pentikäinen, M. Ala-Korpela, P.T. Kovanen, Aggregation, fusion, and vesicle formation of modified low density lipoprotein particles: molecular mechanisms and effects on matrix interactions, J. Lipid Res., 41 (2000) 1703-1714.

[48] R.C. Murphy, P.H. Axelsen, Mass spectrometric analysis of long-chain lipids, Mass Spectrom. Rev., 30 (2011) 579-599.

[49] F. Gao, J. McDaniel, Y.E. Chen, H. Rockwell, D.M. Lynes, Y.-H. Tseng, R. Sarangarajan, R.N. Narain, A.M. Kiebish, Monoacylglycerol analysis using MS/MSALL quadrupole time of flight mass spectrometry, Metabolites, 6 (2016) 25.

[50] 1. Patti GJ, Woo H-K, Yanes O, Shriver L, Thomas D, Uritboonthai W, Apon J, Steenwyk R, Manchester M, Siuzdak G, Detection of Carbohydrates and Steroids by Cation-Enhanced Nanostructure-Initiator Mass Spectrometry (NIMS) for Biofluid Analysis and Tissue Imaging, Anal Chem, 82 (2010) 121–8.

[51] M.A. López-Bascón, M. Calderón-Santiago, J. Sánchez-Ceinos, A. Fernández-Vega, R. Guzmán-Ruiz, J. López-Miranda, M.M. Malagon, F. Priego-Capote, Influence of sample preparation on lipidomics analysis of polar lipids in adipose tissue, Talanta, 177 (2018) 86-93.