Short communication

Tip-tip filtration ameliorates single-phase extraction methods for plasma large-scale lipidomics analysis

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Abstract

We evaluated the performance of three different single-phase extraction methods to be used before untargeted lipidomics analysis by Liquid Chromatography High-Resolution Mass Spectrometry. Lipids were extracted from a pool of healthy human donors' plasma in triplicates and run in both positive and negative ESI. The most satisfactory results were attained using methanol/chloroform (2:1, v/v) mixture. Eventually, we evaluated whether a filtration of the samples could be beneficial to yield cleaner and more mass-friendly extracts. Instead of using syringes, we set up a method we called *tip-tip filtration*, which requires the usage of a filtrating pipette tip. This way of purification led to superior results than the solvent extraction method alone. This additional procedure not only increased reproducibility but also allowed the same lipid coverage. In addition, it permitted to spare time and money, as *tip-tip filtration* is not particularly expensive nor time-consuming and hopefully it may be useful to increase analytical column lifetime.

Keywords

Lipid extraction, mass spectrometry, sample pre-treatment, untargeted lipidomics.

List of abbreviations

BHT, dibutylhydroxytoluene; Car, carnitines; CE, cholesterol esters; Cer, ceramides; CL, cardiolipines; CoQ10, coenzyme Q10; DAG, diacylglycerols; EtherPC, ether-linked phosphatidylcholines; EtherPE, ether-linked phosphatidylethanolamines; EtherTG, ether-linked triacylglycerols; GB3, Globotriaosylceramide; GM3, gangliosides; LacCer, lactosylceramides; LPA, lysophosphatidic acids; LPC, lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; PC, phosphatidylcholines; PC d7, phosphatidylcholine (15:0-18:1) d7; PE, phosphatidylethanolamines; PI, phosphatidylinositoles; OxFA, oxidized fatty acids; OxPC oxidized phosphatidylcholines; OxPE, oxidized phosphatidylethanolamines; SM, sphingomyelins; ST, sterols; ST Sulf, sterol sulfates; Sulf, sulfatides; TAG, triacylgycerols.

1 Introduction

With the development of -*omics* technologies, it is now of general interest to be aware of the challenges regarding the pre-analytical procedures to prepare several biological samples of different origin; particularly, the extraction of lipids is still considered problematic. Lipids are, in fact, biomolecules that have a remarkable complex structure per se and they can combine each other and with different biochemical species, creating even more molecular entities. On the other hand, the human lipidome consists of a very large and complex system as it may even include 100.000 chemical entities, very different from each other, and up to 700 different families of lipids can be found in human plasma in several different concentrations: from millimolar to pico-femtomolar [1,2]. The analysis of the lipidome has gone through many challenges over the years. The first step for the detection of lipids is to perform an efficient separation of the analytes. At first, thin layer chromatography and gas chromatography were the most used separation techniques, but the analytes had to be derivatized on their polar functions, which are not common to all lipids. Eventually, liquid chromatography took place, and it is now commonly coupled to mass spectrometry [3,4]. This represents a new dilemma as it is critical to identify the most accurate detection method. For untargeted analyses, Time of Flight (ToF) mass spectrometers are the most used instruments, as they are capable of distinguishing several analytes with similar structure thanks to a particularly high resolution [5,6]. As the resolution and sensibility of the instruments get sharper, the good performance of the purification of the samples gets more and more crucial. It is evident, then, that the extraction of lipids from human plasma needs to be extremely accurate to have a proper look at the lipidome as a whole, but, nowadays, agreement on a common protocol is far from being reached. Many different single and double phase extraction, such as the Folch and the Bligh & Dyer [7,8], techniques have been proposed, using different percentages of many solvents. Single-phase extraction is of particular interest as it reduces the manipulation of the samples and increases the speed of the entire procedure. The previously mentioned protocols have been modified across the years leading to the generation of new improved single-phase methods, such as the one proposed by Pellegrino et al. [9], to produce cleaner samples and to provide a better extraction of the whole set of lipids, with a better representation of all lipid classes.

Our work aimed to compare three different commonly performed methods for the extraction of lipids from human plasma using different percentages of methanol and chloroform, that still represent the gold standard solvents for the extraction of non-polar compounds: (1) methanol/chloroform (2:1, v/v); (2) methanol/chloroform (1:1, v/v); and (3) methanol/chlorofrom/tert-butyl methyl ether (1.5/1/1, v/v). Furthermore, we evaluated whether an additional filtration of the extract could provide a more accurate and reproducible analysis and help to overcome the typical interferences attained from high-resolution mass spectrometry. In the end, we investigated the advantage of using an internal standard normalization with respect to simple LOWESS normalization.

2 Materials and Methods

2.1 Chemicals and reagents

The chemicals acetonitrile, 2-propanol, methanol, chloroform, tert-butyl methyl ether, formic acid, ammonium acetate, ammonium formate, dibutylhydroxytoluene (BHT), phosphatidylcholine (15:0–18:1) d7 (PC d7) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sterile aerosol pipette tips 1-20 µL, pore size 10 µm (cat. no. 89174-524) were purchased from VWR (Radnor, PA, USA). All aqueous solutions were prepared using purified water at a Milli-Q grade (Burlington, MA, USA).

2.2 Plasma samples from healthy volunteers

All subjects, who voluntarily accepted to participate in the study, were informed and authorization was obtained by signing a letter of consent. These subjects were chosen among those who participated in a larger clinical study [10] approved by the institutional local ethical committee (Ospedale San Paolo, Milano, Italy). Blood from ten volunteers was collected in the fasting state using K_2EDTA as an anticoagulant, and the resulted plasma was obtained by centrifugation 15 min at 3000 rpm. The recruitment criteria were 1) aged between 18–85 years and 2) any pathological conditions; each volunteer was tested for complete blood count and, for being included in this study, their values needed to be within the physiological ranges of the medical laboratory. The pool obtained was aliquoted and stored at -80 °C. All the procedures adopted in the present study were respectful of the ethical standards in the Helsinki Declaration.

2.3 Single-phase extractions for the analysis of lipids

Lipids were single-phase extracted from the diluted pool of healthy human donors' plasma EDTA (25 µL of plasma + 75 µL water) in replicates (n=3) following these protocols: (A) methanol/chloroform (2:1, v/v, 850 µL); (B) methanol/chloroform (1:1, v/v, 850 µL); (C) methanol/chloroform/tert-butyl methyl ether (1.5:1:1, v/v/v, 850 µL). Before the extraction, samples were added with the internal standard (phosphatidylcholine (15:0-18:1) d7 25 µg/mL, 6 µL) then ice-sonicated (30 min), oscillated in a thermomixer (1h, 800 rpm, 5°C), centrifuged (10 min, 13200 rpm) and the organic phase evaporated under nitrogen. The extracts were dissolved in isopropanol/acetonitrile (2:1, v/v) + 0.1 mM BHT. The protocol (D) was essentially the same as (A) with the addition of a tip-tip filtration step of the redissolved sample before LC-MS/MS injection. Essentially, the tip-tip protocol consists of the aspiration of the lipid extract through a 20 µL tip polyethylene filter followed by the withdrawal of the clarified solution from the part overlying the filter with another tip directly into the LC-MS/MS vial (see the scheme in Figure 1).

2.4 Untargeted lipidomics

LC-MS/MS analysis was performed onto a Shimadzu UPLC coupled with a Triple TOF 6600 Sciex (Concord, ON, CA). All samples were analyzed in duplicate in both positive and negative

electrospray ionization. The source parameters were CUR 35, GS1 55, GS2 65, capillary voltage 5.5 kV (ESI+) or 4.5 kV (ESI-), and source temperature (TEM) 350 °C. Spectra were contemporarily acquired by full-mass scan from m/z 200-1500 (100 ms TOF MS accumulation time) and top-20 data-dependent acquisition from m/z 50-1500 (40 ms TOF MS/MS accumulation time). Declustering potential was fixed to 50 eV, and the collision energy was 35±15 eV. The chromatographic separation was reached on a reversed-phase Acquity CSH C18 column 1.7 µm, 2.1 × 100 mm (Waters, Franklin, MA, USA) by a gradient between (A) water/acetonitrile (60:40) and (B) 2-propanol/acetonitrile (90:10), both containing 10 mM ammonium acetate and 0.1% of formic acid. The flow rate was 0.4 mL/min, and the column temperature was 55°C. The elution gradient (%B) was set as below: 0–2.0 min (40%), 2.0–2.5 min (40–50%), 2.5–12.5 min (50–55%), 12.5–13.0 min (55–70%), 13.0–19.0 min (70–99%), 19.0–24.0 min (99%), and 24.0–24.2 (99–40%) and kept constant until 30 min. Five microliters of clear supernatant were directly injected into the LC-MS/MS [11].

2.5 LC-HR-MS data processing

The spectra deconvolution, peak alignment, and sample normalization were attained using MS-DIAL (ver. 4.0). MS and MS/MS tolerance for peak profile was set to 0.01 and 0.05 Da, respectively. Identification was achieved matching spectra with LipidBlast database. The analytical drift, which generally occurs over batch analysis, was resolved by LOWESS normalization injecting the QC pool sample every three runs. Analytes with a CV% superior to 30% in the QC pool sample were excluded. Eventually, internal standard normalization was done against the response of PC d7 (m/z 753.61).

2.6 Statistics and data visualization

Graphs and statistical analyses were prepared with GraphPad Prism 7.0 (GraphPad Software, Inc, La Jolla, California, USA). Univariate statistical analysis was performed using paired t-test for two-groups comparison or paired one-way ANOVA with Bonferroni post-hoc test for more than two groups. $p<0.05$ was considered statistically significant. Data are shown as mean ± SD or median-interquartile range.

3 Results and Discussion

After the LC-MS/MS lipidomic analysis and the data processing as described in Materials and Methods, 317 single lipids species were recognized in the plasma pool from healthy volunteers used for this research, clustered in 28 lipid classes.

3.1 Concordance between methods

At first, we carried out a Deming Linear Regression test with uncertainty in both X and Y [12], investigating three comparisons: methods A vs B, A vs C and B vs C. For each lipids class, the sum of all the peaks intensities of the recognized components was compared among the three methods. This way, each curve point represented the intensity of that lipid class in the analyzed methods. The two protocols employing methanol:chloroform mixture in a different ratio (A, B) gave comparable results (R^2 = 0.98). Nonetheless, the results attained from protocol C, which included tert-butyl methyl ether in the organic mixture, were not as much comparable with the ones attained from both the above-mentioned protocols (R^2 = 0.81 A vs C, 0.80 B vs C).

3.2 Yield of Extraction

In Figure 2A the yields of total lipids obtained with the three extraction protocols are shown in both positive and negative ESI modes detections. Even though there is no statistically significant difference between them all, a slightly higher yield can be attained using protocol A, regarding ESI positive mass spectrometry detection. The recovery of the internal standard PC d7 (Figure 2B) mirrors the behavior of all lipid species, whereas protocol A gives back a more substantial extraction, but still with no statistical difference from the other methods. The heatmap in panel C presents more meaningful information comparing for each lipids class individually the yields from the three extractions. It appears evident that methods A and B satisfy almost all lipids classes ~~in the examination~~, with carnitines (CAR), lysophosphatidylcholines (LPC), and oxidized phosphatidylethanolamines (OxPE), better extracted with protocol A. On the other hand, protocol C seems to have generally lower performances, with a better accomplishment only with OxPE.

3.3 Reproducibility of the different protocols

Reproducibility of the three extraction methods was evaluated comparing the percent Coefficients of Variations (CV%) calculated on the triplicate extractions for each protocol of both the sum of the whole sets of lipids (n=317, Figure 3A), and of the individual lipids classes. In panels B and C the mean CVs% from each lipid class are compared. Overall, it is indeed protocol A the most promising, as it lends the minor CVs% in both positive (Grand mean A vs B and C: 21% vs 23% and 26%) and negative (A vs B and C: 5% vs 15% and 17%) ionizations.

It is now fundamental to distinguish the different normalizations carried out on this batch of analysis. LOWESS normalization is generally used to evaluate and counterbalance the analytical drift, while

the internal standard normalization is required to appraise the pre-analytical variability, especially in targeted mass spectrometry methods. Furthermore, MS Dial only allows to set up an internal standard normalization while using either a single labeled standard or a commercial labeled standards mix. Of course, the latter could be a very efficient way to effectuate an internal standard normalization, but, on the other hand, it is a particularly expensive procedure. As our work focuses on the comparison of three different methods that could be routinely and cheaply applicable in all kinds of research laboratories, we decided to normalize the attained results using a single internal standard, whose recovery was previously displayed (Figure 2B). Unexpectedly, when normalized, repeatability of all the three protocols appeared to be worsened (mean CVs% > 30%, Figure 3D).

3.4 Innovative tip-tip filtration

To reduce the variability, we evaluated whether a filtration step before LC-MS/MS injection could bring any benefit to the analyses. On the other hand, we decided to perform an innovative filtration, using only pipettes tips (Figure 1), avoiding the usage of syringes, whose application is quite expensive, time-consuming, and needs high volume samples. The plasma pool in use for protocol D was extracted in triplicates using protocol A and then underwent tip-tip filtration. As it can be seen in Figure 4A, the extraction yield calculated on total lipids slightly improved in ESI +, while it remained the same in ESI -, even though some phospholipids and glycosphingolipids classes displayed a minimal loss (< 20%).

When analyzing the single classes CVs% (Figure 3B) an improvement in the variability can be appreciated, especially as far as it regards the ESI+ ionization (Grand mean 21% vs 13%).

Furthermore, also the recovery of the internal standard (Figure 3C) is remarkably improved, and the internal standard normalization now gives satisfactory results (Figure 3D).

In the end, the benefits from the use of tip-tip filtrations are summarized as follows: (1) high purification of lipid extracts from cell debris and particulates, which cannot be extensively eliminated by centrifugation; (2) money-saving filter technique: the cost of a tip filter is about 30 times lower than the cost of syringe coupled with a membrane filter (0.05€ vs 1.44€, respectively per each sample) (3) any loss of significant amounts of lipids from biological extracts: the use of tip-tip filtration produces a loss of 10% of lipids in respect to the original single-phase 2:1 methanol/chloroform extraction method (4) more intra-subject reproducibility; (5) increased analytical column lifetime; (6) time-saving in respect to syringe filtration; (7) maximum recovery of the extract with a total loss of less than 10 µL.

4 Conclusion

In this work, we evaluated which of the most used methods for lipid extraction, namely (1) methanol/chloroform (2:1, v/v); (2) methanol/chloroform (1:1, v/v); and (3) methanol/chloroform/tert-butyl methyl ether (1.5/1/1, v/v), could be more advantageous to perform an untargeted lipidomics analysis. Our results demonstrate a remarkable superiority of the first method above the other two proposed, as previously hypothesized by other authors [13,14], in terms of both better reproducibility and rate of extraction. We also believe that the use of an extraction phase richer in chloroform should be specifically dedicated to the study of frankly apolar lipids, such as steroids and triacylglycerols. Furthermore, we proposed an innovative solution for the recovery of lipids and the attainment of clearer samples by using tip-tip filtration, which led to improved results compared to the method requiring a 2:1, v/v methanol/chloroform mixture. With a minimal loss of lipids, tip-tip filtration allows a higher purification of the samples, an increased column lifetime, and a sharp cut in the cost. We strongly believe that this method could provide significant results even in polar small molecules extraction and analysis.

Author contributions

Conceptualization: CM and MDC; Investigation: CM and MDC; Visualization: CM and MDC; Writing – original draft: CM and MDC; Writing – review & editing: GR and RP.

Ethics approval and consent to participate

All experimental protocols regarding human materials were conducted according to the Declaration of Helsinki. Authorization was obtained from all subjects by signing a letter of consent.

Disclose of interest.

The authors have declared that no conflict of interest exists.

Acknowledgments

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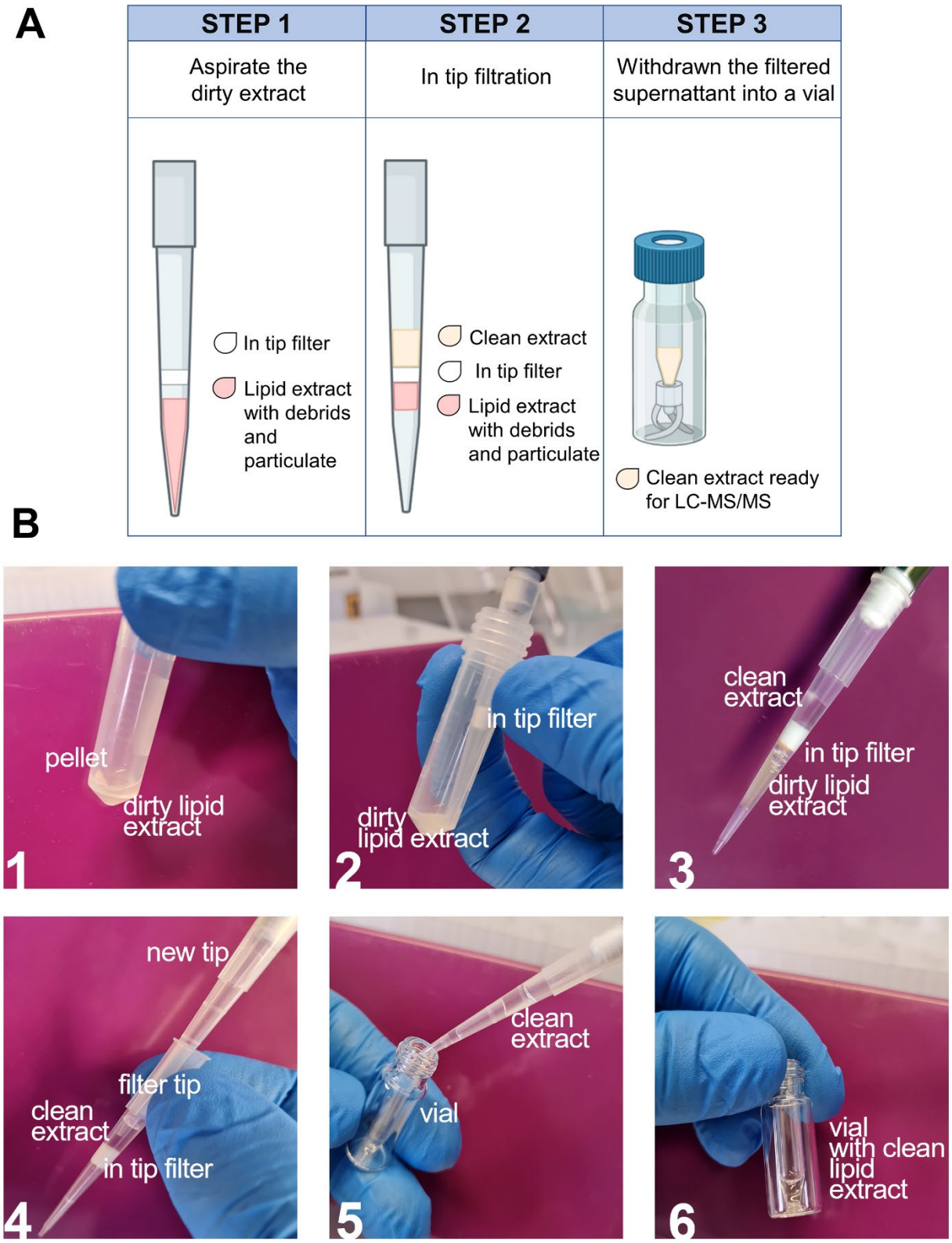
278 **Figures.**



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280 **Figure 1.** (A) Scheme for tip-tip filtration protocol of lipid extracts. Sterile aerosol pipette tips 1-20 µL (cat. no.
281 89174-524) were purchased from VWR (Radnor, PA, USA). (B) Step by step tip-tip filtration protocol.

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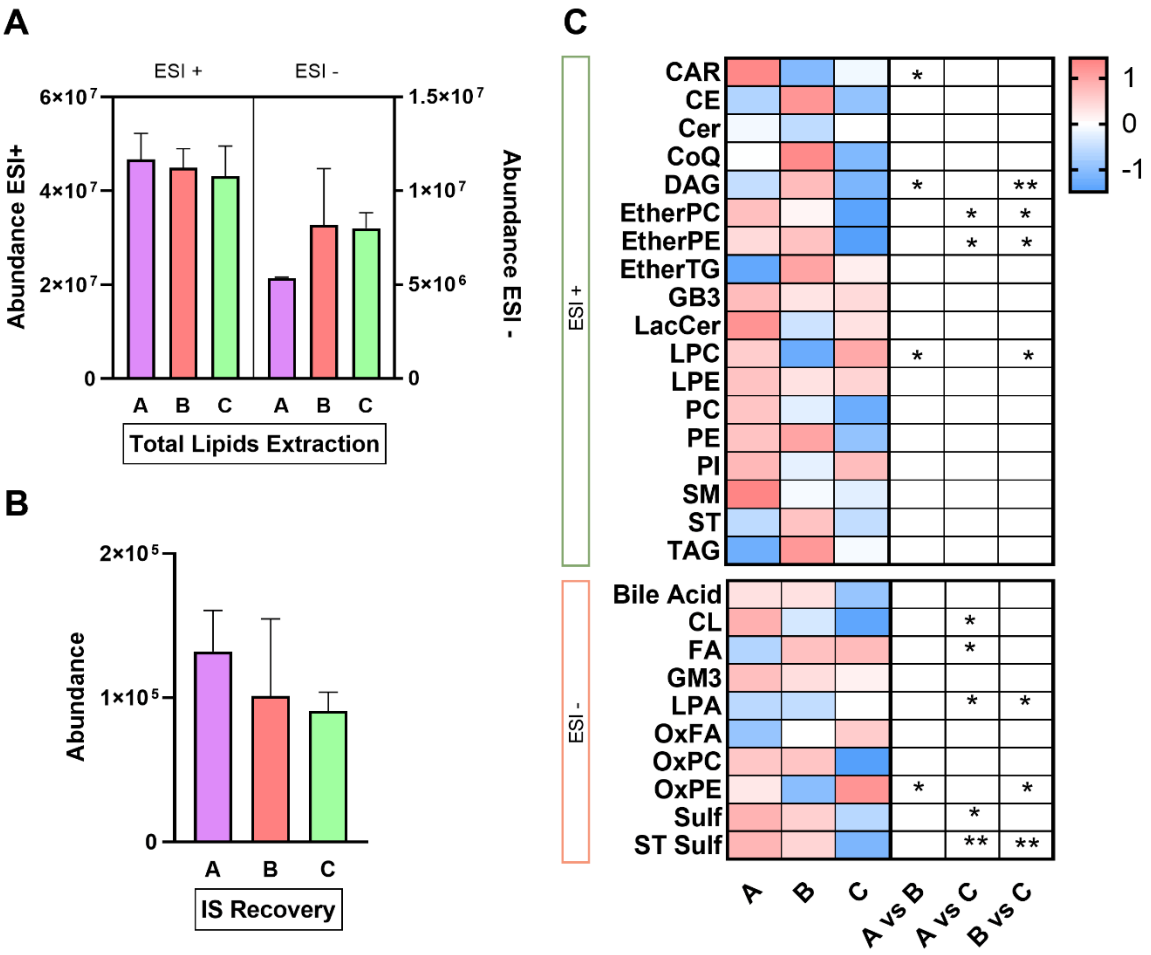


Figure 2. Rate of lipids extraction by using (A) methanol/chloroform (2:1, v/v); (B) methanol/chloroform (1:1, v/v); (C) methanol/chloroform/tert-butyl methyl ether (1.5:1:1, v/v/v). For details to the single extraction protocols see Material and methods. **Panel A:** Sum of total lipids abundance in both the polarities. **Panel B:** Recovery of internal standard (PC d7, 1.5 ug/µl) under the three different extraction methods. **Panel C:** Heatmap of the recovery of each lipid class across the different extraction protocols. Statistical significance was evaluated by paired one-way ANOVA (Bonferroni post hoc test). For the lipids classes nomenclature, see List of Abbreviations.

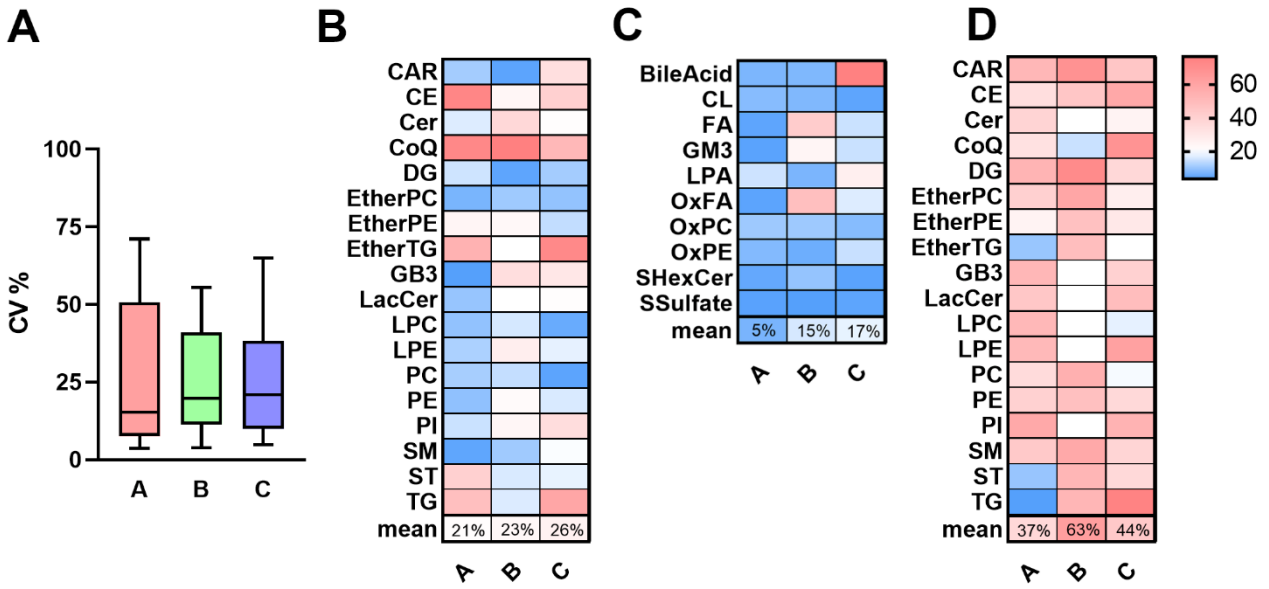
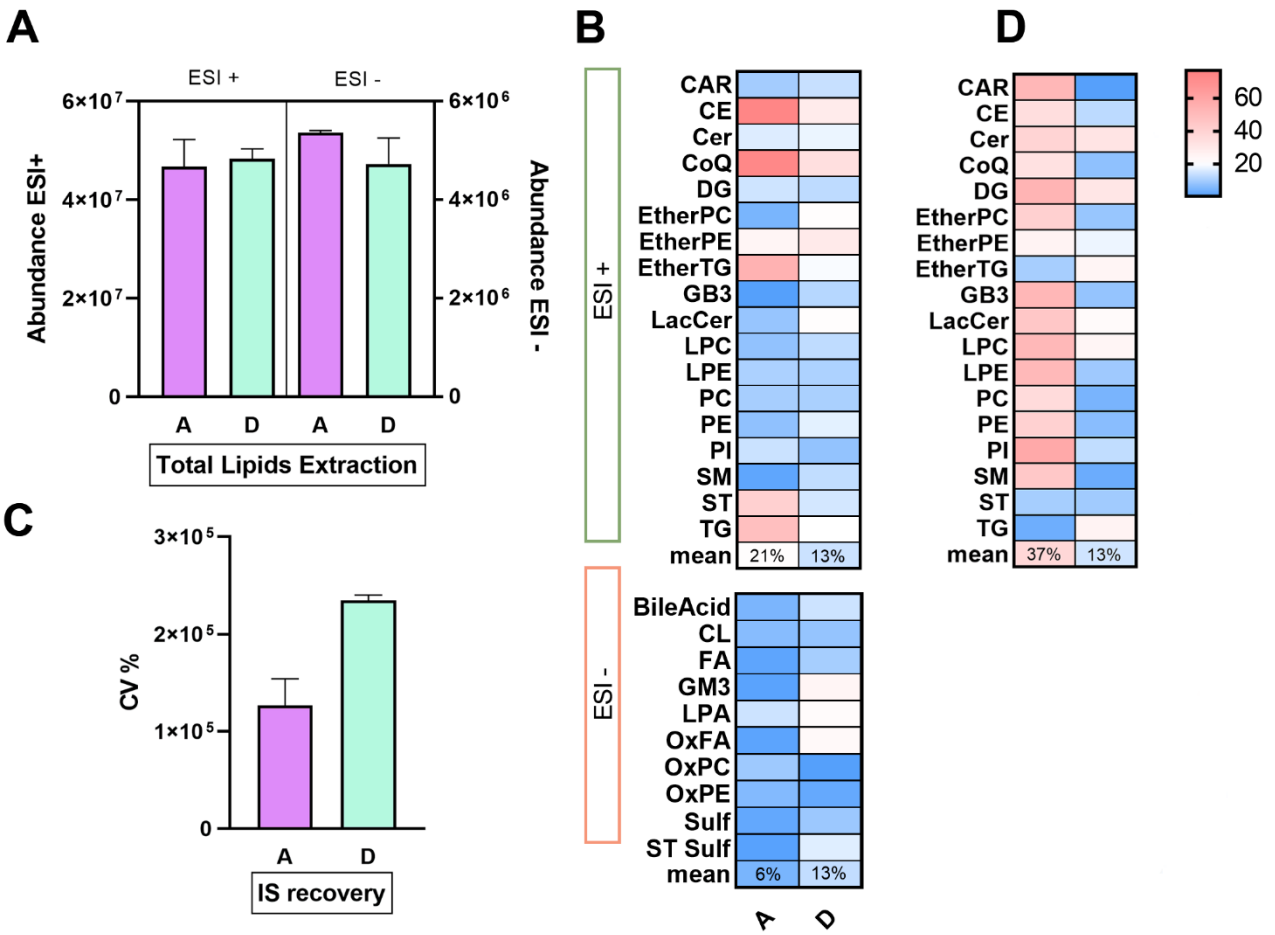


Figure 3. Panel A: The experimental variability of 317 lipids species extracted from plasma in triplicates with (A) methanol/chloroform (2:1, v/v); (B) methanol/chloroform (1:1, v/v); (C) methanol/chloroform/tert-butyl methyl ether (1.5:1:1, v/v/v). The median and 10-90 percentiles of the CVs% distribution for all single lipid species are represented in box-plots. **Panels B, C:** Heatmaps of the CVs% of the entire lipid profile in ESI+ and ESI-, grouped in classes without IS-based normalization. **Panel D:** CVs% of the entire lipid profile in ESI+ after IS-based normalization. For the lipids classes nomenclature, see List of Abbreviations.



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313 **Figure 4.** The impact of tip-tip filtration on protocol A extraction. **Panel A:** Mean of total lipids abundance in
314 both the polarities. **Panel B:** Heatmap of the coefficient of variation (CV%) of the same sample extracted in
315 triplicate. Each lipid class was represented.**Panel C**: Variation of the recovery of internal standard (PC d7, 1.5
316 ug/µl) without (A) and with (D) the tip-tip step. **Panel D:** The influence of internal standard normalization on
317 CVs% in each lipid class. Statistical significance was evaluated by paired t-test. For the lipids classes
318 nomenclature, see List of Abbreviations.

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