

Benchmarking One-Phase Lipid Extractions for Plasma Lipidomics

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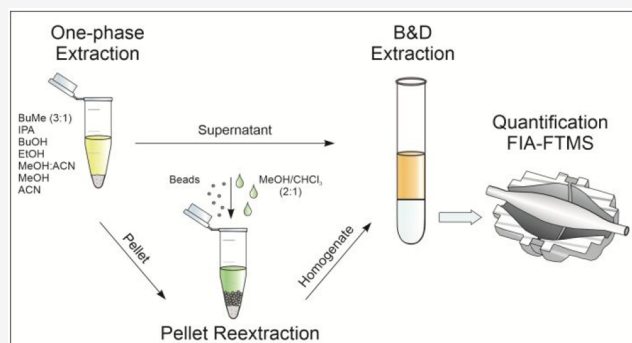


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ABSTRACT: A key element of successful lipidomics analysis is a sufficient extraction of lipid molecules typically by two-phase systems such as chloroform-based Bligh and Dyer (B&D). However, numerous metabolomics and lipidomics studies today apply easy to use one-phase extractions. In this work, quantitative flow injection analysis high-resolution mass spectrometry was applied to benchmark the lipid recovery of popular one-phase extraction methods for human plasma samples. The following organic solvents were investigated: methanol (MeOH), ethanol (EtOH), 2-propanol (IPA), 1-butanol (BuOH), acetonitrile (ACN) and the solvent mixtures BuOH/MeOH (3:1) and MeOH/ACN (1:1). The recovery of polar lysophospholipids was sufficient for all tested solvents. However, nonpolar lipid classes such as triglycerides (TG) and cholesteryl esters (CE) revealed extraction efficiencies less than 5% due to precipitation in polar solvents EtOH, MeOH, MeOH/ACN, and ACN. Sample pellets also contained a substantial amount of phospholipids, for example, more than 75% of total phosphatidylcholine and sphingomyelin for ACN. The loss of lipids by precipitation was directly related to the polarity of solvents and lipid classes. Although, lipid recovery increased with the volume of organic solvent, recovery in polar MeOH remains incomplete also for less polar lipid classes such as ceramides. Addition of stable isotope-labeled internal standards prior to lipid extraction could compensate for insufficient lipid recovery for polar lipid classes including lysolipids and phospholipids but not for nonpolar CE and TG. In summary, application of one-phase extractions should be limited to polar lipid classes unless sufficient recovery/solubility of nonpolar lipids has been demonstrated. The presented data reveal that appropriate lipid extraction efficiency is fundamental to achieve accurate lipid quantification.



Lipidomics is a rapidly growing field that aims to identify and to quantify a broad spectrum of lipid molecules.^{1,2} Recent multicenter studies demonstrated the power and reliability of lipidomic analysis related to clinical questions.^{3,4} Clinical lipidomics relies on accurate and reproducible quantification of lipid molecules typically from plasma samples.⁵

The first step of quantitative lipidomic workflows is a sufficient extraction of lipid molecules. Traditionally, biphasic lipid–lipid extractions are applied such as chloroform-based Bligh and Dyer⁶ or Folch⁷ or less toxic alternatives using MTBE.⁸ Over the past decade, many studies applied monophasic extraction methods to combine the analyses of polar and nonpolar metabolites. Compared to biphasic extractions, the application of monophasic extractions is faster, cheaper, and less complex. However, monophasic extracts may contain salts and other polar metabolites and hence represent less clean lipid mixtures compared to biphasic extracts. These protocols used a variety of organic solvents including acetonitrile (ACN),^{9,10} methanol (MeOH),^{9–11} ethanol (EtOH),¹¹ and isopropanol (IPA),^{9–11} as well as mixtures

such as methanol:acetonitrile (Me:ACN),¹⁰ isopropanol:acetonitrile,^{9,10} and butanol:methanol (BuMe).¹²

There are multiple reports comparing different extractions methods for plasma lipids which typically evaluated the number of detected lipids as well as signal intensities.^{9–11,13,14} However, reports of quantitative lipid recovery for monophasic lipid extractions are not available as of now. Therefore, we applied quantitative flow injection analysis Fourier-transform mass spectrometry (FIA-FTMS) to benchmark lipid recovery of one-phase extractions for human plasma samples. As a reference extraction, we used Bligh and Dyer (B&D)⁶ that has been applied successfully over decades in lipid research.¹

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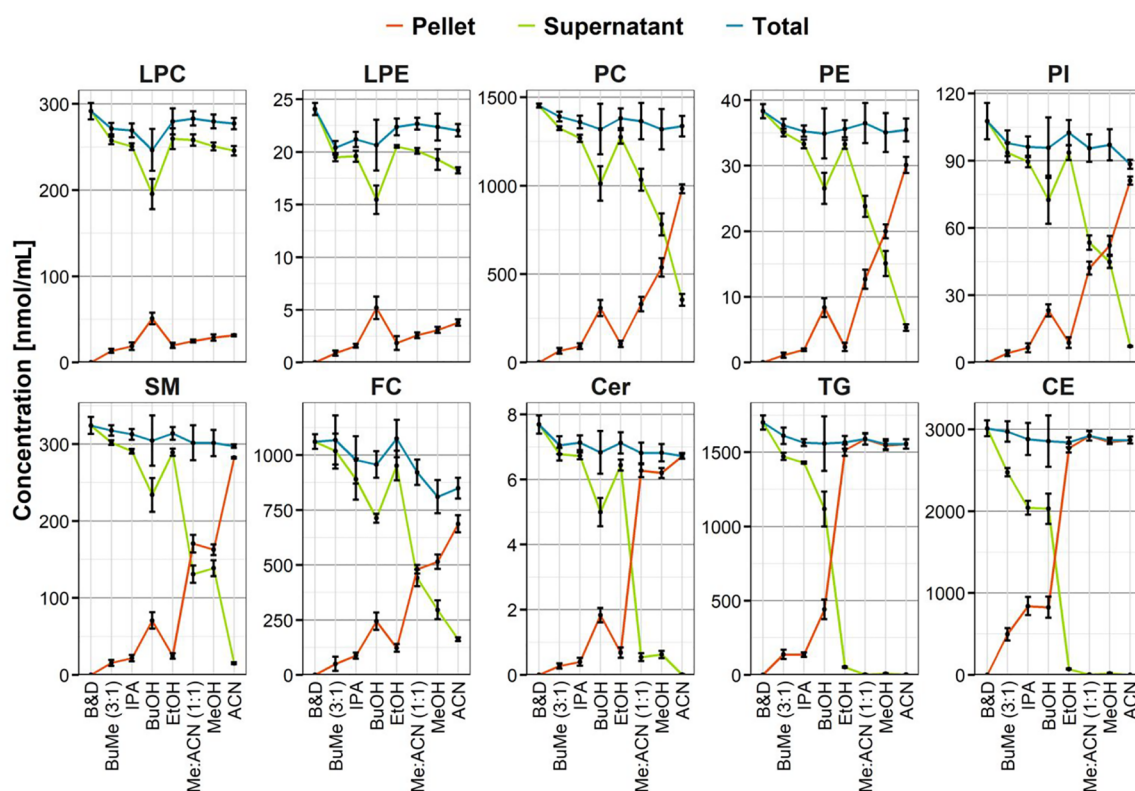


Figure 1. Lipid concentrations of supernatant (green), pellet (red), and sum of pellet and supernatant (blue) recovered after one-phase extraction of human plasma. Sample to solvent ratio was 1:3, and 1-butanol:methanol (BuMe (3:1)), isopropanol (IPA), 1-butanol (BuOH), ethanol (EtOH), methanol:acetonitrile (Me:ACN (1:1)), methanol (MeOH), and acetonitrile (ACN) were used for one-phase extraction. Bligh and Dyer extraction is shown as the reference (B&D). Displayed are mean and SD ($n = 3$) for lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), sphingomyelin (SM), free cholesterol (FC), ceramide (Cer), triglyceride (TG), and cholesteryl ester (CE).

EXPERIMENTAL SECTION

Reagents and Lipid Standards. Chemicals and lipid standards were used as described previously.¹⁵ The compositions of applied internal standard mixtures are listed in Table S1.¹⁶ The EquiSplash mixture was provided by Avanti Polar Lipids (Alabaster, Alabama, USA). Organic solvents had a purity of at least 99.5%.

Plasma Samples. Human EDTA plasma samples were residual samples from routine laboratory diagnostics. Sample pools were collected in an anonymized not traceable way and used for testing of extractions. The material was collected with ethical compliance for human research adhering to the standards of the local regulatory authorities.

One-Phase Extractions. A volume of 10 μL of plasma was mixed with the respective organic solvents ACN, EtOH, IPA, MeOH, 1-butanol (BuOH), BuMe (3:1, v/v), and Me:ACN (1:1, v/v) in a sample to solvent ratio of 1:3, 1:4, or 1:5. Samples were vortexed for 20 s and then incubated for 1 h in an ice-cooled ultrasonic bath. After centrifugation for 15 min at 16,000 rcf, the supernatant was carefully recovered with a syringe (Hamilton) and subjected to B&D extraction. The pellet was homogenized in 1 mL of methanol:chloroform (2:1, v/v) after addition of 1.4 mm of zirconium oxide beads and bead-beating with a Precellys 24 homogenizer (Bertin Technologies SAS, Montigny-le-Bretonneux, France). The resulting suspension was subjected to B&D.

Bligh and Dyer (B&D) Extraction. A mixture of nonendogenous internal standards (Table S1) was added

prior to extraction. Lipids were extracted according to Bligh and Dyer⁶ as described previously.¹⁵

Flow Injection Analysis Fourier-Transform Mass Spectrometry (FIA-FTMS). Lipid quantification by FIA-FTMS was performed on a hybrid quadrupole-Orbitrap mass spectrometer QExactive (Thermo Fisher Scientific, Bremen, Germany) as described previously.¹⁵

RESULTS AND DISCUSSION

Lipid Recovery of One-Phase Extractions. First, we tested the extraction efficiency of monophasic extractions for various organic solvents by monitoring the distribution of lipids, i.e., their concentrations in pellet and supernatant. To cover a broad range of polarities, we analyzed the following extraction solvents: ACN, MeOH, EtOH, 1-butanol (BuOH), and IPA and the solvent mixtures 1-butanol:methanol (3:1, BuMe) and methanol:acetonitrile (1:1, Me:ACN). The plasma was mixed at a solvent ratio of 1:3, and FIA-FTMS^{15,17} was applied to quantify the lipids in the respective sample fractions. The extracts of monophasic extractions still contain salts and other polar metabolites and were therefore subsequently subjected to B&D to obtain lipid extracts suitable for direct analysis. Of note, pellet fractions were extracted after bead-beating in methanol:chloroform (2:1) to increase sample dispersion, which is required for sufficient lipid extraction as demonstrated previously for tissue homogenates.¹⁸ To ensure that B&D could be used as a reference, stable isotope-labeled standards were applied to examine the extraction efficiency of

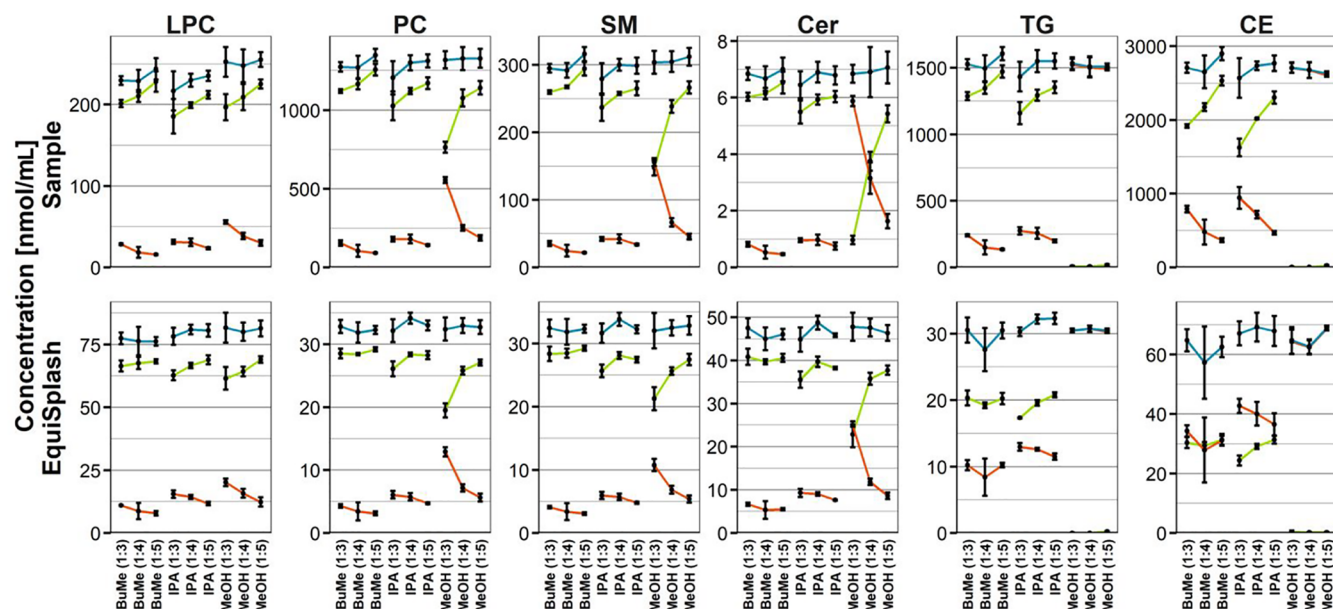


Figure 2. Effect of sample-to-solvent ratios of 1:3, 1:4, and 1:5 on lipid recovery. One-phase extraction was performed with BuMe (3:1), IPA, and methanol. (Upper panels) Concentrations of supernatant (green), pellet (red), and sum of pellet and supernatant (blue) recovered after one-phase extraction of human plasma. (Lower panels) Concentrations of stable isotope-labeled internal standard (EquiSplash) detected in the respective sample fractions. EquiSplash (250 ng of each lipid class, Table S1) was placed into the sample tube and dried prior to one-phase extraction. Displayed are mean and SD ($n = 3$).

the analyzed lipid classes. Recoveries were above 90% for phospholipids, triglycerides (TG), and cholesteryl esters (CE) and about 80% for lysophosphatidylcholines (LPC) and lysophosphatidylethanolamines (LPE) (Figure S1), which could be considered as sufficient for benchmarking one-phase extractions.

The extraction efficiency of monophasic extractions is shown in Figure 1. The recovery of lipids in the supernatant was directly related to the polarities of the lipid class and extraction solvent. While polar lipolipids (LPC, LPE) showed acceptable extraction efficiencies in all tested solvents, nonpolar lipid classes (TG, CE) were practically not extracted and accumulated in the pellet fraction when polar solvents like ACN, MeOH, EtOH, and Me:ACN were applied (Figure 1). Phospholipids with intermediate polarities followed this trend and showed acceptable recoveries for EtOH, IPA, and BuMe. For BuOH, lower extraction efficiencies and higher variations were observed, which may be related to the high viscosity of this solvent resulting in an incomplete retrieval of the supernatant. For all solvents, total recoveries, i.e., sum of lipid content in supernatant and pellet, were in good agreement with reference concentrations (B&D). This provides evidence for both accurate quantification and negligible loss of lipids during sample processing.

We analyzed the recovery of lipid species that closely resembled that of the respective lipid class (Figures S2–S6). Shifts in species profiles were observed when polarity of the solvent causes insufficient extraction, and profiles were shifted toward shorter species, for example, SM and Cer species for MeOH and ACN (Figure S4). In addition, ACN seems to preferentially extract species with a high number of double bonds. In summary, in case of insufficient extraction efficiency, structural variations within lipid classes like number of double bonds or acyl chain length, which alter the polarity of the lipid molecule, may lead to shifts in lipid species profiles.

Influence of Sample to Solvent Ratio. In a next step, we evaluated the impact of the sample to solvent ratio on the lipid recovery. Analysis was focused on solvents with reasonable (IPA and BuMe) and insufficient (MeOH) extraction efficiencies and prototypic lipid classes spanning the polarity range. In general, raising the solvent fraction from 1:3 to 1:4 to 1:5 enhances lipid recovery (Figure 2, upper panel). In particular, moderate extraction efficiencies, for example, for PC, SM, and Cer in MeOH, improved substantially at higher solvent volumes by decreasing the lipid precipitation. However, the recovery of lipid classes practically insoluble in an organic solvent could not be enhanced by changing the sample to solvent ratio; for example, nonpolar TG and CE were almost completely precipitated in MeOH.

Application of Internal Standards. To monitor and address for the loss of lipids, it is recommended to add internal standards (IS) prior to lipid extraction.^{1,19} Therefore, a mixture of stable isotope-labeled lipid species was added to the extraction tube and vacuum dried prior to one-phase extraction. As expected, similar recoveries of endogenous and isotope-labeled lipids (Figure 2) were observed. However, with decreasing polarity of the lipid class and increase of solvent polarity, these labeled standards exhibited lower recovery compared to the endogenous lipids. In particular, these differences were observed for sphingomyelins (SM) and ceramides (Cer) extracted with MeOH as well as TG and CE extracted with BuMe and IPA. Most likely this relates to an insufficient solubility of the dried IS in the selected solvents, despite extensive sonication and agitation of the samples. In this case, it is expected that the application of IS cannot accurately compensate for lipid losses.

Influence of Plasma Lipid Content. Next, we asked whether the overall lipid contents of plasma samples influence the extraction efficiencies of monophasic extractions. Pooled plasma samples with low, intermediate, and high contents of

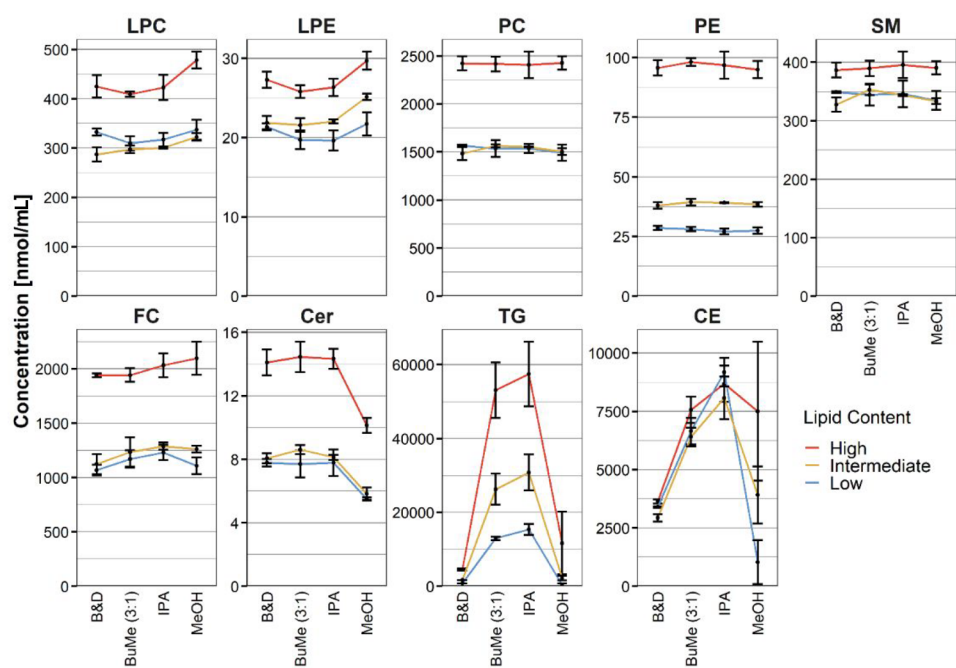


Figure 3. Influence of plasma lipid content. Lipid concentrations in the supernatants for each lipid class and selected extraction methods with different plasma TG contents. IS mixture¹⁶ was added prior to one-phase extraction with BuMe (3:1), IPA, and MeOH at a sample to solvent ratios of 1:5. Displayed are mean and SD ($n = 3$) concentrations of the low (blue), medium (yellow), and high (red) TG content samples.

TG were extracted with IPA, BuMe, and MeOH at a sample to solvent ratio of 1:5. Good agreement was observed for lysolipids, phospholipids, sphingolipids, and free cholesterol (Figure 3). Deviations from B&D were less than 10%, with the exception of LPC and LPE, where we observed slightly higher concentrations (12%–15%) in the medium and high plasma pool upon MeOH precipitation. Additionally, for MeOH, we found about 30% lower ceramide (Cer) concentrations in all sample pools.

Quantifications of nonpolar CE and TG showed substantial deviations from B&D reference concentrations. Although reproducibility was acceptable for IPA and BuMe, CE and TG concentrations were about 2-fold and 15-fold higher compared to B&D, respectively. For MeOH, a trend from underestimation to overestimation was observed for both CE and TG with increasing lipid contents of the plasma samples. In summary, these data indicate that quantification of nonpolar lipid classes by one-phase extraction with the tested conditions fails despite addition of lipid class-specific IS prior to extraction.

Influence of Additional B&D Extraction. Finally, we analyzed whether re-extractions of one-phase extracts by B&D affect lipid concentrations. Therefore, we subjected one-phase extracts after addition of IS and dilution with mobile phase to FIA-FTMS analysis. Concentrations with and without re-extraction were comparable (Figure S7). However, lower concentrations were determined for LPC and LPE without B&D. Similar trends, but less pronounced, were observed for PC, SM, and PI concentrations. All of these lipid classes were analyzed in negative ion mode. The observed deviations may be related to poor spectra quality in the negative ion mode of one-phase extracts without B&D that exhibit huge signal suppressions and numerous additional signals (Figures S8 and S9) which, on the contrary, were not observed in the positive ion mode (Figure S10). These data confirm that direct analysis

of one-phase extracts could not be recommended due to the presence of nonlipid contaminants like salts and polar metabolites.

CONCLUSION

The present study demonstrates that fundamental chemical knowledge of “similar substances will dissolve similar substances” holds true also for lipid extraction. Our data underline that the polarities of organic solvents in one-phase extractions need to be suitable to dissolve the respective lipid class. While all tested organic solvents that are frequently applied for protein precipitation in metabolomics studies are sufficient for the analyses of polar lysolipids and phospholipids, they are inappropriate for the analyses of nonpolar lipid classes like CE and TG. Consequently, extraction efficiencies of one-phase extractions, in particular for lipid classes with low polarity, should be carefully evaluated including testing of the lipid to solvent ratios and lipid contents of samples.

The considerations presented here fit very well to the aims of the Lipidomics Standards Initiative (LSI; <https://lipidomics-standards-initiative.org/>) which covers all aspects related to the quality of lipidomics analyses in a community-based effort.²⁰ Because still too many studies report lipidomics data with insufficient quality, standards and quality checks are urgently needed.²¹

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.2c02117>.

Figures S1–S10 and Table S1 (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Author Contributions

Marcus Höring and Christoph Stieglmeier contributed equally.

Notes

The authors declare no competing financial interest.

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