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ARTICLE *in* ANALYTICAL CHEMISTRY · MAY 2014

Impact Factor: 5.64 · DOI: 10.1021/ac500317c · Source: PubMed

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Objective Set of Criteria for Optimization of Sample Preparation Procedures for Ultra-High Throughput Untargeted Blood Plasma Lipid Profiling by Ultra Performance Liquid Chromatography–Mass Spectrometry

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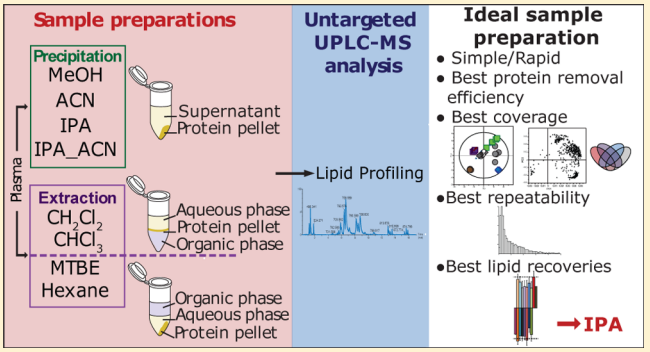
Supporting Information

ABSTRACT: Exploratory or untargeted ultra performance liquid chromatography–mass spectrometry (UPLC–MS) profiling offers an overview of the complex lipid species diversity present in blood plasma. Here, we evaluate and compare eight sample preparation protocols for optimized blood plasma lipid extraction and measurement by UPLC–MS lipid profiling, including four protein precipitation methods (i.e., methanol, acetonitrile, isopropanol, and isopropanol–acetonitrile) and four liquid–liquid extractions (i.e., methanol combined with chloroform, dichloromethane, and methyl-*tert* butyl ether and isopropanol with hexane). The eight methods were then benchmarked using a set of qualitative and quantitative criteria selected to warrant compliance with high-throughput analytical workflows: protein removal efficiency, selectivity, repeatability, and recovery efficiency of the sample preparation. We found that protein removal was more efficient by precipitation (99%) than extraction (95%). Additionally, isopropanol appeared to be the most straightforward and robust solvent (61.1% of features with coefficient of variation (CV) < 20%) while enabling a broad coverage and recovery of plasma lipid species. These results demonstrate that isopropanol precipitation is an excellent sample preparation procedure for high-throughput untargeted lipid profiling using UPLC–MS. Isopropanol precipitation is not limited to untargeted profiling and could also be of interest for targeted UPLC–MS/MS lipid analysis. Collectively, these data show that lipid profiling greatly benefits from an isopropanol precipitation in terms of simplicity, protein removal efficiency, repeatability, lipid recovery, and coverage.



Metabolic profiling and phenotyping provide a global understanding of complex metabolism using two major analytical techniques: nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS).[1−3] Because of the complex composition of blood, the characterization of this biofluid greatly benefits from the multidimensional separation and sensitive detection provided by ultra-high performance liquid chromatography coupled to MS (UPLC–MS). UPLC columns can operate with higher flows and pressures, which directly results in shorter acquisition times for similar resolution to HPLC.[4] However, because of the large diversity in physicochemical properties of the metabolites found in plasma, polar metabolites, and lipids are often analyzed separately by two different LC–MS methods.[5−10] For untargeted UPLC–MS profiling, nonselectivity during sample preparation is critical for successful analysis of these two molecular classes since sample preparation issues may impact the final quality of the data. However, sample preparation is key yet often overlooked step for the successful UPLC–MS based lipid profiling and can have a strong impact on the quality of subsequent spectral data. Given the importance of lipidomics in systems biology and in various disease areas such as obesity and metabolic syndrome,[11−14] there is a strong analytical need for developing robust and efficient methods fit-for-purpose in large-scale

Received: January 23, 2014
Accepted: May 12, 2014
Published: May 12, 2014

   dx.doi.org/10.1021/ac500317c | *Anal. Chem.* 2014, 86, 5766–5774

epidemiological and clinical studies, requiring optimized profiling of thousands of samples.[15]

Typically, two types of sample preparation strategies can be considered for lipid sample preparation: protein precipitations (monophasic) and lipid extractions (biphasic). In this study we evaluate eight conventional sample preparation methods for plasma lipid analysis by UPLC−MS. The four precipitation methods evaluated here were based on the use of organic solvents such as methanol (MeOH), acetonitrile (ACN), isopropanol (IPA), and IPA/ACN. The role of these solvents is to precipitate proteins and solubilize lipids.

The four liquid−liquid extraction methods evaluated were based on MeOH combined with various amounts of chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2), methyl-*tert* butyl ether/H_2O (MTBE), and hexane/IPA (Hexane) mixtures. These extraction methods were evaluated as they partition lipids from polar metabolites. The Folch and Bligh−Dyer methods involving MeOH/$CHCl_3$ are widely used for lipid extraction,[16−18] but since the lipid extract is the lower phase due to the high density of halogenated solvents, this can lead to contamination of the organic extract by proteins at the interface between the two phases. MeOH/hexane/IPA and MeOH/MTBE/H_2O are alternative methods making the organic phase collection easier due to the lower density of the organic solvents and allow higher-throughput protocols.[19−21]

Yet, despite the availability of well-documented sample preparation protocols, there is still a need to identify one of these preparation methods, as efficient, robust, repeatable, cost-efficient, and reflecting the original sample composition for global lipid profiling analysis.[22] In the present study, the benefits and limitations for each protocol were evaluated with respect to the subsequent results generated by UPLC−MS analysis.[23,24] The eight protocols were benchmarked using the following assessment criteria: simplicity (pipetting, drying, storage, cost, and safety), protein removal efficiency, lipid coverage, repeatability of lipids measurements, and recovery efficiency, which are necessary for integration in high-throughput workflows.

EXPERIMENTAL SECTION

Materials. Pooled human plasma was purchased from Sigma-Aldrich (Dorset, U.K.). Organic solvents used for the extractions and precipitations were HPLC grade and obtained from Sigma-Aldrich (Dorset, U.K.). All mobile phases were prepared with LC−MS grade solvents, formic acid and ammonium formate from Sigma-Aldrich (Dorset, U.K.). Bradford reagents were obtained for protein quantification from BioRad (Hertfordshire, U.K.). The 10 lipids standards C17:0, LPC(15:0/0:0), PG(15:0/15:0), PC(15:0/15:0), PE(15:0/15:0), SM(d18:1/17:0), PS(17:0/17:0), Cer(d18:1/17:0), DG(17:0/0:0/17:0)D5, TG(15:0/15:0/15:0) were purchased from Avanti Polar Lipids (Alabaster, Alabama).

Sample Preparation for UPLC−MS: Precipitation. For each precipitation condition, 10 replicate plasma samples (200 μL each) were precipitated by addition of 3 volumes of organic solvent precooled to −20 °C. Selected solvents were MeOH, ACN, IPA, and IPA_ACN (1:2 v:v). Samples were vortex mixed for 1 min. After 10 min of incubation at room temperature, samples were stored overnight at −20 °C to improve protein precipitation and then centrifuged at 14 000*g* for 20 min. The supernatant was collected (600 μL) and stored at −80 °C awaiting MS analysis. The sample was diluted to adjust the water content at 50% and analyzed by UPLC−MS. The analytical workflow is summarized in Figure S-1a in the Supporting Information.

Sample Preparation for UPLC−MS: Extraction. For each extraction condition, 10 replicate plasma samples (200 μL each) were extracted in glass tubes by addition of the following organic solvents: 600 μL of MeOH/$CHCl_3$ (2:2 v:v), 600 μL of MeOH/CH_2Cl_2 (2:2 v:v), 1.5 mL of MeOH/MTBE/H_2O (1:5:1.5 v:v:v) and 1.1 mL of MeOH/hexane/IPA (3:7:1 v:v:v). Samples were vortex mixed for 1 min. After 10 min of incubation at room temperature, samples were stored overnight at −20 °C to improve protein precipitation and then centrifuged at 14 000*g* for 20 min. Organic phases were collected, and 200 μL was dried in a vacuum centrifuge. The samples were stored at −80 °C awaiting MS analysis. Organic phases were reconstituted in 200 μL of IPA/ACN/H_2O (2:1:1 v:v:v). The analytical workflow is summarized in Figure S-1b in the Supporting Information.

Protein Quantification. Proteins were quantified using the Bradford method.[25] Protein concentration was estimated for each extraction and precipitation method. A standard curve of bovine serum albumin was prepared as a quantitative reference. Precipitated or extracted samples were first dried in the vacuum centrifuge and solubilized with water in order to avoid solvent incompatibility with the Bradford reagent. Samples were incubated at room temperature for at least 5 min. Absorbance was measured at 595 nm.

Ultra Performance Liquid Chromatography. Chromatographic analysis was performed using an Acquity UPLC system (Waters Ltd., Elstree, U.K.). Precipitated and extracted samples (organic phase) were injected onto a C_{18} CSH column (100 mm × 2.1 mm, 1.7 μm; Waters) at 55 °C. Flow rate was 400 μL/min. The mobile phase A consists of ACN/H_2O (60:40, v:v) mixed with 10 mM ammonium formate and 0.1% formic acid and mobile phase B IPA/ACN (90:10, v:v) mixed with 10 mM ammonium formate and 0.1% formic acid. The injection volume was 5 μL. This chromatographic approach allowed an effective separation of the different lipid species. Table S-1 in the Supporting Information shows the gradient used for the lipid profiling by UPLC−MS.[26]

Lipid Profiling by UPLC−Quadrupole-Time-of-Flight (Q-TOF) Mass Spectrometry. After separation by UPLC, mass spectrometry was performed using a Q-TOF Premier (Waters, Manchester, U.K.) for global lipid profiling and a Xevo G2-S Q-TOF for the recovery study with an electrospray ionization (ESI) source (Waters, Manchester, UK). Dynamic range enhancement was implemented to the mass spectrometry method of the Xevo G2-S Q-TOF in order to improve isotopic distribution and mass accuracy and reduce high ion intensities. In positive ion-mode, MS parameters were as follows: capillary voltage was set at 2.5 kV, cone voltage at 30 V, source temperature at 120 °C, desolvation temperature at 400 °C, desolvation gas flow at 800 L/h, and cone gas flow at 20 L/h. Acquisition was performed from m/z 100 to 1500. In negative ion mode, MS parameters were as follows: capillary voltage was set at 2.5 kV, cone voltage at 25 V, source temperature at 120 °C, desolvation temperature at 500 °C, desolvation gas flow at 800 L/h, cone gas flow at 25 L/h. Acquisition was performed from m/z 100 to 1500. For both ionization modes, leucine enkephalin (m/z 556.2771 in ESI+, m/z 554.2615 in ESI−) was continuously infused at 30 μL/min and used as lock mass correction.

Structural Identification. Metabolite annotation was made by searching m/z ratios against online databases such as

dx.doi.org/10.1021/ac500317c | *Anal. Chem.* 2014, 86, 5766−5774

METLIN (http://metlin.scripps.edu), Lipidmaps (http://www.lipidmaps.org), and HMDB (http://www.hmdb.ca).[27−30] The mass error used was 5 ppm. Further structural elucidation was performed using collision-induced dissociation experiments, with (data-dependent analysis) and without (MS^E) selection of the precursor ion by the quadrupole of the Q-TOF mass spectrometer.[31,32] Diagnostic fragments of the polar headgroup or the fatty acyl chains were investigated to confirm the annotation proposed by the databases and discriminate isomers.

MS Data Preprocessing. MassLynx software version 4.1 was used for data acquisition and analysis. Waters raw data files were converted to NetCDF format and the metabolite features reported hereafter were entirely generated using the freely available data analysis software package R (v2.11)/XCMS (v1.24.1) to preprocess the raw data. MassLynx software version 4.1 was used for data acquisition and analysis. Target Lynx 4.1 was used to process the data from the lipid standards for the recovery experiment.[33]

Multivariate Statistical Analysis. Principal component analysis (PCA) was carried out on the XCMS extracted intensities using SIMCA P+ v13 (Umetrics, Umeå, Sweden) and Matlab (The Math-Works, Natick, MA). Data sets were scaled to unit-variance. Discriminating features between lipidic profiles were identified for each model by displaying loadings plots (model coefficients vs covariance).

Univariate Statistical Analysis. Variability of sample preparation was also assessed by univariate statistics. The coefficient of variation defined as the standard deviation divided by the mean (CV) was calculated from the mean intensities of each feature detected in the 10 replicates. A histogram of the CV values was plotted for each sample preparation protocol. Instrumental variability was estimated by computation of the CV in quality control (QC) samples, constituted by pooling 10 μL of each of the 80 extracts. Standard deviations were also calculated for each lipid standard in the recovery study.

Lipid Recovery. Recovery for each of the eight methods was evaluated by spiking a 20 ng/μL mixture of the 10 nonendogenous lipid standards into the sample before (prespiked) and after (postspiked) the sample preparation method. The repeatability of the method was confirmed with 6 prespiked samples and 6 postspiked samples. Prior analysis of the 10 standards by MS^E and data-dependent analysis modes allowed the characterization of m/z and retention time of the molecular ion adducts and fragments. For each standard, the most intense ion peak was selected for the recovery calculations.

RESULTS AND DISCUSSION

To identify the most suitable procedure for plasma sample preparation for UPLC−MS lipid profiling, we developed an exhaustive assessment of quantitative criteria for benchmarking the simplicity, protein removal efficiency, lipid coverage, repeatability, and recovery of each sample preparation method. Together, these criteria were used to select the optimal method from the eight sample preparations studied herein.

Simplicity of Protocols. Careful inspection of protocol steps showed that biphasic extractions are more time- and resource-consuming than precipitation methods (Figure S-1 in the Supporting Information).

Pipetting. For extraction methods based on halogenated solvents, collection of the lipid extract fraction required careful pipetting of the lower organic phase while avoiding any contamination by proteins at the interface, which could cause instrumental failure and decrease throughput. MeOH/MTBE/H_2O and MeOH/hexane/IPA protocols make this step slightly easier, since the lipid extract is on top of the aqueous phase and the protein pellet at the bottom of the tube after centrifugation. These methods still require one not to disturb the interface of the aqueous and lipid layer while collecting the lipid extract. Protein precipitation protocols result in a monophasic mixture of solvents with a protein pellet at the bottom of the tube, thus avoiding all the issues associated with the presence of an interface and decreasing risks of contamination of the lipid extract with proteins. In the current study, pipetting was manual, but these pipetting steps are critical when robotic sample preparation is considered. For instance, handling low viscosity solvents used in biphasic extraction protocols is not straightforward, as is the collection of organic phases located at the bottom of the container.

Drying. Extraction protocols also involve a drying step followed by a reconstitution by a mixture of solvents close to the initial mobile phase of the chromatographic system. This is due to the fact that direct injection of chlorinated solvents can result in poor chromatographic separation of lipid classes and can be detrimental to some parts of the autosampler and cause leakage of contaminants from polyetheretherketone (PEEK) tubing. This solvent exchange extends the time of sample preparation and increases the risk of introducing procedural error or altering the metabolic profile by loss of volatile metabolites or degradation of labile molecules.

Storage. In case immediate analysis is not possible, one must also consider the stability of the extract when stored at low temperature.[34] Loss of lipids only occurs with MeOH and ACN precipitation; chromatogram inspection showed no significant loss of lipids for IPA, IPA_ACN, CH_2Cl_2, $CHCl_3$, MTBE, and hexane sample preparations. This has also been reported in the literature. One of the direct advantages is that the IPA precipitation supernatant is stable until injection, therefore bypassing unnecessary sample preparations before reinjection in the case of instrumental failure for instance. (Figure S-2 in the Supporting Information).[24,35,36]

Cost. Aiming at deploying these methods in an ultrahigh throughput workflow, particular attention needs to be paid to solvent cost and their impact on the environment (i.e., preference for "green" solvents). First, extraction solvents are twice as expensive as solvents for precipitations and ACN is even three-times more expensive than IPA and MeOH used for precipitation. Second, compared to precipitation methods, biphasic extraction methods require greater solvent volumes, longer labor time, use of glass vials and dedicated instrumentation for the drying step (centrifugal vacuum concentrator or under nitrogen to evaporate organic phases). For instance, we estimated that consumables for extraction methods are roughly 5 times more expensive than precipitation methods, while the total time for manual interventions can be 2 to 3 times longer.

Safety. Finally, the solvents used in extraction and in several precipitation protocols (MeOH and ACN) are particularly harmful to human health: chloroform is known to be carcinogenic and *n*-hexane is neurotoxic.[37−39] In contrast, an alcohol such as isopropanol is reasonably safe making its handling easier.

Altogether, the addition of a protein precipitant solvent and the removal of the total liquid contents from a solid pellet is a simple and straightforward procedure, which is suitable for

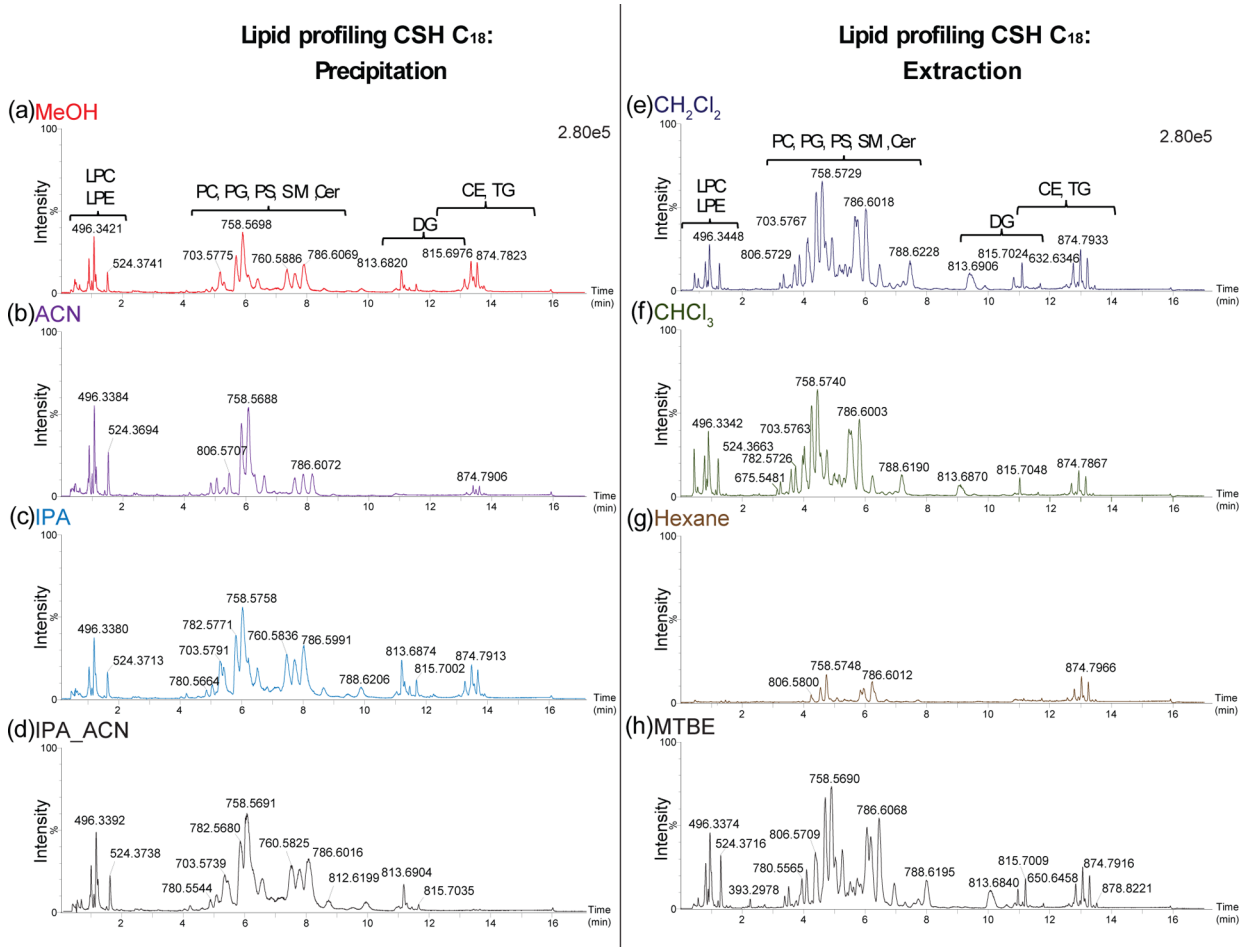


Figure 1. Representative chromatograms of plasma lipid profiling in positive mode. Precipitations: methanol (MeOH) (a), acetonitrile (ACN) (b), isopropanol (IPA) (c) and IPA combined with ACN (d). Extractions: MeOH combined with dichloromethane (CH_2Cl_2) (e), chloroform ($CHCl_3$) (f), hexane/IPA (Hexane) (g), and methyl-*tert* butyl ether/H_2O (MTBE) (h).

high-throughput workflows. However, a quantitative assessment is needed to make a clear recommendation.

Protein Removal Efficiency. Plasma composition is complex and the predominant critical step for sample preparation is to remove proteins. The presence of proteins in a liquid sample poses a challenge to the analytical instrumentation, decreasing column lifetime, causing ion suppression, and thus impacting on data quality.[40] After each procedure, the percentage of proteins remaining in the supernatant was evaluated with the Bradford method (Table S-2 in the Supporting Information).[5,25] The residual amount of proteins in samples was measured at ∼1% for precipitations and for MeOH/MTBE/H_2O and MeOH/hexane/IPA extractions in the organic phase. However, MeOH/CH_2Cl_2 and MeOH/$CHCl_3$ extractions were less efficient as ∼5% of proteins still remain in the organic phase. These results are in general agreement with previous studies on sample preparation for plasma.[5,40,41] MeOH precipitation is the standard method of plasma sample preparation because of the efficient protein precipitation (98%). To improve protein precipitation, the solvents were kept at 4 °C and added slowly to the sample.[8,10,42] It was also important that the mixture samples/solvent were stored overnight at −20 °C before centrifugation in order to maximize the precipitation of the large amount of proteins.

Lipid Coverage. Inspection of chromatograms indicated that plasma samples precipitated with ACN did not achieve a suitable detection of sphingomyelins (SM), diacylglycerols (DG), and triacylglycerols (TG) (Figure 1b). Likewise, we observed that MeOH/hexane/IPA extraction did not allow the detection of lysophospholipids (Figure 1h). However, samples precipitated with MeOH (Figure 1a) and IPA (Figure 1c) or samples extracted with MeOH associated by CH_2Cl_2 (Figure 1e), $CHCl_3$ (Figure 1f), and MTBE/H_2O (Figure 1h) offered broader lipid coverage.

PCA was used to evaluate the differences between the sample preparation methods in ESI+ and ESI− modes (Figure 2). The data set was acquired on 40 aliquots (five sample preparation replicates for each sample preparation method). First, the repeatability of the UPLC−MS run was verified by multiple injections of the pooled quality control (QC) sample constituted by pooling a 10 μL aliquot from all the samples: QCs were tightly clustered in the middle of the PCA scores plots which confirmed the reliability of the analytical platform.

As anticipated after initial inspection of the chromatograms, the unsupervised PCA model clearly indicated that the eight plasma sample preparation methods generated different LC−MS profiles. Moreover, in negative mode PC1 showed a separation between extractions and precipitations. We cannot rule out a confounding effect of the drying step in the extraction protocol, which could potentially result in loss of

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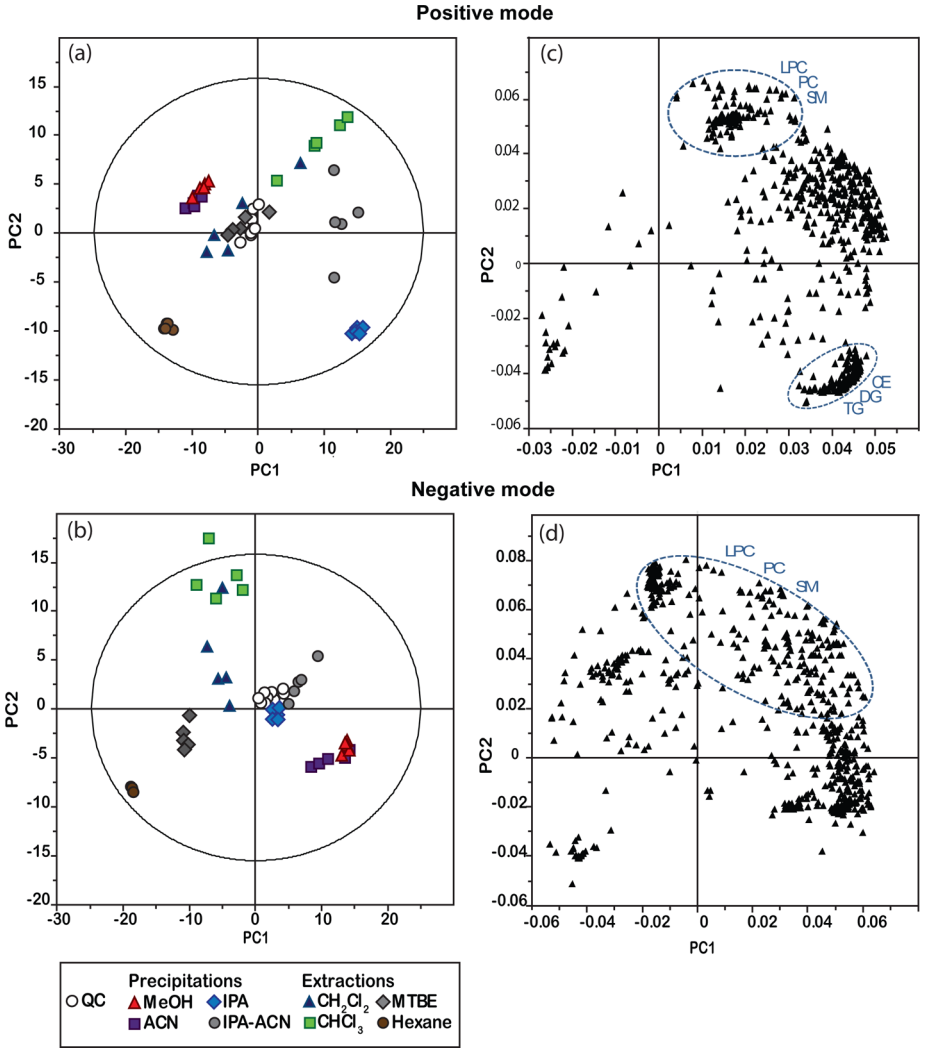

Figure 2. Scores plots (a and b) and loading plots (c and d) obtained from precipitations and extractions samples analyzed by lipid profiling in positive mode and negative mode. In PC1 and PC2 precipitation protocols lead to tight clustering of the samples while extraction protocols exhibit larger variability. Abbreviations: LPC, lysophophocholines; PC, phosphocholines; SM, sphingomyelines; TG, triacylglycerols; DG, diacylglycerols, CE cholesteryl esters.

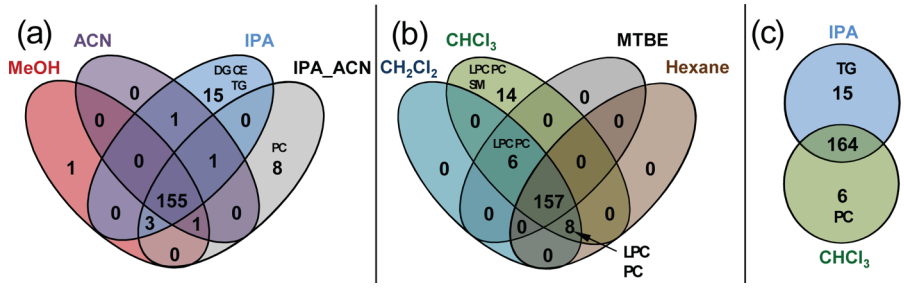

Figure 3. Venn diagrams show the overlapping selectivity between extractions (a), precipitations (b), and IPA vs MeOH combined with $CHCl_3$ (c) based on identified lipids. Lipid species detected after using the IPA protocol are highly similar to those detected after using the well-established $CHCl_3$/MeOH-based protocols.

analytes. The MeOH/MTBE/H_2O and MeOH/hexane/IPA extracted samples were better clustered than MeOH/CH_2Cl_2 and MeOH/$CHCl_3$ extracted samples in the score plot of PC1 vs PC2. This difference may be due to the physical location of the lipids during the biphasic extraction: withdrawing the organic phase directly at the top can confer a better clustering (for MeOH/MTBE/H_2O and MeOH/hexane/IPA). Precipitation results in tighter clustering in PCA than extraction. We interpret this broad-sense repeatability as being related to the simplicity of the protocols (which only involves the addition of single solvent). We then investigated the main sources of variation in the PCA, by inspecting score plots (Figure 2a,b) and loadings plots (Figure 2c,d)

To interpret the loadings, putative identification from the different databases of the features detected was carried out. Detailed inspection of features resulted in including parent ions

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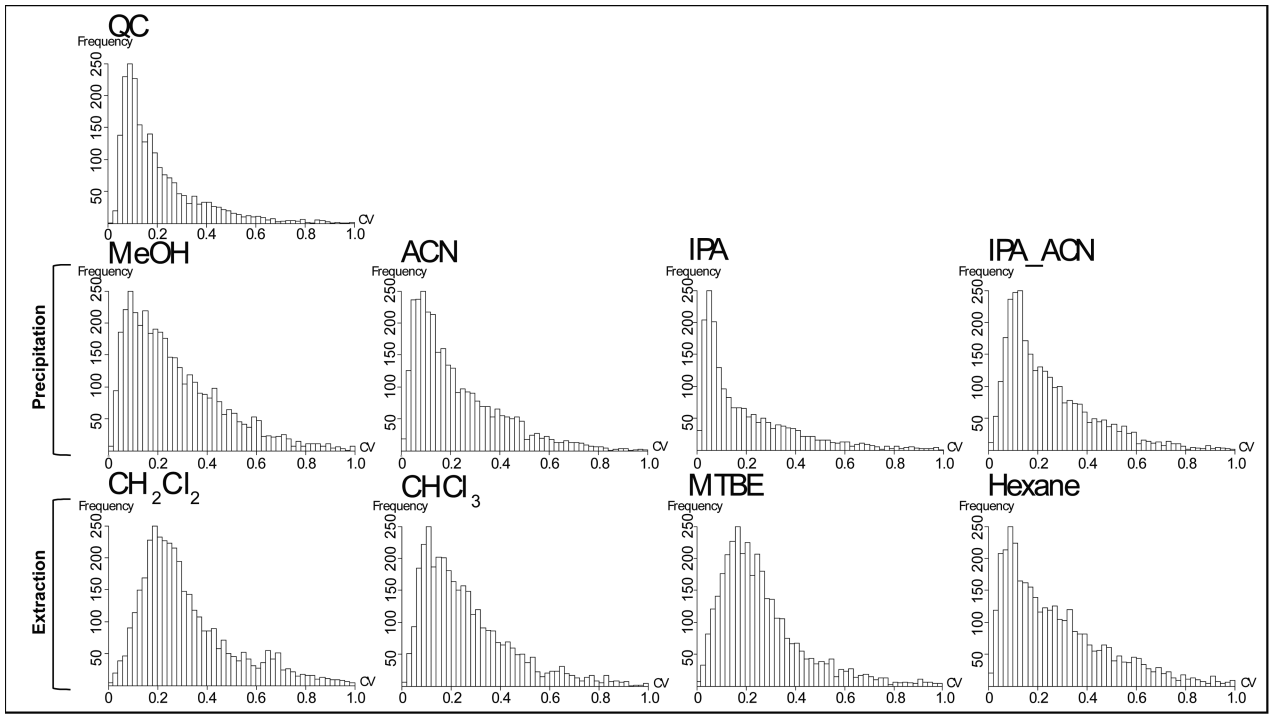

Figure 4. Histograms of the CVs to assess repeatability of the eight methods. Quality control, QC. Precipitations: MeOH, ACN, IPA, IPA_ACN. Extractions: MeOH combined with CH_2Cl_2 or $CHCl_3$, MTBE/H_2O (MTBE) and, hexane/IPA (Hexane).

but filtering out isotopes or adducts and also noisy features.[10,28] Data-dependent analysis (DDA) and MS^E result in the characterization of lipid species in both positive and negative ion modes. Numerous lipid classes were identified such as lysophosphatidylcholines (LPC), lysophosphatidylethanolamines (LPE), phosphatidylinositol (PI), phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidylglycerols (PG), sphingomyelins (SM), ceramides (Cer), diacylglycerols (DG), triacylglycerols (TG), and cholesteryl esters (CE). Altogether, the PCA loadings plots (Figure 2c,d) show that the main difference between sample preparation methods is due to the intrinsic lipid selectivity of each solvent.

The Venn diagrams in Figure 3 summarize the lipid selectivity of each sample preparation method. Analysis for both polarities lead to the detection of 724 features including 185 identified lipids. The peak intensities of the 185 lipids were compared between each method of sample preparation. As demonstrated in Figure 3a, IPA precipitation enables the inclusion of all classes of lipids and in particular improved TG detection compared to the other precipitations. These results also demonstrate that MeOH associated with $CHCl_3$ extraction result in an increase of lysophospholipids and phospholipids compared to the other extraction methods (Figure 3b). Moreover, it appears that MeOH combined to $CHCl_3$ only extracted 6 more phosphocholines than IPA, whereas the IPA precipitation protocol considerably increased our ability to study TG (Figure 3c). Clearly each lipid species have a specific interaction with the solvent according to their solubility. This may explain the differences of extraction observed between phospholipids (amphipathic) and TG (neutral). However, PCs are more observed than TGs as PCs well ionized in both modes and are more represented than TGs in plasma. Altogether, these results imply that IPA has a similar lipid selectivity and coverage as MeOH combined to $CHCl_3$ and is clearly superior in terms of standard recovery when comprehensive analysis of all lipid classes is required.

Repeatability. Untargeted lipid profiling is based on the relative comparison of spectral profiles and therefore a systematic QC strategy is necessary to assess repeatability of analytical workflows. All sample preparation methods were tested for their ability to provide similar profiles between replicates under the same conditions: each sample was split into five aliquots, which were extracted or precipitated independently. Stable retention times were obtained after conditioning the column with multiple (n = 15) injections of QC samples.[43] Typical variation of the retention times with an Acquity UPLC under our lipid profiling conditions did not exceed 0.3 s (CV around 0.16%) which is critical for correct data preprocessing and feature identification. The CV distribution for the detected features (retention time (RT) and m/z) in the pooled QC samples indicated that the instrument and samples were remarkably consistent over time. In fact 66.6% of the features from the pooled QC have a CV lower than 20%, which defines the instrumental repeatability estimate. To compare the CV distributions, we implemented a t test on CV distribution frequencies (Table S-3 in the Supporting Information) and demonstrated that all sample preparation methods are significantly different from the sample preparation QC (p < 0.05), apart from IPA (p = 0.892) and IPC_ACN (p = 0.360).

To evaluate the repeatability of each sample preparation method, the CVs for sample preparation were calculated for features detected in the five replicates and independently prepared in different batches for each method (Figure 4 and Table S-4 in the Supporting Information). The results provide compelling evidence that IPA enabled a robust lipid extraction since 61.1% of the features had a CV lower than 20%. The IPA repeatability at 20% threshold (61.1% of features with a CV < 20%) is similar to the intrinsic instrumentation repeatability determined with the pooled QC (66%), whereas the other

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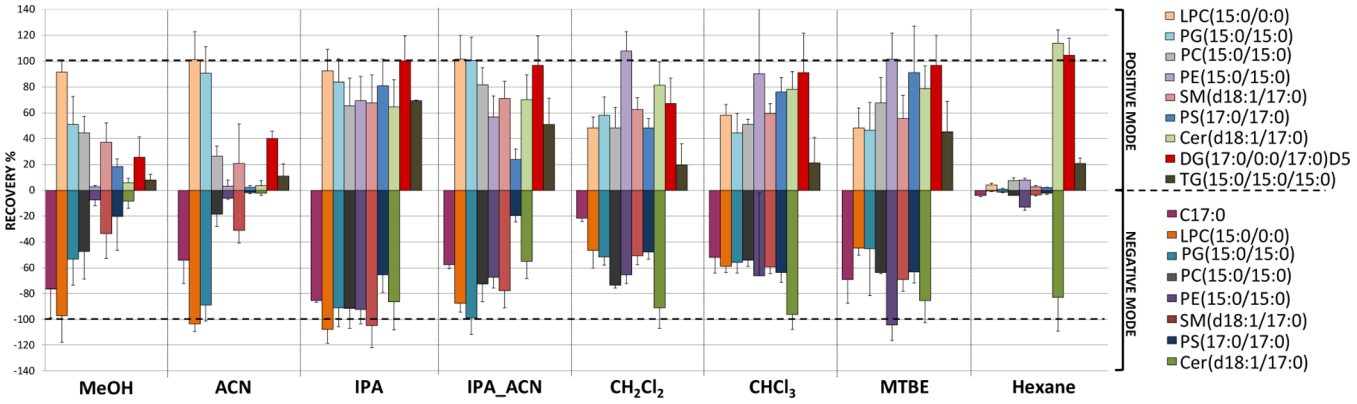

Figure 5. Histogram summarizing the data obtained from the lipid recoveries. Samples are compared according to the ESI± mode and sample preparation method. The error bars show the standard deviation of the replicates recovery.

Table 1. Synopsis of the Criteria Evaluated for the Eight Sample Preparation Methods[a]

Methods		Advantages			Constraints		
Precipitation	MeOH	Two steps - CV <20% - 99% protein removal		Broad lipid coverage	Loss of diacylglycerols and triacylglycerols		Poor recoveries > 30-40%
	ACN			-	Loss of sphingomyelins, diacylglycerols and triacylglycerols		
	IPA			Best repeatability 61.1% features <20% - Broad lipid coverage - Excellent recoveries > 60-80%	-		
	IPA_ ACN			Recoveries ˜ 70%	Loss of triacylglycerols		
Extraction	$CHCl_3$	CV <25%	-	Broad lipid coverage - Recoveries ˜ 60%	Multiple steps	95% protein removal	Organic phase on bottom
	CH_2Cl_2						-
	MTBE		Organic phase on top			99% protein removal	Loss of lysophospholipids, phospholipids and sphingomyelins - Poor recoveries >20%
	Hexane			-			

[a]Precipitations: MeOH, ACN, IPA, and IPA combined with ACN. Extractions: MeOH combined with CH_2Cl_2, $CHCl_3$, hexane/IPA (Hexane), and MTBE/H_2O (MTBE).

methods are less repeatable (<55.4%). Interestingly, IPA protocol is not only the best precipitation method for lipid profiling but also presented a better CV distribution than the well-established MeOH/MTBE/H_2O and MeOH/$CHCl_3$-based extraction protocols.

Lipid Recovery. The efficiency of each one of the 8 sample preparation methods was then tested for the capacity to quantitatively recover 10 lipid standards spiked into the samples before and after the treatment (Table S-5 in the Supporting Information). Lipid standards selected for this purpose were nonendogenous lipids (i.e., odd carbon chains), and their structures were similar to endogenous lipids found in human plasma.[44] The recovery was calculated as the ratio of peak areas of the internal standards in sample spiked before sample-preparation (prespiked samples, defining the amount of exogenous standard detected) to those added after sample-preparation (postspiked samples used to define the amount of detectable exogenous standard, see Figure 5 and Table S-6 in the Supporting Information).

As initially expected, the area under the peak for internal standards in prespiked samples is lower than in postspiked samples (~12% difference), which allowed the derivation of a sample preparation yield. However, some of the standards are only observed in one mode. This is typically the case for the C17:0 standard, which we only observed in ESI−. Likewise, the standards for TG(15:0/15:0/15:0) and DG(17:0/0:0/17:0)D5 were only observed in positive mode. The other lipid families (lysophosphocholines, phosphoglycerols, phosphatidylcholines, phosphatidylethanolamines, sphingomyelins, phosphatidylserines, and ceramides) can ionize in both positive and negative modes and in this case, there are little variations but we did not find them significant between positive and negative modes. Finally, as for variations between sample preparation methods, our paper provides key under-reported data and fills a huge gap in terms of sample preparation yield. As previously observed in the chromatograms (Figure 1g), MeOH/hexane/IPA extraction only allowed a selective extraction, which resulted in losing lysophospholipids, phospholipids, and SMs, as demonstrated by their poor recovery (<20%). Similar conclusions were drawn for ACN sample preparation methods regarding the SM, DG, and TG lipid species (Figure 1b). Traditional MeOH precipitation presented comparable recoveries to the ACN precipitation. However, the PC recovery appeared to be better with ACN (>80%) compared to MeOH method (<60%). Lipid extraction by MeOH/CH_2Cl_2, MeOH/$CHCl_3$, and MeOH/MTBE/H_2O yielded a recovery higher than 50% for most of

dx.doi.org/10.1021/ac500317c | Anal. Chem. 2014, 86, 5766−5774

Table 2. Eight Methods Were Scored against the Evaluation Criteria[a]

		MeOH	ACN	IPA	IPA_ACN	CH_2Cl_2	$CHCl_3$	MTBE	Hexane
simplicity (1 mark each)	pipetting	1	1	1	1	0	0	0.5	0.5
	drying	1	1	1	1	0	0	0	0
	storage	0	0	0.5	0.5	0.5	0.5	0.5	0.5
	cost	1	0	1	0	0.5	0.5	0.5	0.5
	safety	0	0	1	0	0	0	0	0
protein removal efficiency (3 marks)		3	3	3	3	1.5	1.5	3	3
repeatability (5 marks)		2.5	2.5	5	2.5	0	2.5	2.5	2.5
lipid coverage (2 marks)		2	1	2	2	1	2	1	1
lipid recovery (5 marks)		0	0	5	2.5	2.5	2.5	2.5	0
total scores		11.5	9	20.5	13.5	6.5	10.5	11	8

[a]Scores are in the range from 0 to 1:0 if the method does not meet evaluation criteria, 0.5 if the method partially meets evaluation criteria, and 1 if the method fully meets evaluation criteria.

the lipid species but some exceptions such as TG recovery decrease the universality of these methods. Conversely, IPA precipitation offered a highly consistent and reliable recovery (>60−80%) for the 10 lipids standards tested. These results show that IPA precipitation had significantly higher recovery than the other methods.

Isopropanol Precipitation and Its Suitability for High-Throughput Workflows and Molecular Epidemiology. In this study we evaluated several sample preparation methods for lipid profiling using various assessment criteria: throughput, protein removal efficiency, selectivity, repeatability, and recovery (Tables 1 and 2). The number of steps involved in each preparation method varied between two and four for precipitations and extractions, respectively. As a result, protocols can be ranked by decreasing throughput as follow: MeOH ≈ ACN ≈ IPA ≫ IPA_ACN ≫ MeOH/MTBE/H_2O ≈ MeOH/hexane/IPA > MeOH/$CHCl_3$ ≈ MeOH/CH_2Cl_2. Moreover, severe variations in selectivity were observed by multivariate analysis between the different methods, providing evidence of significant loss for some lipid species. In a metabonomic and metabolic phenotyping context, repeatability is one of the most important criteria to improve the reliability of the various analytical assays leading to the identification of potential biomarkers. Performance of analytical methods is evaluated using the U.S. Food and Drug Administration (FDA) or European Medicine Agency (EMA) guidances. For absolute quantification of xenobiotics, the current threshold for repeatability is 15%, but for endogenous biomarker discovery, more flexibility is allowed (currently 30%); it has finally been suggested that a threshold of 20−25% is adequate to evaluate the CV of the assay.[45,46] In this respect, precipitation methods have proven to be valuable alternatives to extraction protocols.

Protein removal procedures involving acid, heat, and organic solvents were extensively compared in the literature.[5,29,43,47−49] Generally, denaturation of proteins by acid or heat is limited in reproducibility of the features and metabolome coverage. Want et al. and Bruce et al. studied the impact of different plasma to organic solvent ratios on chromatograms. These studies concluded that organic solvent precipitations are more efficient and more robust compared to acid or heat removal. A plasma to organic solvent ratio of 1:3 was found to be the upper limit in terms of protein removal efficiency.[40] We therefore selected this ratio for the four precipitation methods.

Our current findings expand prior work as we conducted an extensive comparison between extraction and precipitation method.[8,20,23,43] Folch or Bligh and Dyer protocols have long been the preferred method of plasma sample preparation for LC−MS analyses.[16,17] However, there are concerns about safety issues with extraction solvents and requirement of less toxic solvent for plasma sample preparation. In addition, our results clearly show that liquid−liquid extraction methods are complex to handle and less repeatable than precipitation methods.

IPA precipitation enables protein removal in a single step procedure prior to lipid profiling. The presence of remaining metabolites in the monophasic solution does not affect UPLC−MS detection of lipids as the chromatographic gradient starts with aqueous solvent, which results in elution of the polar metabolites.

CONCLUSIONS

Ultimately, taking into account the above-mentioned criteria we found the most suitable sample preparation for lipid profiling was isopropanol precipitation (Table 1). Table 2 presents a weighted score system for all criteria. High-throughput Phenome centers operate with a certain set of constraint such as running thousands of samples per year and therefore requires simplicity, protein removal efficiency, lipid coverage, repeatability of lipids measurements, and recovery efficiency. However, research environments with less emphasis on sample throughput may settle with different analytical settings. We provide compelling evidence that isopropanol precipitation method is a straightforward one-step procedure. Isopropanol sample preparation allows the analysis of a wide range of lipids, which is critical for optimal biological interpretation. We conclude that isopropanol precipitation is a promising sample preparation method for large-scale lipidomic studies and is easily amenable to molecular epidemiology studies involving thousands of samples in high-throughput analytical workflows.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors would like to thank Dr. Panagiotis Vorkas for helpful discussions on lipidomic analysis. M.H.S. is funded by the Nestlé-Imperial College Alliance (Grant RDLS015375). M.G. was funded by Technologie Servier (Grant P25106). This work was supported by the Medical Research Council and National Institute for Health Research (Grant Number MC_PC_12025).

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