In-Vial Dual Extraction for Direct LC-MS Analysis of Plasma for Comprehensive and Highly Reproducible Metabolic Fingerprinting.

ARTICLE *in* ANALYTICAL CHEMISTRY · JUNE 2012

Impact Factor: 5.64 · DOI: 10.1021/ac300716u · Source: PubMed

5 AUTHORS, INCLUDING:

Luke Whiley
Defence Science and Technology Laboratory …
11 PUBLICATIONS **88** CITATIONS
SEE PROFILE

Joanna Barbara Godzien
University Foundation San Pablo CEU
13 PUBLICATIONS **107** CITATIONS
SEE PROFILE

Cristina Legido-Quigley
King's College London
63 PUBLICATIONS **1,187** CITATIONS
SEE PROFILE

Coral Barbas
University Foundation San Pablo CEU
190 PUBLICATIONS **3,990** CITATIONS
SEE PROFILE

analytical chemistry

Article

pubs.acs.org/ac

In-Vial Dual Extraction for Direct LC-MS Analysis of Plasma for Comprehensive and Highly Reproducible Metabolic Fingerprinting.

Luke Whiley,[†,‡,§,⊥] Joanna Godzien,[†,∥,⊥] Francisco J Ruperez,[†] Cristina Legido-Quigley,*[,‡] and Coral Barbas*[,†]

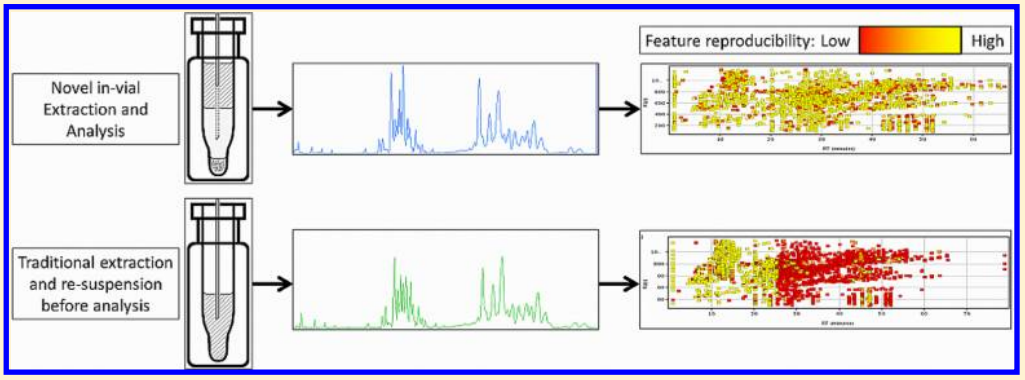
[†]Center for Metabolomics and Bioanalysis (CEMBIO), Facultad de Farmacia, Campus Monteprincipe, Universidad CEU San Pablo, 28668 Boadilla del Monte, Madrid, Spain

[‡]Institute of Pharmaceutical Sciences, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, United Kingdom

[§]Institute of Psychiatry, King's College London, De Crespigny Park, London, SE5 8AF, United Kingdom

[∥]Department of Molecular Biology, Faculty of Mathematics and Natural Sciences, The John Paul II Catholic University of Lublin, Krasnicka 102, 20-718 Lublin, Poland

Supporting Information



ABSTRACT: Metabolic fingerprinting of biological tissues has become an important area of research, particularly in the biomarker discovery field. Methods have inherent analytical variation, and new approaches are necessary to ensure that the vast numbers of intact metabolites present in biofluids are detected. Here, we describe an in-vial dual extraction (IVDE) method and a direct injection method that shows the total number of features recovered to be over 4500 from a single 20 μL plasma aliquot. By applying a one-step extraction consisting of a lipophilic and hydrophilic layer within a single vial insert, we showed that analytical variation was decreased. This was achieved by reducing sample preparation stages including procedures of drying and transfers. The two phases in the vial, upper and lower, underwent HPLC-QTOF analysis on individually customized LC gradients in both positive and negative ionization modes. A 60 min lipid profiling HPLC-QTOF method for the lipophilic phase was specifically developed, enabling the separation and putative identification of fatty acids, glycerolipids, glycerophospholipids, sphingolipids, and sterols. The aqueous phase of the extract underwent direct injection onto a 45 min gradient, enabling the detection of both polarities. The IVDE method was compared to two traditional extraction methods. The first method was a two-step ether evaporation and IPA resuspension, and the second method was a methanol precipitation typically used in fingerprinting studies. The IVDE provided a 378% increase in reproducible features when compared to evaporation and a 269% increase when compared to the precipitate and inject method. As a proof of concept, the method was applied to an animal model of diabetes. A 2-fold increase in discriminant metabolites was found when comparing diabetic and control rats with IVDE. These discriminant metabolites accounted for around 600 entities, out of which 388 were identified in available databases.

Because of vast variations in the physicochemical properties of analytes, current methodology can only identify subsets of the metabolic content of biological samples. Most comprehensive approaches use a combination of technologies, for example LC-MS, NMR, and GC-MS. Recent work by the Human Metabolome Project utilized these approaches to comprehensively profile human serum metabolites (<1500 Da), resulting in the confirmation of 4600 individual components.[1]

Although this multi-pronged approach can provide a wealth of data, analysis time and cost are greatly increased. Along with this and perhaps of more important, in many cases the quantity of biofluid is limited. Therefore, for the metabolic fingerprinting

Received: March 13, 2012
Accepted: June 15, 2012
Published: June 16, 2012

   dx.doi.org/10.1021/ac300716u | *Anal. Chem.* 2012, 84, 5992–5999

field to progress, efforts must be made to increase metabolite coverage within each analytical technique.

LC-MS (along with NMR) is currently one of the main techniques used to profile metabolites from biological samples. The technique can boast the benefit of on column separation of molecules prior to mass spectral analysis, ensuring it can provide a broad metabolic picture of the sample.[2] However, issues still arise; for example, components that are not fully resolved can undergo ion suppression in the mass spectrometer source, meaning nonseparated components compete with one another for ionization and therefore detection, reducing selectivity and accuracy. Along with this, matrix components, e.g., endogenous phospholipids, are a significant source of imprecision in analyses conducted by LC-MS/MS. Present at relatively high concentrations they are heavily associated with influencing ion suppression and enhancement effects and, thus, introduce analytical variation.[3]

LC-MS approaches to metabolic fingerprinting typically tend to employ a simple protein precipitation extraction designed to remove protein, avoiding column degradation and blockage. Following this, extracts are injected onto an LC system and analyzed by a reversed phase gradient.[4−7] However, when utilizing this general approach, the extremes of the metabolite spectrum are overlooked, and potentially important biomarker candidates are lost. For example, highly polar compounds wash off in the chromatographic conditions, which front the chromatogram as they undergo little to no retention, while highly nonpolar species can be insoluble in the extraction solvents used in metabolite studies, e.g., methanol and acetonitrile, and are either not efficiently extracted or remain in-column, unable to undergo elution.

Increasing metabolite coverage by optimization of sample pretreatment has been investigated previously. For example, one recent publication by Yanes et al.[8] examined a range of extraction protocols, combined with a range of modified chromatographic and MS detection methods. An improved single-phase "all-in-one" extraction was developed from *E.coli* samples whereby the coverage of analyzed molecules was increased.

Increased coverage by the completion of dual extractions, one designed for polar molecules and the other for nonpolar, has previously been investigated by Masson et al.[9] A number of methods underwent comparison that either extracted polar metabolites prior to nonpolar extraction or used a two-phase extraction using two solvents that separate (dichloromethane and methanol). Analysis was then completed via UPLC-MS with the findings suggesting that the optimal protocol for profiling both polar and nonpolar metabolites utilized an aqueous extraction with water/methanol prior to an organic extraction with dichloromethane/methanol.

Lipidomics, a subset of metabolomics, is particularly prevalent in biomarker studies as a large number of disease states are associated with irregularities in lipid metabolism, including neurological Alzheimer's,[10−13] schizophrenia,[14,15] Parkinson's,[16,17] cardiac (atherosclerosis[18]), viral,[19] and bacterial infection[20] as well as obesity[21] and insulin-resistant diabetes.[22] Furthermore, it is well-known that lipids play vital roles in cellular functions, including membrane regulation,[23] source of reserve energy within vacuoles,[24] and cell signaling transduction processes.

Along with the problems of sample extraction, it is also of critical importance that when extracting metabolites for fingerprinting that no "false" metabolites are introduced to the sample via the pretreatment process. An effect that highlights this is the fact that it is commonly known that unsaturated fatty acids and lipid species readily undergo oxidation when exposed to atmospheric conditions, so care must be taken when drying or transferring any extracts. Other systematic errors introduced by extraction protocols are loses of sample when transferring sample between vials and tubes during extraction; the more stages of transfer within a procedure, the more chances of introducing these errors.

Therefore, the aim of this work was the development of a novel two-phase metabolic fingerprinting method that results in both a lipophilic nonpolar as well as a hydrophilic polar extract from just 20 μL of plasma. The complete extraction and analysis was designed to be completed within the same LC vial. Each phase was individually analyzed using LC-QTOF-MS, using an optimized gradient for the metabolites present in each layer. The method was designed to increase the component coverage from small sample size and to improve the reproducibility.

With these factors considered, the method presented within this paper is capable of analysis within a single sample vial, which involves no sample transfer and hence reduces preparation time and analytical variation. Along with this, metabolites remain constantly suspended in solution, therefore, reducing the risk of modification due to external influences such as drying loses.

The result is a novel "in-vial dual extraction" (IVDE) and direct injection metabolic fingerprinting method that both increases metabolite coverage while decreasing the analytical variability, making it ideal for comprehensive metabolic profiling and biomarker discovery.

MATERIALS AND METHODS

Chemicals and Reagents. Ultrapure water, used to prepare all the aqueous solutions, was obtained "in-house" from a Milli-Qplus185 system (Millipore, Billerica, MA, USA). LC-MS grade methanol, acetonitrile, and analytical grade formic acid were purchased from Fluka Analytical (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Analytical grade ammonia hydroxide (30% ammonium in high purity water) was acquired from Panreac Quimica SA (Barcelona, Spain) and analytical grade methyl-*tert*-butyl-ether (MTBE) from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). A C15 triacylglycerol (tripentadecanoin) with mass 764.6894 (C48O6H92) was purchased from Larodan Fine Chemicals AB (Malmö, Sweden) and was used as an internal standard. Chromacol 03-FIV HPLC Vials with fixed 0.3 mL glass inserts (Chromacol, Welwyn Garden City, UK) were chosen.

Analytical Setup. The analysis was completed using an HPLC system (1200 series, Agilent Technologies, Waldbronn, Germany) coupled to an Agilent QTOF (6520) with electrospray ionization source. The HPLC system consisted of a degasser, two binary pumps, temperature controlled autosampler, and column oven. During all analysis, two reference masses were used: m/z 121.0509 ($C_5H_4N_4$) and m/z 922.0098 ($C_{18}H_{18}O_6N_3P_3F_{24}$) for positive ionization mode and m/z 112.9856 ($C_2O_2F_3(NH_4)$) and m/z 1033.9881 ($C_{18}H_{18}O_6N_3P_3F_{24}$) for negative ionization mode. These masses are continuously infused to the system to allow constant mass correction.

Samples. The extraction method and analytical conditions for analysis were developed using human plasma from a healthy volunteer. The study designed to test the biomarker discovery

dx.doi.org/10.1021/ac300716u | *Anal. Chem.* 2012, 84, 5992−5999

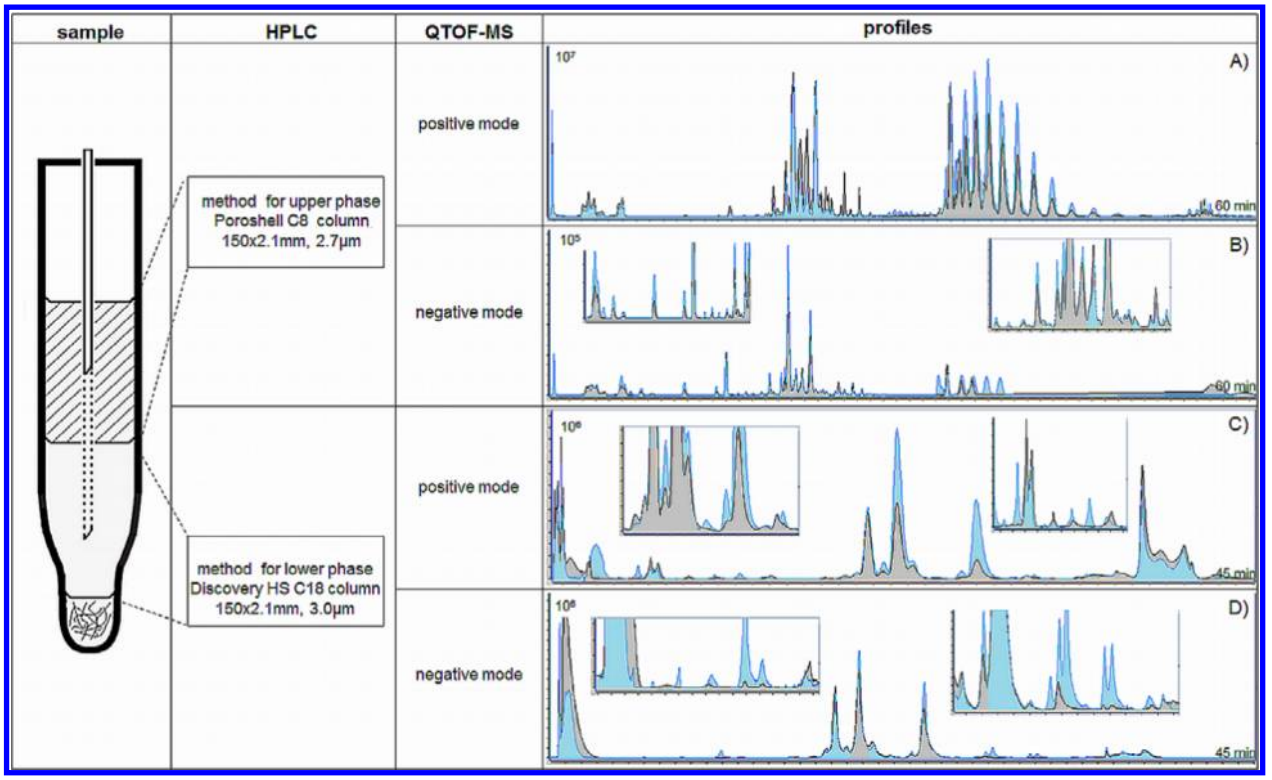


Figure 1. Schematic of the IVDE method with two different specialized chromatographic conditions and two polarity modes. Extracted compound chromatograms (ECC) for upper phase analyzed (A) in positive mode, (B) in negative mode, (C) for lower phase in positive mode, and (D) for lower phase in negative mode. Chromatograms in gray are representative for controls and in blue for diabetics.

capabilities of the obtained method was performed using rat plasma from streptozotocin (STZ) diabetic and control (age and sex matched) Sprague–Dawley adult rats from the animal quarters of University San Pablo-CEU. More details about animals and samples can be found in the Supporting Information as was described previously.[26]

IVDE Method Extraction. The IVDE method uses methyl-*tert*-butyl ether (MTBE) as an organic phase solvent previously used by Matyash et al.[21] During this procedure, the whole plasma extraction and separation of the two phases (ether and water) was performed within a HPLC vial. The volumes of the method were adapted to suit the size of the vial inserts. A total of 20 μL of plasma was mixed with 10 μL of Milli-Q water. Proteins were precipitated with 40 μL of methanol, vortex-mixed for 2 min, and then 200 μL of MTBE containing 10 μg/mL of internal standard was added prior to mixing of the whole mixture via vortex at room temperature for 1 h. After adding 50 μL of miliQ water, the sample was mixed and centrifuged at 3000*g* for 10 min. The upper MTBE and lower methanol–water phases were then injected onto the LC-MS system directly from the vial by adjustment of the instruments needle height in two separate runs, at 5 or 15 mm from the bottom (positions −5 and +5 in autosampler settings) with the same drawing and ejecting speed (200 μL/min).

Evaporation Method. Conditions were as previously described by Matyash et al.[21] Briefly, in glass tubes, 20 μL of plasma, 10 μL of MS-grade water, and 40 μL of MS-grade methanol were vortex-mixed for 2 min, and then 200 μL of MTBE (containing 5 μg/mL internal standard) was added. The capped tube was vortex-mixed for 1 h, and then 50 μL of MS-grade water was added and vortex-mixed for 2 min. Tubes were centrifuged at 3000*g*. From the upper phase, 50 μL was removed and transferred to new glass tubes. Samples were evaporated to dryness at 35 °C in a vacuum evaporator (Thermo Fisher Scientific, Wathmam, MA, USA) and finally resuspended (vortex for 2 min) in 50 μL of isopropanol (IPA) before being transferred to a HPLC vial and injected onto the LC-MS system.

LC-MS for IVDE Upper Phase. Analysis was completed at 60 °C using an Agilent Poroshell 120 EC-C8 column (150 mm × 2.1 mm, 2.7 μm). A gradient was employed consisting of mobile phase A (10 mM ammonium formate in Milli-Q water) and mobile phase B (10 mM ammonium formate in methanol) pumped at 0.5 mL/min. Initial conditions at time 0 were 75% B, increasing to 96% B in 23 min. This was then held until 45 min. The gradient then increased to 100% B by 46 min and held until 50 min. Starting conditions were returned by 51 min, and a 9 min re-equilibration time was included, taking the total run time to 60 min. Chromatographic conditions were previously optimized as described below (Figure 1).

Mass spectrometry detection was performed in both positive and negative ESI mode in full scan from 50 to 1200 m/z. The mass spectrometer source conditions consisted of a capillary voltage of 3500 V (positive mode) or 4500 V (negative mode), while both ionization modes used a scan rate of 1.02 scans per second, nebulizer gas flow rate of 10.0 L/min, source temperature of 350 °C, and source pressure of 40 psig. The same method was used when analyzing the evaporation method described above.

LC-MS Settings for IVDE Lower Phase. Conditions were as previously described:[6,28] 10 μL of extracted sample was injected onto a reversed-phase C18 column (Discovery HS C18 150 mm × 2.1 mm, 3 μm; Supelco) with a guard column (Discovery HS C18 20 mm × 2.1 mm, 3 μm; Supelco).

dx.doi.org/10.1021/ac300716u | *Anal. Chem.* 2012, 84, 5992–5999

Separation was performed at 40 °C at the flow rate 0.6 mL with solvent A composed of water with 0.1% formic acid, and solvent B consisted of acetonitrile also with 0.1% formic acid. Metabolites were eluted using general gradient started from 25% B to 95% B in 35 min, and returned to initial conditions in 1 min, with 9 min re-equilibration time (Figure 1).

Mass spectrometry detection was completed in both positive and negative ESI mode in full scan from 50 to 1000 m/z for positive mode and from 50 to 1050 m/z for negative mode. The capillary voltage was accordingly 3000 V for positive polarity and 4000 V for negative ionization. The scan rate of 1.02 scan per second, nebulizer gas flow rate of 10.5 L/min, temperature 325 °C, and pressure 52 psig were the same for both ionization modes.

Precipitation and Injection Method. *Extraction.* The extraction method has been previously employed by the CEMBIO division for plasma metabolic fingerprinting.[6,28] This method consisted of protein precipitation with 3 volumes of cold (frozen) methanol:ethanol mixture (1:1) to 1 volume of plasma. Samples were vortex-mixed and then incubated on ice for 5 min and centrifuged at 16000g at 4 °C for 20 min. Collected supernatant was filtered through a 0.22 μm nylon syringe filter and passed to a HPLC vial for the analysis.

LC-MS. Chromatographic and mass spectrometric conditions were the same as described in the previous paragraph for the lower hydrophilic phase.

Data Treatment. Data analysis was completed using the same approach for all data collected. The resulting data file was cleaned of background noises and unrelated ions by the Molecular Feature Extraction (MFE) tool in MassHunter Qualitative Analysis Software (B.04.00, Agilent). The MFE then creates a listing of all possible components as represented by the full TOF mass spectral data. The software settings for data reprocessing are in the Supporting Information.

Data pretreatment, including alignment and filtering, was performed in MassProfiler Professional (B.02.01, Agilent). Data was filtered by choosing the data that were present in all samples under any condition. Precise software details and settings regarding the data processing are in the Supporting Information.

Databases for Identification. Metabolites were identified by searching by mass accuracy against the online available databases such as the METLIN (http://metlin.scripps.edu), HMDB (http://hmdb.ca), KEGG (http://genome.jp/kegg), and lipidMAPS (http://ipidMAPS.org). KEGG and lipidMAPS were accessed for batch search through the MassTRIX (Mass Translator into Pathways, http://metabolomics.helmholtz-muenchen.de/masstrix2/), and in this case, the error mass used for this database was adjusted to 7 ppm (the highest available). For features that returned no batch match, individual searching in databases was performed, increasing the error to 20 ppm.

RESULTS AND DISCUSSION

IVDE extraction consists of adding two nonmiscible solvents to 20 μL of plasma in a 300 μL vial insert. The procedure leads to a separation of analytes that disperse into either phase, meaning successive re-extractions with solvents of different polarities will not be necessary to improve the detection of less polar compounds. Two different chromatographic methods optimized for each type of metabolites were employed with the aim of decreasing matrix effects, increasing resolution, and compound identification.

Selection of Solvents for IVDE. The IVDE method selected methyl-*tert*-butyl ether (MTBE) as extraction solvent over a number of published alternatives, especially the more commonly accepted Folch method,[29] because of its density and polarity. The Folch method separates with a layer of protein intersecting the phases, making the collection of both layers challenging. Figure 1S of the Supporting Information demonstrates the advantages of a MTBE-based extraction when used in this IVDE approach. The adapted MTBE extraction produced two layers of solvent above the protein precipitate, which formed a solid pellet of protein in the base of the vial. Conversely, it is shown that the traditional Folch lipid extraction, which employs chloroform as a solvent, resulted in the lipid phase sitting below a floating protein precipitate due to the higher density of chloroform. Traditional analysis of this dense phase requires careful collection, following the removal of both the upper aqueous and proteinacious layers. Working with small volumes, such as the method described here (20 μL of plasma is required), is a delicate operation, increasing the risk of analytical errors and the introduction of variation.

As commented previously, the IVDE method resulted in a protein pellet in the vial enabling easy injection of the two phases, while removing the risk of column blockages caused by the precipitate. The ether extraction method described by Matyash et al.[27] has been extensively tested against the Folch method, resulting in comparable if not favorable extraction recoveries. It includes the transfer of the organic phase, evaporation to dryness, and resuspension in isopropanol, which were avoided here.

A further advantage of the completion of the extraction using the method described here was that MTBE was found to be suitable for direct injection onto the LC-MS gradient. Traditionally, the injection of 100% organic solvent is not advised when completing LC separation because of the adverse effect it can have on early eluting peaks. However, as the separation described here is completed over the course of an hour long gradient, the direct injection of MTBE was found to have no effect on the reproducibility and separation achieved, including the early eluting peaks. Therefore, in using MTBE as an extraction solvent, not only is the extraction method and collection procedure simpler but it removes a drying and resuspension stage from the protocol, reducing the risk of drying losses, analytical variation, and introducing potentially oxidative conditions to dried lipid species.

Arguably the greatest benefit to using the described IVDE extraction is the ability to complete the extraction and analysis in one simple in-vial procedure. It was found that by adding the plasma and extraction solvents to a capped LC vial the extraction could be completed prior to direct injection. The ability to manually adjust the injector needle enabled both the upper and the lower phase to be directly injected onto a column in separate runs.

IVDE Development of LC-MS Conditions for the Upper and Lower Phases. Different columns, gradients, and temperatures were tested to optimize the chromatographic separation for the upper phase. Starting conditions were adapted from Sandra et al.[30] who recently developed a method for plasma lipidomics. Results were compared in terms of (i) visual inspections of chromatograms looking at intensity of the signal and number and aspect of the peaks, (ii) total number of features obtained, (iii) number of features detected in every sample of triplicate injections and Relative Standard Deviation (RSD%) to check method repeatability, (iv) quality of

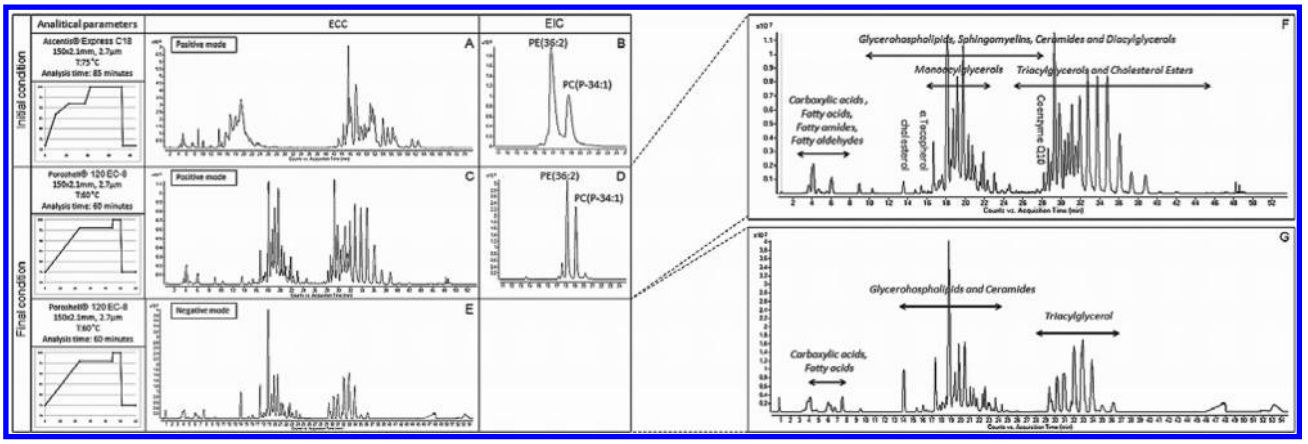


Figure 2. Initial and optimized chromatographic conditions for the IVDE upper phase extract of human plasma. (A) Extracted compound chromatograms (ECC) for initial conditions in positive mode. (B) Extracted ion chromatogram (EIC) for PE(36:2) and PC(P-34:1). (C) ECC for optimized conditions in positive mode. (D) EIC for PE(36:2) and PC(P-34:1). (E) ECC for optimized conditions in negative mode. (EIC) showing the traces for PE(36:2) and PC(P-34:1). (F) Enlarged C. (G) Enlarged E.

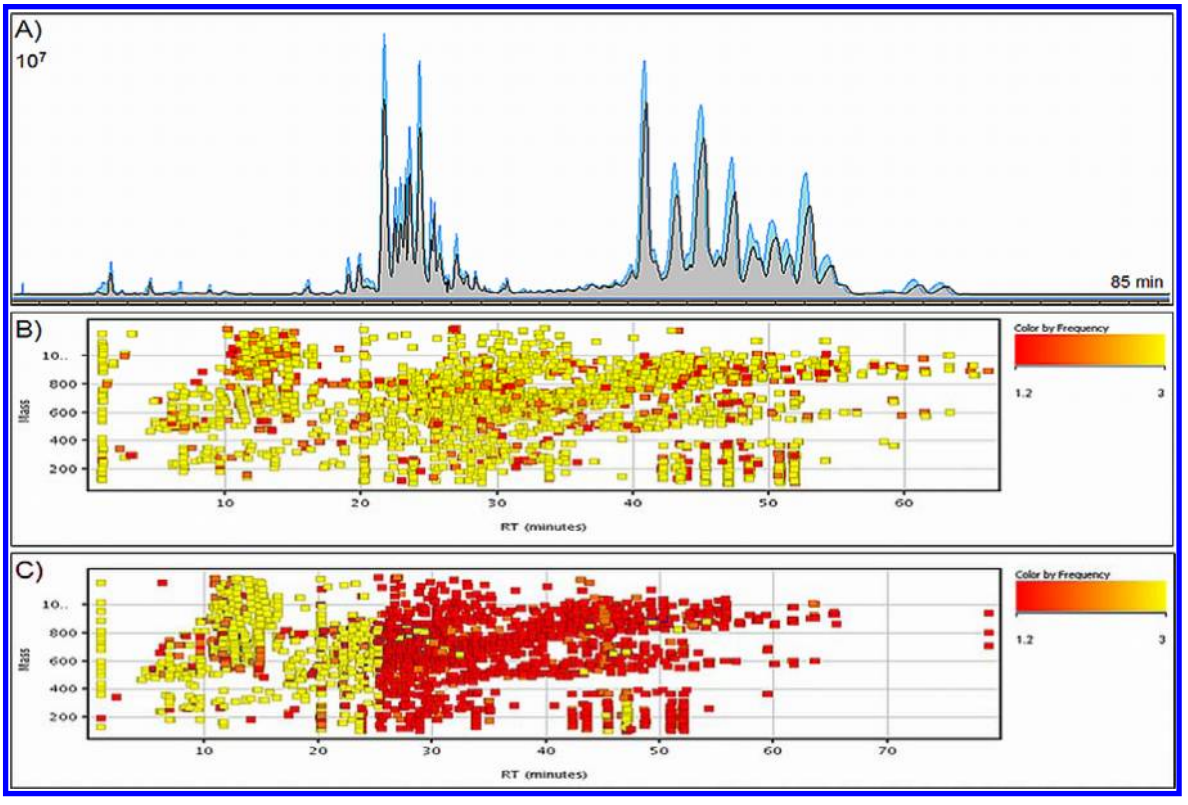


Figure 3. (A) Extracted compound chromatograms (ECC) for IVDE (blue) and for the evaporation approach (gray). LC-MS feature maps colored by frequency of three plasma replicates obtained by IVDE (B) and evaporation approach (C).

separation for two phospholipids: PE(36:2) and PC(P-34:1) that differ in mass by 0.0364 Da to inspect resolution, and (v) number of features identified in databases from those identified in total to test the real usefulness of the method.

Figure 2 summarizes initial and final conditions as well as the extracted compound chromatograms (ECC) (Figure 2A,C,E) and extracted ion chromatograms (EIC) showing PE(36:2) and PC(P-34:1) traces (Figure 2B,D). Many modifications were made from the original conditions. The change in type of particles from totally porous to superficially porous enabled the reduction of column temperature from 75 to 60 °C, while still providing an efficient separation of lipid features, protecting column lifetime. Along with this, the optimized gradient developed on the octylsylane phase enabled a reduction in the overall runtime from 85 to 60 min.

For the LC-MS analysis of the IVDE lower phase, a standard gradient with reversed-phase chromatography previously optimized for total plasma[4,6] was employed. That was done under the hypothesis that after eliminating nonpolar compounds, ion suppression would decrease and a higher number of compounds could be identified with an appropriate sensitivity and reproducibility.

Comparison of IVDE with Evaporation of the Upper Phase. Once the chromatographic conditions were optimized, a separate experiment compared a direct injection of the IVDE upper phase with a traditional transfer, evaporation, resuspen-

dx.doi.org/10.1021/ac300716u | Anal. Chem. 2012, 84, 5992−5999

sion approach common in many profiling methods.[30] It was found that the IVDE direct injection provided a higher and more reproducible signal, illustrated in Figure 3.

Reproducibility of both methods was examined by comparing the RSD% values for features detected in all three replicate extractions of the same plasma pool. Table 1 (panel A and B)

Table 1. Comparison between Samples Prepared with the In-Vial Dual Extraction (IVDE) Method and Evaporation and Resuspension Approach[a]

parameter	panel A evaporation	panel B IVDE
number of features present in all samples	603	2280
RSD < 30%	338	1912
RSD < 20%	280	1738
RSD < 10%	182	1358
RDS < 5%	86	821
RSD < 30% [%]	56.05	83.86
RSD < 20% [%]	46.43	76.23
RSD < 10% [%]	30.18	59.56
RDS < 5% [%]	14.26	36.01
TS	2.24×10^9	2.95×10^9
TUS	1.95×10^8	2.88×10^9

[a]TS, total signal: sum of signals for all features detected in all replicates. TUS, total useful signal: sum of signals only for features common for all three samples. [%]: percent of features with expected RSD among number of features present in all samples.

clearly demonstrates a higher number (378% increase) of molecules extracted using the IVDE method with an acceptable (below 30%) RSD% value as compared to the evaporation approach.

Along with this, a comparison between methods was then made by examining the RSD% of those metabolites putatively identified via database matching in both the IVDE and evaporation methods. The results of this are listed in Table 1, with the IVDE approach showing increased total metabolite features as well as a higher rate of reproducibility, represented by more features with a lower RSD%.

Other comparison parameters that were used to examine the methods were: Total Signal (TS), which is the sum of signals for all features detected in all three replicates, and Total Useful Signal (TUS), which is the sum of signals only for common features found in all three samples. TS obtained with both types of sample treatments is comparable (Table 1), while the TUS differs significantly. The difference between TS and TUS is very small for the IVDE approach, but for the evaporation method, the difference is comparatively large, further confirming the superior reproducibility of sample treatment when performing IVDE direct injection.

In addition, both methods were compared in terms of number of metabolites found and identified in databases, as well as their biochemical classes (Table 1S in the Supporting Information). It was observed that in all categories of lipids more compounds were identified within the IVDE method. More specifically, glycerophospholipids and glycerolipids show the largest differences (from 15 identified with evaporation in comparison to 256 with the IVDE aproach). Only the number of features identified as peptides was higher using the evaporation method.

This benefit observed when completing the IVDE analysis is believed by the authors to be a result of the reduction of sample preparation steps and, therefore, reducing the number of instances that variation can be introduced to samples. The approach also avoids drying and transfer losses; however, the exact reason for this loss of metabolites is difficult to identify.

Comparison of IVDE with Precipitation and Injection Approach. Further testing and validation of the IVDE method was demonstrated by the completion of an evaluation study designed to compare the novel IVDE approach to that of a currently accepted method employing a single-phase precipitate and inject project.

As listed in Table 2, splitting the sample extraction into two phases using the IVDE approach gave a 256% increase in

Table 2. Panel A: Comparison of Coverage between Human Plasma Prepared Using the In-Vial Dual Extraction (Ivde) Method and Precipitate and Inject Method. Panel B: Comparison of Coverage between Controls and Diabetic Rats Prepared Using the In-Vial Dual Extraction (IVDE) Method and Precipitate and Inject Method[a]

	panel A			
	IVDE upper + lower phase		precipitate and inject	
parameter	positive mode	negative mode	positive mode	negative mode
number of features present in all samples	3636	826	1020	636
RSD < 30%	3120	696	916	418
RSD < 20%	2762	556	852	310
RSD < 10%	1738	348	698	126
RDS < 5%	637	210	489	38
RSD < 30% [%]	85.81	84.26	89.80	65.72
RSD < 20% [%]	75.96	67.31	83.53	48.74
RSD < 10% [%]	47.80	42.13	68.43	19.81
RDS < 5% [%]	17.52	25.42	47.94	5.97
number of identified features	1425	314	493	283
	panel B			
	IVDE upper + lower phase		precipitate and inject	
parameter	positive mode	negative mode	positive mode	negative mode
number of features present in all samples	3568	908	1361	446
number of statistically significant masses	536	165	140	79
number of identified features	293	102	99	72
number of pathways	46	33	28	16
number of significant pathways	13	16	11	5

[a]"Significant pathways" describes pathways where more than three metabolites are identified via database searching of MassTrix.

reproducible features (positive ionization) and a 30% increase (negative ionization) compared to the precipitate and inject method, meaning a total increase of 269%.

Comparison of IVDE with Precipitation and Injection Approach in a Biomarker Discovery Model. The IVDE method was developed for future biomarker identification studies; therefore, its capabilities for this role had to be examined. Plasma samples were obtained from rats in two groups; rats that received streptozotocin (D group) and their corresponding sex and age-matched controls (C group). It is known that streptozotocin selectively destroys the beta cells of the Langerhans islets from the pancreas, and therefore, insulin

dx.doi.org/10.1021/ac300716u | Anal. Chem. 2012, 84, 5992−5999

cannot be further synthesized, mimicking the complications of type 1 diabetes.[31]

Table 2 (panel B) shows the results for a comparison of coverage between the two groups of samples. Again, the samples were prepared via both IVDE and the precipitate and inject method. In the IVDE method, the results from upper and lower phase were combined to provide a comprehensive overview.

To evaluate the two analytical approaches, the total number of features present in all samples for each group was compared, with results showing an increase in the number of statistically significant and identified features in the IVDE method than for the one prepared with the precipitate and inject method. The IVDE method increased the number of detected reproducible features by 162% in positive MS and 103% for negative MS. The total number of statistically significant features found to be responsible for the separation between samples from control and diabetic animals also increased in the IVDE method by 283% in positive MS and 109% in negative MS. The statistically significant molecules then underwent putative database identification, with the IVDE method again providing an increase of 196% and 42% more features identified in positive MS and negative MS, respectively.

In addition to this, the MassTrix metabolic pathway translator was employed to analyze both approaches, with the two-phase method providing information on approximately twice the number of pathways than the traditional single method.

Table 2S in the Supporting Information provides a list of the features acquired with the IVDE method when applied to a biomedical study. This data was collated, providing more than 600 different features that were expressed different between diabetics and controls.

The table's aim is to enable this method to be easily assessed and provide information for its implementation. It is out of the scope of this methodology-based paper to discuss in detail all the changes that were observed. The table presented allows the reader to see putative metabolites that show changes between the sample groups. Some of them coincide with others previously described, and there are some previously unreported that may therefore present promising areas for further research.

CONCLUSIONS

Over the course of this study, an IVDE fingerprinting method has been developed that was designed to decrease analytical variation and expand the global coverage of metabolite identification resulting in >4500 reproducible features from a 20 μL plasma extract. This was achieved by the development of an in-vial two phase extraction, which was then able to undergo direct analysis on a LC-QTOF-MS. The method was exhaustively tested and provided data that demonstrated an increase in the overall number of features and a decrease in the variability observed between injections when compared to two off-line methods. The method was applied to an animal model of diabetes, and data mining confirmed its advantages for fingerprinting.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: +[34]913724711 (C.B.), fax: +[34]913724712 (C.B.), e-mail: cbarbas@ceu.es (C.B.), cristina.legido_quigley@kcl.ac.uk (C.L.-Q.).

Author Contributions

⊥These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

J.G., F.J.R., and C.B. acknowledge the EADS-CASA and Spanish Ministry of Science and Innovation (MICINN) MICINN CTQ2011-23562.

L.W. and C.L.-Q. acknowledge the NHS National Institutes of Health Research (NIHR) Biomedical Research Centre (BRC) for mental health at the South London and Maudsley (SLaM).

REFERENCES

(1) Psychogios, N.; Hau, D. D.; Peng, J.; Guo, A. C.; Mandal, R.; Bouatra, S.; Sinelnikov, I.; Krishnamurthy, R.; Eisner, R.; Gautam, B.; Young, N.; Xia, J.; Knox, C.; Dong, E.; Huang, P.; Hollander, Z.; Pedersen, T. L.; Smith, S. R.; Bamforth, F.; Greiner, R.; McManus, B.; Newman, J. W.; Goodfriend, T.; Wishart, D. S. *PLoS One* **2011**, *6* (2), e16957.

(2) Wilson, I. D.; Plumb, R.; Granger, J.; Major, H.; Williams, R.; Lenz, E. M. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2005**, *817* (1), 67−76.

(3) Chambers, E.; Wagrowski-Diehl, D. M.; Lu, Z.; Mazzeo, J. R. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2007**, *852* (1−2), 22−34.

(4) Ciborowski, M.; Martin-Ventura, J. L.; Meilhac, O.; Michel, J. B.; Ruperez, F. J.; Tunon, J.; Egido, J.; Barbas, C. *J. Proteome Res.* **2011**, *10* (3), 1374−1382.

(5) Greenberg, N.; Grassano, A.; Thambisetty, M.; Lovestone, S.; Legido-Quigley, C. *Electrophoresis* **2009**, *30* (7), 1235−1239.

(6) Ciborowski, M.; Ruperez, F. J.; Martinez-Alcazar, M. P.; Angulo, S.; Radziwon, P.; Olszanski, R.; Kloczko, J.; Barbas, C. *J. Proteome Res.* **2010**, *9* (8), 4131−4137.

(7) Loftus, N.; Barnes, A.; Ashton, S.; Michopoulos, F.; Theodoridis, G.; Wilson, I.; Ji, C.; Kaplowitz, N. *J. Proteome Res.* **2010**, *10* (2), 705−713.

(8) Yanes, O.; Tautenhahn, R.; Patti, G. J.; Siuzdak, G. *Anal. Chem.* **2011**, *83* (6), 2152−2161.

(9) Masson, P.; Alves, A. C.; Ebbels, T. M.; Nicholson, J. K.; Want, E. J. *Anal. Chem.* **2010**, *82* (18), 7779−86.

(10) Pettegrew, J. W.; Panchalingam, K.; Hamilton, R. L.; McClure, R. J. *Neurochem. Res.* **2001**, *26* (7), 771−782.

(11) Han, X. L.; Holtzman, D. M.; McKeel, D. W.; Kelley, J.; Morris, J. C. *J. Neurochem.* **2002**, *82* (4), 809−818.

(12) Pratico, D.; Clark, C. M.; Lee, V. M. Y.; Trojanowski, J. Q.; Rokach, J.; FitzGerald, G. A. *Ann. Neurol.* **2000**, *48* (5), 809−812.

(13) Montine, T. J.; Kaye, J. A.; Montine, K. S.; McFarland, L.; Morrow, J. D.; Quinn, J. F. *Arch. Pathol. Lab. Med.* **2001**, *125* (4), 510−512.

(14) Dietrich-Muszalska, A.; Kontek, B. *Psychiatry Clin. Neurosci.* **2010**, *64* (5), 469−475.

(15) Fenton, W. S.; Hibbeln, J.; Knable, M. *Biol. Psychiatry* **2000**, *47* (1), 8−21.

(16) Cheng, D.; Jenner, A. M.; Shui, G.; Cheong, W. F.; Mitchell, T. W.; Nealon, J. R.; Kim, W. S.; McCann, H.; Wenk, M. R.; Halliday, G. M.; Garner, B. *PLoS One* **2011**, *6* (2), e17299.

(17) Huang, X.; Chen, H.; Miller, W. C.; Mailman, R. B.; Woodard, J. L.; Chen, P. C.; Xiang, D.; Murrow, R. W.; Wang, Y. Z.; Poole, C. *Mov Disord* **2007**, *22* (3), 377−81.

(18) Kreisberg, R. A.; Oberman, A. *J. Clin. Endocrinol. Metab.* **2002**, *87* (2), 423−37.
(19) Negro, F. *Gut* **2010**, *59* (9), 1279−87.
(20) Zaas, D. W.; Duncan, M.; Rae Wright, J.; Abraham, S. N. *Biochim. Biophys. Acta, Mol. Cell Res.* **2005**, *1746* (3), 305−313.
(21) Nicholas, S. B. *Curr. Hypertens. Rep.* **1999**, *1* (2), 131−6.
(22) Savage, D. B.; Petersen, K. F.; Shulman, G. I. *Physiol. Rev.* **2007**, *87* (2), 507−20.
(23) van Meer, G.; Voelker, D. R.; Feigenson, G. W. *Nat. Rev. Mol. Cell Biol.* **2008**, *9* (2), 112−124.
(24) Zweytick, D.; Athenstaedt, K.; Daum, G. *Biochim. Biophys. Acta, Rev. Biomembr.* **2000**, *1469* (2), 101−120.
(25) Pawson, T.; Nash, P. *Science* **2003**, *300* (5618), 445−52.
(26) Godzien, J.; Ciborowski, M.; Angulo, S.; Ruperez, F. J.; Paz Martínez, M.; Señorans, F. J.; Cifuentes, A.; Ibañez, E.; Barbas, C. *J. Proteome Res.* **2011**, *10* (2), 837−844.
(27) Matyash, V.; Liebisch, G.; Kurzchalia, T. V.; Schevchenko, A.; Schwudke, D. *J. Lipid Res.* **2008**, *49* ((5)), 1137−1146.
(28) Ciborowski, M.; Teul, J.; Martin-Ventura, J. L.; Egido, J.; Barbas, C. *PLoS One* **2012**, *7* (2), No. e31982, DOI: 10.1371/journal.-pone.0031982.
(29) Folch, J.; Ascoli, I.; Lees, M.; Meath, J. A.; LeBaron, N. *J. Biol. Chem.* **1951**, *191* (2), 833−41.
(30) Sandra, K.; Pereira Ados, S.; Vanhoenacker, G.; David, F.; Sandra, P. *J. Chromatogr., A* **2010**, *1217* (25), 4087−99.
(31) Ganda, O. P.; Rossini, A. A.; Like, A. A. *Diabetes* **1976**, *25* (7), 595−603.