analytical chemistry

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# Systematic Evaluation of Extraction Methods for Multiplatform-Based Metabotyping: Application to the *Fasciola hepatica* Metabolome

Jasmina Saric,[†] Elizabeth J. Want,[†] Urs Duthaler,[‡] Matthew Lewis,[†] Jennifer Keiser,[‡] John P. Shockcor,[§] Gordon A. Ross,[#] Jeremy K. Nicholson,[†] Elaine Holmes,*[,†] and Marina F. M. Tavares*[,†,∥]

[†]Biomolecular Medicine, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, Sir Alexander Fleming Building, South Kensington, London SW7 2AZ, United Kingdom

[‡]Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, CH-4002 Basel, Switzerland and University of Basel, CH-4003 Basel, Switzerland

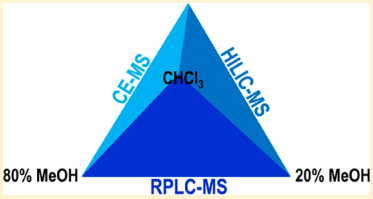
[§]Pharmaceutical Business Operations, Waters Corporation, Milford, Massachussetts 01757, United States

[#]Agilent Technologies Life Sciences and Chemical Analysis, Lakeside, Cheadle Royal Business Park, Stockport, Cheshire, SK8 3GR, United Kingdom

[∥]Institute of Chemistry, University of Sao Paulo, P.O. Box 26077, 05513-970, Sao Paulo, SP, Brazil

Supporting Information

**ABSTRACT:** Combining data from multiple analytical platforms is essential for comprehensive study of the molecular phenotype (metabotype) of a given biological sample. The metabolite profiles generated are intrinsically dependent on the analytical platforms, each requiring optimization of instrumental parameters, separation conditions, and sample extraction to deliver maximal biological information. An in-depth evaluation of extraction protocols for characterizing the metabolome of the hepatobiliary fluke *Fasciola hepatica*, using ultra performance liquid chromatography and capillary electrophoresis coupled with mass spectroscopy is presented. The spectrometric methods were characterized by performance, and metrics of merit were established, including precision, mass accuracy, selectivity, sensitivity, and platform stability. Although a core group of molecules was common to all methods, each platform contributed a unique set, whereby 142 metabolites out of 14,724 features were identified. A mixture design revealed that the chloroform:methanol:water proportion of 15:59:26 was globally the best composition for metabolite extraction across UPLC-MS and CE-MS platforms accommodating different columns and ionization modes. Despite the general assumption of the necessity of platform-adapted protocols for achieving effective metabotype characterization, we show that an appropriately designed single extraction procedure is able to fit the requirements of all technologies. This may constitute a paradigm shift in developing efficient protocols for high-throughput metabolite profiling with more-general analytical applicability.




Metabolic profiling using mass spectrometry (MS) coupled with ultra performance liquid chromatography (UPLC) or gas chromatography (GC) and nuclear magnetic resonance (NMR) spectroscopy have been successfully applied to the characterization of systemic responses of organisms to disease, pharmaceutical intervention, and dietary modulation.[1−3] In such studies, the adequacy of a given analytical platform is typically dependent upon the class of chemical compounds under investigation, the cost of analysis, the ease of sample preparation, and the requirement for sensitivity, specificity, and robustness. No single method enables complete coverage of the entire metabolic information and, increasingly, metabolic profiling studies are adopting more than one analytical platform to augment the number of metabolites identified and thereby enhance the extraction of biological information.

Although the literature is scattered with platform-specific sample preparation procedures,[4] there is a paucity of studies reporting the systematic evaluation of sample preparation across multiple platforms.[5] Despite the recent technological developments in the field of sample preparation of biofluids, spanning from the more-traditional protein precipitation methods,[6−9] liquid−liquid or solid-phase extractions[10,11] and microextractions[12] to the more-sophisticated use of molecularly imprinted polymers[13] and restricted-access materials,[14] sample extraction continues to be a crucial and time-consuming step of any analytical method, with important implications on the information recovered and analytical interpretation. This is particularly true for comprehensive metabolic profiling, where the chemical complexity, sample heterogeneity, and wide concentration range of endogenous metabolites place a strong demand on the extraction procedure.[15] Metabolite losses, matrix effects, artifacts, and analytical variability are often

**Received:** April 3, 2012
**Accepted:** July 12, 2012
**Published:** July 12, 2012

   dx.doi.org/10.1021/ac300586m | *Anal. Chem.* 2012, 84, 6963−6972

inevitable,[16] which indicate that the ultimate goal of full characterization of a metabolome will be a challenging task for many biological samples.

To preserve sample integrity, methods with minimal sample treatment are desirable and often applicable for liquid biological samples, such as urine or plasma, where simple dilution followed by filtration and/or centrifugation are the norm. However, for solid or semisolid biological samples, such as faeces and tissues, more-elaborate sample preparation procedures are required. Tissue extraction is usually performed by the cooled homogenization of tissue, followed by the stepwise addition of reagents and solvents of differing polarities such as perchloric acid or methanol−chloroform mixtures.[17] Other experimental approaches include the use of automated homogenizers,[18−20] microdialysis tissue sampling,[21] and solid-phase extraction with a large variety of sorbents.[22]

Here, we apply a multiplatform strategy for a more-comprehensive assessment of the metabolic composition of tissues using the parasitic hepatobiliary trematode *Fasciola hepatica* as a model system. *F. hepatica* infects livestock and imposes a considerable economic burden across the globe linked to decreased productivity of the affected animals.[23] Because of its zoonotic character, fascioliasis has emerged as human infection in the last two decades, whereby an estimated 91 million people are at risk. Although some host spots of prevalence have been identified, such as western Europe, the Andes, and Egypt, human cases have been reported from as many as 51 countries.[24] Disease management is currently suboptimal since diagnosis is largely based on microscopic examination of helminth eggs in stools, whereby detection capacity is limited in early and light infection. The first-line therapeutic intervention is limited to one main compound, namely triclabendazole, whereby resistances have already been reported.[25] Metabolic characterization of the fluke may aid in deepening the understanding of the biochemical communication between parasite and host and may provide leads for identifying novel diagnostic markers and drug targets at the metabolic level.

We evaluated the extraction of metabolic information from spectral profiles acquired across five different analytical methods. A quadrupole time-of-flight (Q-ToF) mass analyzer was used for UPLC-MS analysis in combination with two different column chemistries, i.e. reversed-phase liquid chromatography (RPLC) using a C18 column and hydrophilic interaction liquid chromatography (HILIC), in both positive and negative electrospray ionization modes (ESI+ and ESI−, respectively). Capillary electrophoresis coupled with mass spectroscopy (CE-MS) was applied in ESI+ mode only. A mixture design was used to define the optimal solvent composition for the global platform combination, and thus derive a single procedure that can be applied generically for tissue extraction across a range of biomedical samples and analytical platforms. In addition to providing an optimized and augmented metabolic screening capacity, such an approach would also facilitate the mathematical modeling across different analytical platforms, since the preparation parameters are maintained regardless of platform, thereby reducing additional sources of variation introduced by differential extraction efficiency of metabolites.[26]

## MATERIALS AND METHODS

**Materials.** Water, acetonitrile, methanol, and chloroform (chromatography-grade) used in the tissue extractions were obtained from Sigma (Gillingham, U.K.), as well as most of the standards used to confirm the identity of the chromatographic peaks. Cholic acid derivatives were acquired from Steraloids, Inc. (Newport, RI, USA) and phospholipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) (see Table S-1 in the Supporting Information). Stock standard solutions were prepared at 1 mg/mL in chromatography-purity water. Working solutions used in the identification and MS/MS fragmentation studies were prepared at concentrations of 0.010−0.050 mg/mL.

**Sample Preparation.** Fresh cattle livers were obtained from an abattoir in Basel, Switzerland, and live *F. hepatica* worms were recovered from the bile ducts at the Swiss Tropical and Public Health Institute (Basel, Switzerland). Worms were snap frozen and forwarded on dry ice to Imperial College London and kept at −40 °C.

For characterization of the *F. hepatica* metabolome and for assessment of the methods performance, two batches of six individual flukes (∼80 mg each) obtained from two different livers were thawed and weighed. Each worm was placed into a separate 2 mL plastic tube containing 500 mg of 1 mm-diameter zirconia beads (Stratech Scientific, Ltd., U.K.). One milliliter (1 mL) of 80% methanol, which was previously cooled to 4 °C, was added to each tube. The tubes were processed in a tissue homogenizer (Precellys 24, Peqlab, Ltd., U.K.) in two 30 s cycles of 6,000 rpm and subsequently centrifuged for 5 min at 18,894 *g*. The supernatants were transferred into Eppendorf tubes that were placed on ice. The extraction procedure was repeated once by adding 500 μL of cold methanol to the remaining pellet in each tube, followed by homogenization and centrifugation as described above. Both supernatants were combined and divided in different aliquots for spectral assessment (i.e., equal aliquots of 100 μL for UPLC-MS and CE-MS analysis), and the remainder was retained as a backup. The extracts were dried overnight in a speedvac (Eppendorf concentrator plus, Eppendorf UK, Ltd., Cambridge, U.K.) at 45 °C, under vacuum and a rotational speed of 1,400 rpm (the *g*-force varies according to the position of the tubes in the rotors between 130 *g* and 250 *g*). Dried extracts were stored at −40 °C prior to UPLC-MS and CE-MS analysis.

In order to evaluate the optimal solvent extraction condition to suit multiple analytical platforms, a mixture design study was performed. A total of 13 *F. hepatica* worms (∼400 mg total) were ground together in liquid nitrogen using a mortar and pestle. Samples of the ground tissue (∼30 mg) were transferred into separate Eppendorf tubes and 1 mL of a given proportion of cooled solvent/solution (4 °C) was added to the tissue as follows: (1) = 1,000A, (2) = 1,000B, (3) = 100B + 900C, (4) = 500A + 500B, (5) = 500A + 500C, (6) = 500B + 500C, (7) = 750A + 250B, (8) = 250A + 750B, (9) = 250B + 750C, (10) = 700A + 150B + 150C, (11) = 150A + 700B + 150C, (12) = 150A + 150B + 700C, and (13) = 333A + 333B + 333C (where A = 80% methanol, B = 20% methanol, and C = pure chloroform). Samples were vortexed for 1 min and centrifuged (18,894 *g*; 5 min), and the upper aqueous phases were transferred into new separate Eppendorf tubes. The same amount of the solvent mixture was added to the remaining pelleted biomass and the extraction procedure was repeated. The aqueous phase was again harvested and combined with the previous extract into a single Eppendorf tube. Final volumes were 2 mL after the addition of 80% methanol. The samples were vortexed again and divided into equal aliquots of 200 μL each for UPLC-MS, CE-MS analysis and a backup sample. The

aqueous extracts were dried overnight at 45 °C under vacuum. The organic phase was collected and stored at −40 °C but was not analyzed further here, since the aqueous phase gave information related to both the low-molecular-weight components and lipids.

The dried aliquots for UPLC-MS and CE-MS were dissolved in 200 μL of 50% methanol and 20% methanol, respectively, vortexed for 20 s, and subsequently sonicated for 5 min. A volume of 120 μL was transferred either into the wells of a 96-well plate (Waters, Hertfordshire, U.K.) for UPLC-MS or to the CE-MS sample vials (Agilent Technologies UK, Ltd., Edinburgh, U.K.). A quality control (QC) sample pool was prepared by mixing 5 μL of each aqueous extract sample in a separate Eppendorf tube. At the beginning of each of the chromatographic/electrophoretic runs, five injections from the QC sample pool were made; further QC injections were made after every five samples throughout the run, in order to assess repeatability and platform stability.

**Instrumentation and Data Acquisition.** All extracts were analyzed on a UPLC system (UPLC Acquity, Waters Ltd., Elstree, U.K.) coupled online via electrospray ionization to a Q-ToF Premier mass spectrometer (Waters MS Technologies, Ltd., Manchester, U.K.), using both a Waters Acquity UPLC BEH C18 column (1.8 μm, 2.1 × 100 mm) at 50 °C and a Waters Acquity HILIC BEH column (1.7 μm, 2.1 × 100 mm) at 40 °C, operated under gradient elution, as follows. For C18: A = 0.1% formic acid in water, B = 0.1% formic acid in methanol, under a flow rate of 0.4 mL/min. Gradient elution: 0−2 min, 99.9% A:0.10% B; 6 min, 75% A:25% B; 10 min, 20% A:80% B, 12 min, 10% A:90% B, 21−23 min, 0.10% A:99.9% B, 24−26 min: 99.9% A:0.10% B. For HILIC: A = 95% acetonitrile: 5% 200 mmol/L ammonium acetate, containing a total of 0.1% formic acid; B = 50% acetonitrile:50% 20 mmol/L ammonium acetate, containing a total of 0.1% formic acid, under a flow rate of 1.4 mL/min. Strong wash solvent was 5% acetonitrile, whereas the weak wash solvent was 95% acetonitrile. Gradient elution: 0−1 min, 99% A:1% B; 12 min, 100% B; 12.1−15 min, 99% A:1% B.

Other chromatographic conditions common to both modes include the temperature of the autosampler compartment (4 °C), the injection volume (5 μL), and the injection loop option (partial loop with needle overfill). Capillary voltage was 3,200 V (positive ionization) and 2,400 V (negative ionization), and the sample cone voltage was maintained at 35 V. The desolvation temperature was set to 350 °C and the source temperature was set to 120 °C; the cone gas flow and desolvation gas flow were maintained at 25 and 900 L/h, respectively. The Q-ToF Premier was operated in V optics mode, with a data acquisition rate of 0.2 s and a 0.01 s interscan delay. Leucine enkephalin ($m/z$ 556.2771) was used as lockmass, whereby a solution of 200 pg/μL in 50% acetonitrile was infused into the instrument at a rate of 3 μL/min via an auxiliary sprayer. Data were collected in centroid mode with a scan range of 50−1000 $m/z$, with lockmass scans collected every 15 s and averaged over three scans to perform mass correction.

CE-MS analysis was performed on an Agilent 7100 capillary electrophoresis system coupled to an Agilent 6224 Accurate-Mass time-of-flight mass spectrometer (Agilent Technologies, Waldbronn, Germany) by means of a coaxial spray needle configured to an electrospray ionization source. A fused-silica capillary (Composite Metal Services, Hallow, U.K.) with an internal diameter of 50 μm, an outer diameter of 375 μm, and a total length of 65 cm was used as a separation device for the positive ionization mode. New fused-silica capillaries were conditioned for 30 min with 1 mol/L NaOH at a pressure of 1 bar, followed by 10 min with deionized water and 30 min with a background electrolyte (BGE) composed of 0.80 mol/L formic acid at pH 1.8 containing 20% methanol. Before analysis, the capillary was conditioned by pressure flushes (925 bar) of 1 mol/L NaOH (10 min), 0.10 mol/L NaOH (10 min), deionized water (10 min), and BGE (30 min) at 25 °C. In both cases, the capillary was conditioned with the distal end outside of the MS source. Between runs, the BGE vials were replenished to a height of 1.5 cm and the capillary was flushed with BGE for 5 min. At the end of the day, the capillary was pressure-flushed with deionized water (10 min), methanol (10 min), and air (10 min). Samples were injected hydrodynamically (50 mbar for 10 s), followed by injection of BGE (50 mbar, 5 s). The CE system was operated under a constant voltage of +30 kV, and the cartridge was thermostatized at 25 °C. A sheath liquid (SHL) composed of 70% methanol containing 0.5% formic acid was delivered at a flow rate of 4 μL/min via a 1:100 splitter connected to an isocratic pump (1260 Infinity Series, Agilent Technologies) running at 400 μL/min. The nebullizer was set to 10 psig at 0.5 min after injection and a flow of heated dry nitrogen gas (150 °C) was maintained at a rate of 10 L/min. Transfer capillary voltage was 4500 V, the fragmentor was set to 120 V, the skimmer was set to 65 V, and the ion guide octapole was set to 750 V. The CE unit was operated by the 3D-CE ChemStation Rev B.04.03 software, and MS data were acquired by the MassHunter WorkStation Acquisition B.02.01 in centroid mode with a data acquisition rate of 5 spectra per second. Purine ($C_5H_4N_4$; $[M+H]^+$ 121.05087) and HP921 (hexakis (2,2,3,3,-tetrafluoropropoxy) phosphazine; CAS No.: 58943-98-9; $C_{18}H_{18}O_6N_3P_3F_{24}$; $[M+H]^+$ 922.00980) were used as reference masses, whereby a solution containing 10 mL water, 90 mL methanol, 100 μL formic acid, 1,600 μL of 5 mmol/L purine, and 600 μL of 2.5 mmol/L phosphazine derivative listed above (HP 0921) were infused directly into the ion source. The reference mass spectra were collected simultaneously with the analytical data and used for accurate mass correction.

**Data Processing and Peak Identification.** The raw data files derived from UPLC-MS and CE-MS acquisition were preprocessed, using the publically available XCMS software (version 1.24.1).[27] Isotope peaks, fragments, and adducts were treated as separate features. For the dataset containing the extracts from individual flukes, the samples were grouped according to the liver from which they were extracted, whereby QC samples were treated as a separate group. Default settings were employed in XCMS, with the exception of the width of overlapping $m/z$ slices used for creating peak density chromatograms and grouping peaks across samples (mzwid = 0.025), the bandwidth for the grouping performed after retention time correction (bw = 10 s), and the degree of smoothing for local polynomial regression fitting (span = 0.3). A table of time-aligned detected features containing the retention times, $m/z$ ratio, and intensities of each sample was then obtained. Median normalization was performed using an in-house-developed R script (Dr. K. Veselkov, Imperial College London),[28] followed by tissue weight normalization. Output tables containing information on the average retention time and average $m/z$ were also prepared as data input of an in-house-developed MATLAB script (Dr. P. Masson, Imperial College London) that allowed searching of candidate metabolites in an in-house-built UPLC-MS database within specified errors; in


dx.doi.org/10.1021/ac300586m | Anal. Chem. 2012, 84, 6963−6972

this work, initial values of 0.05 Da for $m/z$ and 1 min for retention time were used. A set of candidate compounds was then generated as a first pass and errors on the $m/z$ and retention time assignments were provided for each compound. Compounds with $m/z$ and retention time differing by more than 20 ppm and 0.5 min, respectively, were disregarded. Eventually, a mass chromatogram of the corresponding authentic standard and the sample were acquired and the peak submitted to fragmentation in a MS/MS experiment. If the fragmentation pattern of both standard and sample peak matched, the peak was then assigned to the alleged compound with improved reliability.

## RESULTS AND DISCUSSION

**Analytical Platform Selectivity for *F. hepatica* Metabolites.** Mass spectroscopy (MS) has the necessary sensitivity to assess low-abundance molecular species in biological matrices. When combined with appropriately designed extraction methods and any gas- or liquid-based separation technique, MS is capable of analyzing a plethora of metabolites of different chemical classes. Moreover, the variety of separation modes, ionization schemes, and mass analyzers qualifies the use of coupled MS platforms as a resourceful approach to metabolic profiling.[29−32]

We compared individual *F. hepatica* extracts at 80% methanol prepared from flukes obtained from two different cow livers, using analytical conditions and instrumental parameters provided in previous work for UPLC-MS,[18,33] and a method developed specifically for CE-MS.

Typical base peak mass chromatograms acquired using ESI+ and ESI−, respectively, on the C18 column are depicted in Figure 1A and Figure S-1A in the Supporting Information. As expected, many polar components exhibited poor retention, coeluting close to the column dead volume. Therefore, assignments for peaks eluting by less than 0.94 min ($k$ < 1.0) were not considered to be reliable. In the positive ionization C18 mass chromatogram of Figure 1A, a few amino acids exhibited moderate retention (retention factors ($k$) in the range of 1.0−6.0): tyrosine, isoleucine/leucine, phenylalanine, and tryptophan. The mass chromatogram presented retention time windows for other interesting chemical classes: the elution of cholic acid derivatives centered roughly at 11 min ($k \approx 14$), the monosubstituted glycerophosphocholine derivatives eluting at 12−15 min ($k \approx 17$), and the disubstituted phosphatidylcholine homologues eluting after 17 min ($k$ > 22). A polyethylene glycol peak envelope (~8 min) was observed in the mass chromatogram, but it was also detected in the pure water and therefore attributed to contamination.

Similar features were observed in the negative ionization C18 mass chromatogram of Figure S-1A in the Supporting Information. In addition to phenylalanine and tryptophan, a few carboxylic acids (methylmalonic and pantothenic acids), adenosine monophosphate (AMP), and inosine comprise the early eluting polar compounds, whereas, in the cholic acid region, deoxycholic acid was further detected. Similar compounds were identified in the phospholipid region for both positive and negative ionization modes, although the monosubstituted glycerophosphocholine derivatives exhibited much higher ionization yields in negative ion mode, when compared to the disubstituted phosphatidylcholine homologues, in direct contrast to the observation made from the positive ionization mode data.

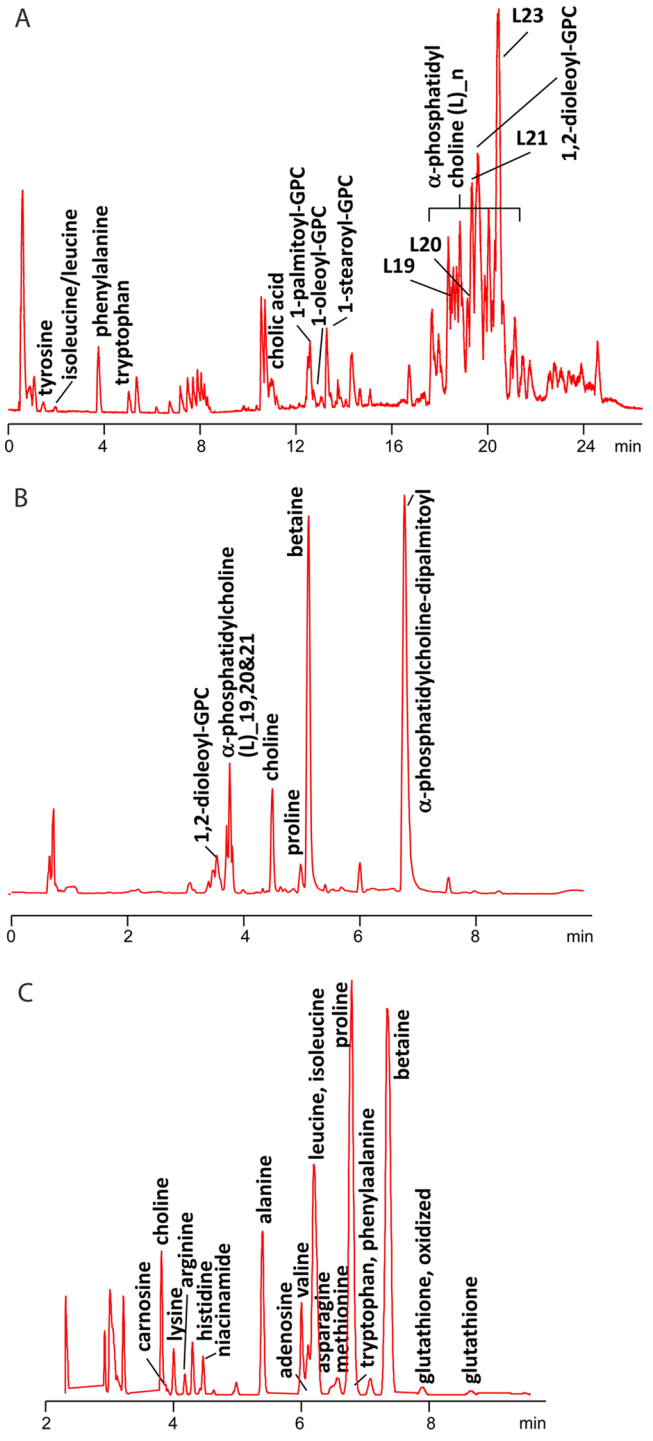



**Figure 1.** Typical mass chromatograms and electropherograms of 80% methanol extracts of *F. hepatica* flukes acquired at ESI+ mode in (A) C18 column, (B) HILIC column, and (C) CE capillary. Key: GPC, glycerophosphocholine; the remaining terms have the nomenclature α-phosphatidylcholine (L)_$n$ (which represents a mixture of α-phosphatidylcholines (L)_$n$ numbered 1 to 23 by order of elution in the RPLC-MS method (refs 18 and 33); tentative identification based on $m/z$ searches at http://www.lipidmaps.org/data/structure/LMSDSearch.php): L19, 1-hexadecanoyl-2-octadecadienoyl-sn-glycero-3-phosphocholine; L20, 1-nonanoyl-2-tricosanoyl-sn-glycero-3-phosphocholine; L21, 1-hexadecanoyl-2-octadecenoyl-sn-glycero-3-phosphocholine; and L23, 1-octadecanoyl-2-octadecenoyl-sn-glycero-3-phosphocholine.


dx.doi.org/10.1021/ac300586m | *Anal. Chem.* 2012, 84, 6963−6972

Table 1. Evaluation of the Metabolic Coverage of the Proposed Methods for *F. hepatica* Tissue Samples

| | peaks per sample | metabolite features | identified peaks | retained peaks[a] | small polar metabolites[b] |
|---|---|---|---|---|---|
| RPLC-MS, ESI+ | 5653 | 5183 | 82 | 49 | 19 |
| HILIC-MS, ESI+ | 2562 | 2281 | 94 | 88 | 63 |
| CE-MS, ESI+ | 126 | 114 | 37 | 37 | 37 |
| RPLC-MS, ESI− | 5981 | 5420 | 70 | 35 | 22 |
| HILIC-MS, ESI− | 1882 | 1726 | 102 | 92 | 67 |

[a] $k > 1$, among identified peaks. [b] Among retained peaks.

As the results of Figure 1A and Figure S-1A in the Supporting Information testify, the choice of C18 columns for UPLC-MS studies allows immediate visualization of nonpolar and moderately polar compounds. However, its use discriminates instantly against highly polar compounds. Recently, HILIC has been offered as a powerful alternative to compensate for the poor RPLC retention of highly polar compounds, ubiquitous components of many biological fluids.[34−36] HILIC is a form of normal-phase liquid chromatography (NPLC) in the sense that uses polar stationary phases, usually water-rich layers immobilized onto silica particles. However, unlike NPLC, HILIC employs aqueous mobile phases; during gradient elution, the polarity is increased from a low organic content to a high-water-content mobile phase in order to promote the elution of polar compounds. HILIC columns have found application in numerous areas, including metabolic profiling of various pathologies, but as yet a systematic evaluation of their performance as a metabolic profiling tool is lacking.[37,38]

Typical base peak mass chromatograms are shown in Figure 1B and Figure S-1B in the Supporting Information, acquired under positive and negative ionization modes, respectively, on the HILIC column. In the positive ionization mode (Figure 1B), prominent peaks from a few quaternary ammonium compounds (choline and betaine) and proline (a cyclic aminoacid) were bracketed by moderately retained phospholipids ($4 < k < 5$) and $\alpha$-phosphatidylcholine-dipalmitoyl ($k = 9$). The negative ionization mode mass chromatogram (Figure S-1B in the Supporting Information) was richer, in terms of polar compounds: succinic acid and AMP constitute the acidic components, whereas tryptophan, phenylalanine, taurine, glutamine, glutamic acid, and histidine constitute the amino acids components. Proline and betaine were also identified in the mass chromatogram. Similar to the observation from the C18 column, in negative ionization mode, a fraction of single substituted glycerophosphatidylcholine homologues emerged.

Although we expected to visualize a much larger variety of polar compounds with the HILIC column, that was not the case for the tissue samples under investigation. This might be due to the fact that these samples were prepared in high concentrations of methanol, resulting in a preferential extraction of phospholipids, which outnumbered the polar components. Since the phospholipids exhibited an extensive retention in chromatography, even in HILIC columns, the information on polar compounds was somewhat compromised.

Considering the orthogonal separation mechanism provided by CE when compared to LC, CE has emerged as a promising complementary technique for metabolic profiling.[39−41] Small cationic and anionic charged species are expected to be the target metabolites of CE separations. CE-MS metabolic profiling studies are often conducted under electrospray ionization (ESI) and triple coaxial sheath flow interfaces. Unlike UPLC, the mobile phase or, more precisely, the background electrolyte (BGE) composition changes according to the selected ionization mode. Typically, cationic metabolites are screened in low-pH volatile electrolytes, such as formic acid or acetic acid, whereas anionic metabolites are analyzed in high-pH volatile electrolytes, such as ammonia/ammonium salts buffers (ammonium formate, acetate, or carbonate being the most commonly used). The addition of low percentages of organic solvents to the BGE is often required to improve resolution. In addition, a SHL that may be of distinct composition for each ionization mode is also used to promote ionization at the capillary tip.

A typical extracted-compound mass electropherogram acquired under positive ionization mode is shown in Figure 1C. The BGE and SHL composition, as well as instrumental parameters, were previously optimized with mixtures of appropriate standards to provide the highest signal-to-noise (S/N) ratio and best resolution. Peak assignment relied on comparisons of migration times and $m/z$ with an in-house-built database composed of ca. 120 authentic standards, mostly amino acids and substituted amines.

In order to evaluate comparatively the metabolic coverage of each analytical method applied to *F. hepatica* tissue samples, selected statistics were compiled in Table 1. Peaks per sample refers to the number of peaks XCMS delivered after its peak picking algorithm was performed; metabolite features is the number of peak groups after grouping, alignment, and normalization took place, indicating that the parameters chosen in these routines adequately extracted compounds within specified $m/z$ and time slices across all samples.[27]

As observed in Table 1, a total of 14,724 metabolite features were extracted, of which 385 metabolites were tentatively identified, based on in-house databases, comparison with authentic standards, and occasionally MS/MS fragmentation. After eliminating the redundant assignments among methods, 142 unique metabolites resulted. A complete list of assigned metabolites is provided in the Supporting Information (Table S-2). As the data in Table 1 indicates, although the overall number of features is quite large, it does not necessarily translate into information or biomarkers. The tissue samples under examination were quite rich in phospholipids, derivatives, and homologues, which were compounds predominantly visualized by four UPLC-MS out of five protocols. In addition, the fact that no filtering algorithms were applied also contributed to increasing the number of retrieved features. We chose not to use filtering algorithms, because the raw data are the most useful at a general level. For identification purposes, for instance, the related $m/z$ due to isotope patterns, fragments, salt adducts, etc. ultimately improved our ability to identify metabolites. The relative low yield of metabolic features obtained via CE-MS, compared to the UPLC-MS-based methods, is likely to be due to inappropriate original sample dilution and further dilution of the sample within the electrospray interface by the SHL. The initial number of peaks

per sample is already low for CE-MS (Table 1). Furthermore, phospholipids, which are the richest components of the tissue samples under consideration, were not taken into account in the CE-MS method. Online signal enhancement strategies have been invoked to address sensitivity issues of CE-MS metabolic profiling studies;[42] nevertheless, the number of retrieved features when a SHL is employed is usually poor, varying from a few hundreds to ~1000.[8,41,43,44]

Identification of metabolites for UPLC-MS platforms relied solely on in-house databases, which explains the relatively small number of identified metabolites per feature (<2% for RPLC-MS and 6% for HILIC-MS, versus 32% for CE-MS). Each database contains a few hundred metabolites, classified by retention/migration time and $m/z$, and were built using information gathered from different biological samples (serum, urine, tissue, etc) fortified by authentic standards. In a way, these libraries also account for matrix effects and their use was preferred over the use of publicly available databases, because peak assignment could be performed with increased reliability.

Interestingly, Table 1 shows that, for the RPLC-MS method, practically half of the identified compounds were not properly retained by the C18 column, and, among the retained compounds, 39% were small polar metabolites for ESI+ and 63% for ESI−. With the HILIC column, a similar large number of metabolites were extracted in both ionization modes, despite the low complexity of the corresponding mass chromatogram (Figure 1B); practically all identified compounds were retained, and, among them, 63%−67% were small polar compounds. As expected, CE-MS had the best yield of polar compounds over the identified metabolites (100%).

The Venn diagram in Figure 2 summarizes the selectivity differences among the five analytical methods applied. Metabolite identification was not exhaustive but rather

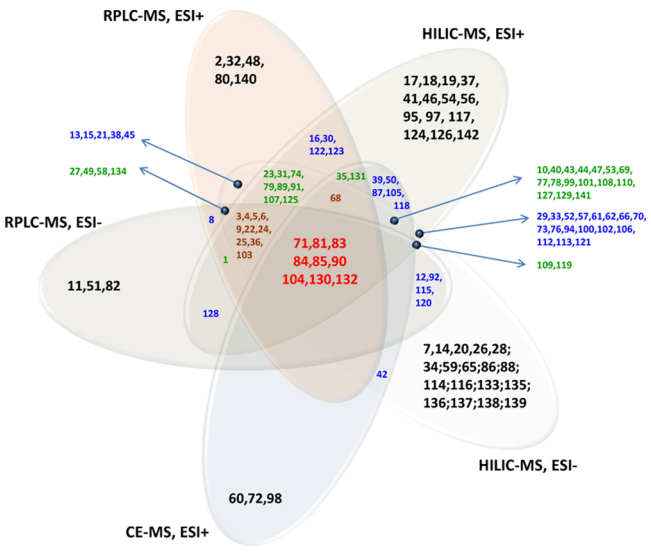



**Figure 2.** Venn diagram depicting the 142 metabolites identified across all analytical platforms. The metabolites are color-coded according to the method by which they were identified: red (five methods), brown (four methods), green (three methods), blue (two methods) and black (one method). Common metabolites to all five methods: (71) glutathione, (81) hypoxanthine, (83) inosine, (84) isoleucine, (85) leucine, (90) methionine, (104) phenylalanine, (130) tryptophan, and (132) tyrosine. (For a complete list of identified metabolites, see the Supporting Information (Table S-2).)

dependent on the variety and quantity of compounds contemplated in our libraries. Nine (9) out of 142 metabolites were identified across all analytical platforms but many metabolites are unique to a given method, denoting complementarity over redundancy of the proposed multiplatform approach. HILIC-MS provided the largest number of unique metabolites in both ionization modes.

**Evaluation of Systems Performance.** Performance characteristic data for selected components found in 12 individual *F. hepatica* extracts prepared at 80% methanol with flukes obtained from two different liver sources have been compiled in Table 2 and include retention/migration times and peak area precision, as well as mass accuracy, signal-to-noise (S/N) ratios, plate numbers per meter (N/m), retention factor ($k$) and effective mobility ($\mu_{eff}$) in Tiselius units (TU). For both UPLC and CE, peak areas were normalized by the corresponding worm weight. Each peak area in the CE mass electropherogram was further corrected by the migration time, since peaks migrate at different velocities past the detector.

Peak area ratios of selected compounds in the extracts prepared from worms obtained from livers 1 and 2 did not differ much for the UPLC-MS data (C18 and HILIC columns, both ESI+ and ESI−). Retention time repeatability was found to be remarkably high for both C18 and HILIC columns in ESI + (an average of 0.2% CV) but relatively lower in ESI− (ca. 0.9% CV). CE-MS data presented a much higher CV in migration time when compared to UPLC-MS (average of 2% CV), whereas the precisions for peak area with liver 1 fluke preparations were similar. Samples prepared from liver 2 provided much lower counts for peak area (not shown) and a large within-sample variability (average of 58% CV). In CE-MS, apparent migration times are prone to error, because of electroosmotic flow (eof) variability (surface phenomenon and instrumental variation, such as the SHL and gas flow rates at the interface). Although we found that the precision of the migration time is within acceptable values for CE-MS, precision could be improved using several strategies, including normalization to internal standards,[45] and the use of dynamically coated capillaries.[46] The use of internal standards helps to correct migration time misalignments and it will also improve the efficiency of peak grouping algorithms augmenting the number of retrieved features. However, the migration time variation that we observed in Table 2 was not detrimental to the overall quality of the data processing and classification. By reporting electrophoretic mobilities (after eof correction) instead of migration times, precision can be improved to the level of UPLC-MS. Since the eof value was not measured in each single run, mobility precision could not be estimated; however, an estimate of the effective mobility order of magnitude for selected solutes was included in Table 2, as well as the retention factors for UPLC-MS data. Other features of Table 2 include the following:

- Mass accuracy was better than 5 ppm, with a few exceptions;
- S/N ratios were roughly 10−20 fold smaller for CE-MS data when compared to UPLC-MS data, reinforcing the idea of dilution of sample components at the interface by the sheath liquid; and
- Plate numbers, overall, were larger for phospholipids than for polar compounds in the C18 column, but equivalent high values were obtained in the HILIC column at both ionization modes, even surpassing the


dx.doi.org/10.1021/ac300586m | Anal. Chem. 2012, 84, 6963−6972

**Table 2. Performance Characteristics According to Analytical Platform and Ionization Mode for Selected Compounds**

| | Flukes from Liver 1[a] | | | | | | Flukes from Liver 2[a] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RT %CV | peak area %CV | mass accuracy (ppm) | S/N[c] | plate number per meter, N/m | $k$ or $\mu_{eff}$ (TU) | RT %CV | peak area %CV | mass accuracy (ppm) | S/N[c] | plate number per meter, N/m | $k$ or $\mu_{eff}$ (TU) |
| **RPLC-MS, ESI+** | | | | | | | | | | | | |
| phenylalanine | 0.15 | 19 | 2.5 | 2783 | 22849 | 4.25 | 0.16 | 23 | −1.2 | 1763 | 20254 | 4.22 |
| tryptophan | 0.32 | 13 | 1.5 | 615 | 50064 | 5.87 | 0.19 | 23 | 0.44 | 601 | 46509 | 5.90 |
| 1-palmitoyl-sn-glycero-3-phosphocholine | 0.052 | 17 | 5.9 | 7298 | 220119 | 15.9 | 0.067 | 32 | −0.10 | 3616 | 281862 | 15.9 |
| 1-stearoyl-sn-glycero-3-phosphocholine | 0.058 | 20 | 3.2 | 7290 | 189486 | 16.6 | 0.041 | 33 | 0.10 | 4594 | 245450 | 16.9 |
| α-phosphatidylcholine(L)_23 | 0.078 | 27 | −7.2 | 9403 | 146903 | 26.2 | 0.036 | 16 | −8.5 | 8559 | 172948 | 26.3 |
| **RPLC-MS, ESI−** | | | | | | | | | | | | |
| phenylalanine | 0.90 | 23 | 2.3 | 156 | 15561 | 4.21 | 0.88 | 18 | 5.1 | 196 | 13307 | 4.22 |
| tryptophan | 0.89 | 17 | 3.3 | 591 | 22474 | 5.80 | 0.68 | 18 | 0.82 | 426 | 74712 | 5.90 |
| 1-palmitoyl-sn-glycero-3-phosphocholine | 1.0 | 19 | 2.8 | 3136 | 155991 | 15.7 | 0.88 | 22 | 2.8 | 2065 | 284207 | 16.0 |
| 1-stearoyl-sn-glycero-3-phosphocholine | 0.90 | 28 | −1.2 | 3964 | 218825 | 17.3 | 0.72 | 22 | 3.8 | 3923 | 400980 | 17.5 |
| α-phosphatidylcholine(L)_23 | 0.52 | 20 | −6.4 | 7372 | 198030 | 26.2 | 0.39 | 19 | −9.0 | 6756 | 502369 | 26.4 |
| **HILIC-MS, ESI+** | | | | | | | | | | | | |
| choline | 0.11 | 29 | −2.2 | 10005 | 175393 | 5.08 | 0.12 | 31 | 1.6 | 7486 | 152790 | 5.09 |
| proline | 0.11 | 13 | −9.8 | 2501 | 111882 | 5.77 | 0.081 | 24 | −8.6 | 1924 | 118826 | 5.76 |
| betaine | 0.10 | 21 | 0.85 | 10549 | 62550 | 5.94 | 0.11 | 23 | 0.85 | 9681 | 93618 | 5.93 |
| α-phosphatidylcholine-dipalmitoyl | 0.080 | 16 | 4.5 | 18307 | 133239 | 8.19 | 0.11 | 22 | 4.5 | 19019 | 126417 | 8.18 |
| **HILIC-MS, ESI−** | | | | | | | | | | | | |
| acetate | 0.087 | 18 | 1.6 | 421 | 172215 | 5.29 | 0.12 | 24 | 2.1 | 265 | 206101 | 5.29 |
| betaine | 0.080 | 17 | −0.52 | 789 | 367596 | 5.96 | 0.010 | 19 | −0.52 | 594 | 386839 | 5.96 |
| adenosine monophosphate | 0.73 | 28 | −0.76 | 797 | 12097 | 7.43 | 0.48 | 36 | −3.6 | 394 | 14240 | 7.54 |
| α-phosphatidylcholine-dipalmitoyl | 0.059 | 30 | 3.7 | 23791 | 264843 | 8.16 | 0.059 | 24 | 3.7 | 19055 | 282725 | 8.17 |
| **CE-MS, ESI+**[b] | | | | | | | | | | | | |
| choline | 1.9 | 18 | −0.64 | 514 | 40436 | 78.7 | 0.94 | 51 | −2.5 | 181 | 57299 | 77.7 |
| alanine | 2.5 | 24 | 6.7 | 803 | 37849 | 53.2 | 1.3 | 66 | 4.9 | 225 | 47661 | 52.2 |
| proline | 2.8 | 14 | 0.43 | 1389 | 31713 | 40.5 | 1.5 | 60 | −0.2 | 421 | 40865 | 39.6 |
| betaine | 3.0 | 21 | −4.4 | 1505 | 26683 | 36.6 | 1.6 | 55 | −4.4 | 536 | 30044 | 35.7 |

[a] $n$ = 6, six independent preparations of flukes from each liver at 80% methanol. [b] Peak areas were further corrected by the migration time. [c] Root-mean-square (rms) noise.



dx.doi.org/10.1021/ac300586m | Anal. Chem. 2012, 84, 6963−6972

efficiency obtained by CE-MS when polar compounds were contrasted.

Principal component analysis (PCA) of the QC samples for each analytical platform showed that all QC samples clustered together, except for the first 3−5 injections, which were used for system stabilization and, therefore, were expected to present some scattering. Moreover, it was observed that 64% of the total number of metabolite features presented a CV value of <30%, which denotes a reliable dataset.[47]

**Mixture Designed Extractions.** A number of experimental designs have been developed to address specifically the analysis and modeling of mixtures.[48,49] One common manner in which mixture proportions for three mixture components can be summarized is via triangular graphs. Figure 3 and Figures S-2,

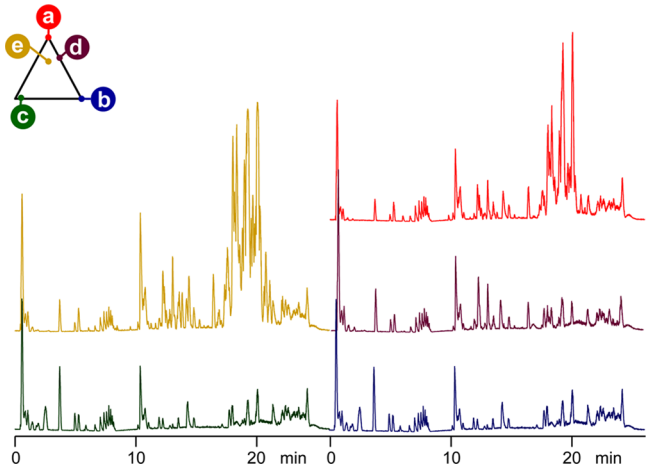



**Figure 3.** Optimization of extraction of a pooled *F. hepatica* sample using a mixture design approach and RPLC-MS analysis in ESI+ mode. Analyses were performed only on the aqueous extracts. Extractions were performed in (a) 80% methanol, (b) 20% methanol, (c) 90:10 chloroform:20% methanol, (d) 65% methanol, and (e) 15:15:70 chloroform:20% methanol:80% methanol.

S-3A, S-3B, and S-4 in the Supporting Information depict a series of mass chromatograms or electropherograms registered for the aqueous phase of mixture designed extracts of a pooled *F. hepatica* tissue sample. The line between (a) and (b) in Figure 3 (apexes of the triangle inserts) corresponds to binary solvent mixtures containing methanol:water in different proportions, ranging from 80% methanol (a) to 20% methanol (b); all other points in the triangle are ternary mixtures, i.e., they contain methanol:water:chloroform as extracting solvents. Since the organic layer was discarded, and only the aqueous layer retained for analysis, all extraction solvents considered in this study must contain a certain amount of water. Thus point (c) is not an apex, but does contain a large amount of chloroform to explore its extraction ability, with respect to more hydrophobic compounds (c is 90:10, where 90 parts correspond to pure chloroform and 10 parts correspond to a 20% methanol solution in water).

Although points (d) and (e) in Figure 3 contain a similar amount of methanol in the aqueous phase (65% for (d) and 69% for (e)), the extraction power of solvents (d) and (e) toward the tissue sample components differs greatly. Solvent (e) is a ternary mixture, i.e., contains chloroform, and therefore a partition equilibrium takes place. The tissue components will distribute between the organic and aqueous phase according to their characteristics and intermolecular interactions will modulate the partition.

In Figure 3, all chromatograms are fairly similar in composition, except for the region comprising 17−21 min. That particular region corresponds to the retention of the more hydrophobic tissue components, i.e., glycerophosphocholine derivatives and homologues. For binary mixtures (line a-d-b), it is clear that a large amount of methanol is necessary to maintain these compounds solubilized in the aqueous phase. For ternary mixtures in the line c-b, the amount of chloroform does not seem to make any impact in the extract composition. Extracts (b) and (c) are quite similar, despite the large amount of chloroform in (c) (90 parts); low amounts of methanol in the aqueous phase, 20% in this case, were not enough to solubilize the phospholipids. However, the situation is strikingly different if larger amounts of methanol are used in the presence of chloroform. If the chromatograms of the tissues extracted by solvents (d) and (e) are inspected (65% methanol, no chloroform for (d); 69% methanol in aqueous phase, 15 parts of chloroform for (e)), a collection of peaks in the phospholipids retention region is readily visualized. Possibly in the case of extract (e), the presence of chloroform and moderate amounts of methanol induce partition of the phospholipids toward the aqueous phase, whereas in the case of extract (c), the aqueous phase was too polar and the phospholipids remained in the chloroform phase.

The HILIC-MS chromatograms corresponding to mixture designed extracts of *F. hepatica* (Figure S-3 in the Supporting Information) repeats the same features presented by the C18 phase, although the phospholipid fractions are much less prominent. However, as observed in the mass electropherograms of Figure S-4 in the Supporting Information, CE-MS appears to be insensitive toward the composition of the extraction medium, since it discriminates only the most polar fraction of the analyzed samples.

Application of mixture design methods can be used to summarize the performance surface of various solvents toward a selected response in geometric graphs. So far, Figure 3 has been generated to explore the data in a qualitative manner, in which the total number of peaks and S/N enhancement were considered guiding responses. However, in quantitative approaches, optimal proportions of a solvent mixture can be sought based on the yield of metabolite extraction. The contour plot in Figure 4A represents the corrected peak area of

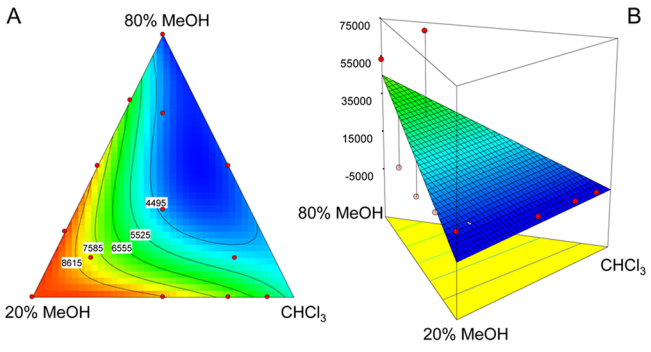



**Figure 4.** Representation of responses as a function of extraction composition for RPLC-MS at ESI+ mode. Responses are computed as the peak area of (A) phenylalanine and (B) α-phosphatidylcholine (L)_23 (1-octadecanoyl-2-octadecenoyl-sn-glycero-3-phosphocholine) corrected by the biomass weight.


dx.doi.org/10.1021/ac300586m | *Anal. Chem.* 2012, 84, 6963−6972

phenylalanine, analyzed by RPLC-MS at ESI+, as a function of solvent composition used in the extraction. A cubic model was fitted to the data using the Design-Expert software, showing significant model terms ($F$ = 12, $p$-value = 0.004, adjusted $r^2$ = 0.85, PRESS = $4.08 \times 10^7$). It is clear from Figure 4A that lower percentages of methanol as the extraction solvent enhances the amount of phenylalanine, which is consistent with the polar character of the compound. Similarly, Figure 4B presents a response surface for the corrected peak area of α-phosphatidylcholine (L)_23 (1-octadecanoyl-2-octadecenoyl-sn-glycerol-3-phosphocholine) analyzed using the same platform, as a function of solvent composition used during extraction. The concentration of α-phosphatidylcholine (L)_23 at the ternary point (composition marked as (e) in the chromatogram of Figure 3) is remarkably high. As a matter of fact, this point is an outlier of the model and cannot be explained by the response surface of Figure 4B. If included, it creates a discontinuity in the model and poor statistical results (with outlier: $F$ = 4.59, $p$-value = 0.039, adjusted $r^2$ = 0.37, PRESS = $5.49 \times 10^9$; without outlier: $F$ = 20, $p$-value = 0.004, adjusted $r^2$ = 0.84, PRESS = $1.44 \times 10^9$). Nevertheless, what can be observed from Figure 4B is that as the methanol content of the solvent increases, the concentration of α-phosphatidylcholine (L)_23 increases, which is consistent with the hydrophobic character of this phospholipid. More importantly, the enrichment of phospholipids in the tissue extract for this particular ternary point solvent combination can be rationalized by the physical chemical properties of chloroform. Chloroform is an unusual solvent in the sense that it exhibits a low dielectric constant (on the order of 4.81; for reference, methanol has a dielectric constant of 32.6 and water has a dielectric constant of 78.5)[50] but, at the same time, it has a measurable effective hydrogen bond acidity (Abrahańs $A$ parameter on the order of 0.15; for comparison, methanol has an $A$ value of 0.43 and water has an $A$ value of 0.82).[51] Thus, chloroform is able to interact with the oxygen atoms in both methanol and the phospholipid molecule via hydrogen bonding. By dissolving into methanol to a certain extent, chloroform serves as a carrier and brings the phospholipid into the methanolic phase. Therefore, even for smaller amounts of methanol, when chloroform is used as part of the extraction medium, an enrichment of phospholipids in the extract results.

## CONCLUSION

The different analytical methods applied, i.e., UPLC-MS (C18 and HILIC) in ESI+ and ESI− modes and CE-MS in ESI+ mode, proved to be highly complementary for gaining maximum metabolite coverage and, hence, offer a real opportunity for planning future multiplatform use in global metabolic profiling. Although a core group of molecules was common to all five methods applied, each chromatographic platform contributed a unique set of metabolites to the final metabolic yield of 142 metabolites (or 14,724 features). The mixture design for tissue extraction, which delivered the best compromise for all five analytical methods, in terms of number of metabolites retrieved and yield, was a 15:59:26 chloroform:methanol:water mix.

The use of a single solvent system and tissue extraction method for all platforms in any given study reduces the complexity of statistical integration of the data from the different analytical platforms, since the variability resulting from differences in extraction efficiency between platform-optimized solvents or sample extraction methods is removed. Thus, the power of using statistical correlation between multiple analytical platforms is enhanced and should result in enhanced biomarker identification and recovery.

The strategy applied here for metabolite extraction from *F. hepatica* samples may be widely applicable to other helminths or mammalian tissue samples.

## ASSOCIATED CONTENT

### Supporting Information

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

## AUTHOR INFORMATION

### Corresponding Author

*E-mails: mfmtavar@iq.usp.br (M.F.M.T.), elaine.holmes@imperial.ac.uk (E.H.).

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

This study received financial support from the Swiss National Science Foundation to J.K. and U.D. (Project No. PPOOA-114941). Additional support was provided for J.S. by the Wellcome Trust (089002/B/09/Z) and for M.F.M.T. by the Fundação de Amparo à Pesquisa do Estado de Sao Paulo of Brazil (Fapesp 2010/01579-4). We are grateful to Waters, Ltd., for advice on the UPLC-MS technology, and to Agilent Technologies for assistance with the CE-MS analysis.

## REFERENCES

(1) Lenz, E. M.; Wilson, I. D. *J. Proteome Res.* **2007**, *6*, 443−458.
(2) Saric, J.; Li, J. V.; Utzinger, J.; Wang, Y.; Keiser, J.; Dirnhofer, S.; Beckonert, O.; Sharabiani, M. T.; Fonville, J. M.; Nicholson, J. K.; Holmes, E. *Mol. Syst. Biol.* **2010**, *6*, 396.
(3) Gavaghan, C. L.; Holmes, E.; Lenz, E. M.; Wilson, I. D.; Nicholson, J. K. *FEBS Lett.* **2000**, *484*, 169−174.
(4) Daykin, C. A.; Foxall, P. J. D.; Connor, S. C.; Lindon, J. C.; Nicholson, J. K. *Anal. Biochem.* **2002**, *304*, 220−230.
(5) Geier, F. M.; Want, E. J.; Leroi, A. M.; Bundy, J. G. *Anal. Chem.* **2011**, *83*, 3730−3736.
(6) Lloyd, D. K. *J. Chromatogr. A* **1996**, *735*, 29−42.
(7) Chambers, E.; Wagrowski-Diehl, D. M.; Lu, Z.; Mazzeo, J. R. *J. Chromatogr. A* **2007**, *852*, 22−34.
(8) Simó, C.; Ibañez, C.; Gómez-Martínez, A.; Ferragut, S. A.; Cifuentes, A. *Electrophoresis* **2011**, *32*, 1765−1777.
(9) Want, E. J.; O'Maille, G.; Smith, C. A.; Brandon, T. R.; Uritboonthai, W.; Qin, C.; Trauger, S. A.; Siuzdak, G. *Anal. Chem.* **2006**, *78*, 743−52.
(10) Rezzi, S.; Vera, F. A.; Martin, F. P. J.; Wang, S.; Lawler, S.; Kochhar, S. *J. Chromatogr. B* **2008**, *871*, 271−278.
(11) Álvarez-Sánchez, B.; Priego-Capote, F.; Mata-Granados, J. M.; Luque de Castro, M. D. *J. Chromatogr. A* **2010**, *1217*, 4688−4695.
(12) Vuckovic, D.; Zhang, X.; Cudjoe, E.; Pawliszyn, J. *J. Chromatogr. A* **2010**, *1217*, 4041−4060.
(13) Turiel, E.; Martín-Esteban, A. *Anal. Chim. Acta* **2010**, *668*, 87−99.
(14) Mullett, W. M. *J. Biochem. Biophys. Methods* **2007**, *70*, 263−273.
(15) Álvarez-Sánchez, B.; Priego-Capote, F.; Luque de Castro, M. D. *Trends Anal. Chem.* **2010**, *29*, 111−119.
(16) Eeckhaut, A. V.; Lanckmans, K.; Sarre, S.; Smolders, I.; Michotte, Y. *J. Chromatogr. B* **2009**, *877*, 2198−2207.
(17) Lindon, J. C.; Nicholson, J. K. *Trends Anal. Chem.* **2008**, *27*, 194−204.
(18) Masson, P.; Alves, A. C.; Ebbels, T.; Nicholson, J. K.; Want, E. J. *Anal. Chem.* **2010**, *82*, 7779−7786.

(19) Masson, P.; Spagou, K.; Nicholson, J. K.; Want, E. J. *Anal. Chem.* **2011**, *83*, 1116−1123.
(20) Wu, H.; Southam, A. D.; Hines, A.; Viant, M. R. *Anal. Biochem.* **2008**, *372*, 204−212.
(21) Price, K. E.; Lunte, C. E.; Larive, C. K. *J. Pharm. Biom. Anal.* **2008**, *46*, 737−747.
(22) Parab, G. S.; Rao, R.; Lakshminarayanan, S.; Bing, Y. V.; Moochhala, S. M.; Swarup, S. *Anal. Chem.* **2009**, *81*, 1315−1323.
(23) Abunna, F.; Asfaw, L.; Megersa, B.; Regassa, A. *Trop. Anim. Health Prod.* **2010**, *42*, 289−292.
(24) Mas-Coma, S.; Bargues, M. D.; Valero, M. A. *Int. J. Parasitol.* **2005**, *35*, 1255−1278.
(25) Keiser, J.; Utzinger, J. *Expert Opin. Pharmacother.* **2004**, *5*, 1711−1726.
(26) Crockford, D. J.; Holmes, E.; Lindon, J. C.; Plumb, R. S.; Zirah, S.; Bruce, S. J.; Rainville, P.; Stumpf, C. L.; Nicholson, J. K. *Anal. Chem.* **2006**, *78*, 363−371.
(27) Smith, C. A.; Want, E. J.; O'Maille, G.; Abagyan, R.; Siuzdak, G. *Anal. Chem.* **2006**, *78*, 779−787.
(28) Veselkov, K. A.; Vingara, L. K.; Masson, P.; Robinette, S. L.; Want, E.; Li, J. V.; Barton, R. H.; Boursier-Neyret, C.; Walther, B.; Ebbels, T. M.; Pelczer, I.; Holmes, E.; Lindon, J. C.; Nicholson, J. K. *Anal. Chem.* **2011**, *83*, 5864−5872.
(29) Roux, A.; Lison, D.; Junot, C.; Heilier, J.-F. *Clin. Biochem.* **2010**, *44*, 119−135.
(30) Theodoridis, G.; Gika, H. G.; Wilson., I. D. *Trends Anal. Chem.* **2008**, *27*, 251−260.
(31) Lu, E.; Bennett, B. D.; Rabinowitz, J. D. *J. Chromatogr. A* **2008**, *871*, 236−242.
(32) Goodacre, R.; Vaidyanathan, S.; Dunn, W. B.; Harrigan, G. G.; Kell, D. B. *Trends Biochem.* **2004**, *22*, 245−252.
(33) Spagou, K.; Wilson, I. A.; Masson, P.; Theodoridis, G.; Raikos, N.; Coen, M.; Holmes, E.; Lindon, J. C.; Plumb, R. S.; Nicholson, J. K.; Want, E. J. *Anal. Chem.* **2011**, *83*, 382−390.
(34) Karatapanis, A. E.; Fiamegos, Y. C.; Stalikas, C. D. *J. Chromatogr. A* **2011**, *1218*, 2871−2879.
(35) Ikegami, T.; Tomomatsu, K.; Takubo, H.; Horie, K.; Tanaka, N. *J. Chromatogr. A* **2008**, *1184*, 474−503.
(36) Hemstrom, P.; Irgum, K. *J. Sep. Sci.* **2006**, *29*, 1784−1821.
(37) Cubbon, S.; Bradbury, T.; Wilson, J.; Thomas-Oates, J. *Anal. Chem.* **2007**, *79*, 8911−8918.
(38) Bajad, S. U.; Lu, W.; Kimball, E. H.; Yuan, J.; Peterson, C.; Rabinowitz, J. D. *J. Chromatogr. A* **2006**, *1125*, 76−88.
(39) Ramautar, R.; Somsen, G. W.; de Jong, G. J. *Electrophoresis* **2009**, *30*, 276−291.
(40) García-Pérez, I.; Vallejo, M.; García, A.; Legido-Quigley, C.; Barbas, C. *J. Chromatogr. A* **2008**, *1204*, 130−139.
(41) Monton, M. R. N.; Soga, T. *J. Chromatogr. A* **2007**, *1168*, 237−246.
(42) Ramautar, R.; Busnel, J.-M.; Deelder, A. M.; Mayboroda, O. A. *Anal. Chem.* **2012**, *84*, 885−892.
(43) Haselberg, R.; Ratnayake, C. K.; de Jong, G. J.; Somsen, G. W. *J. Chromatogr. A* **2010**, *1217*, 7605−7611.
(44) Soga, T.; Ohashi, Y.; Ueno, Y.; Naraoka, H.; Tomita, M.; Nishioka, T. *J. Proteome Res.* **2003**, *2*, 488−494.
(45) Sugimoto, M.; Hirayama, A.; Robert, M.; Abe, S.; Soga, T.; Tomita, M. *Electrophoresis* **2010**, *31*, 2311−2318.
(46) Ramautar, R.; Nevedomskaya, E.; Mayboroda, O. A.; Deelder, A. M.; Wilson, I. D.; Gika, H. G.; Theodoridis, G. A.; Somsen, G. W.; de Jong, G. J. *Mol. Biosyst.* **2011**, *7*, 194−199.
(47) Want, E. J.; Wilson, I. D.; Gika, H.; Theodoridis, G.; Plumb, R. S.; Shockcor, J.; Holmes, E.; Nicholson, J. K. *Nat. Protoc.* **2010**, *5*, 1005−1018.
(48) Ferreira, S. L. C.; Bruns, R. E.; da Silva, E. G. P.; dos Santos, W. N. L.; Quintella, C. M.; David, J. M.; de Andrade, J. B.; Breitkreitz, M. C.; Jardim, I. C. S. F.; Barros Neto, B. *J. Chromatogr. A* **2007**, *1158*, 2−14.
(49) Garcia, L. M. Z.; de Oliveira, T. F.; Soares, P. K.; Bruns, R. E.; Scarminio, I. S. *Chemom. Intell. Lab. Syst.* **2010**, *103*, 1−7.
(50) Sarmini, K.; Kenndler, E. *J. Chromatogr. A* **1997**, *792*, 3−11.
(51) Abraham, M. H. *J. Phys. Org. Chem.* **1993**, *6*, 660−684.