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# Solvent-Dependent Metabolite Distribution, Clustering, and Protein Extraction for Serum Profiling with Mass Spectrometry

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**The aim of metabolite profiling is to monitor all metabolites within a biological sample for applications in basic biochemical research as well as pharmacokinetic studies and biomarker discovery. Here, novel data analysis software, XCMS, was used to monitor all metabolite features detected from an array of serum extraction methods, with application to metabolite profiling using electrospray liquid chromatography/mass spectrometry (ESI-LC/MS). The XCMS software enabled the comparison of methods with regard to reproducibility, the number and type of metabolite features detected, and the similarity of these features between different extraction methods. Extraction efficiency with regard to metabolite feature hydrophobicity was examined through the generation of unique feature density distribution plots, displaying feature distribution along chromatographic time. Hierarchical clustering was performed to highlight similarities in the metabolite features observed between the extraction methods. Protein extraction efficiency was determined using the Bradford assay, and the residual proteins were identified using nano-LC/MS/MS. Additionally, the identification of four of the most intensely ionized serum metabolites using FTMS and tandem mass spectrometry was reported. The extraction methods, ranging from organic solvents and acids to heat denaturation, varied widely in both protein removal efficiency and the number of mass spectral features detected. Methanol protein precipitation followed by centrifugation was found to be the most effective, straightforward, and reproducible approach, resulting in serum extracts containing over 2000 detected metabolite features and less than 2% residual protein. Interestingly, the combination of all approaches produced over 10 000 unique metabolite features, a number that is indicative of the complexity of the human metabolome and the potential of metabolomics in biomarker discovery.**

The measurement of metabolites in biofluids offers unique information concerning metabolism, health, and nutrition.[1−5] This approach has had proven success in drug metabolite analysis[6,7] and the characterization of novel metabolites[8] and holds great promise for disease diagnosis.[9−12] Historically, most metabolite studies were performed using high-resolution capillary gas chromatography/mass spectrometry (GC/MS), enabling the identification of key small molecules, including fatty acids, amino acids, and organic acids in biofluids, and providing diagnostic information for many inherited metabolic disorders.[5] GC/MS is still employed today, particularly in plant metabolism;[13−16] however, there are limitations such as the size and type of metabolite that can be analyzed and the extensive preparation required.[17] This led to the emergence of NMR as a metabolite profiling tool; however, NMR has limited sensitivity, resolution, and dynamic range, resulting in only the most abundant components being observed.[17] In contrast, mass spectrometry, in particular electrospray ionization liquid chromatography/mass spectrometry (LC/ESI-MS), offers the ability to analyze a wide variety of compounds, as well as excellent quantitation, reproducibility, and sensitivity.[18−20] LC/ESI-MS enables the separation of the thousands of metabolites present

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(1) Watkins, S. M.; German, J. B. *Curr. Opin. Mol. Ther.* **2002**, *4*, 224−228.
(2) Watkins, S. M.; German, J. B. *Curr. Opin. Biotechnol.* **2002**, *13*, 512−516.
(3) Nicholson, J. K.; Connelly, J.; Lindon, J. C.; Holmes, E. *Nat. Rev. Drug Discovery* **2002**, *1*, 153−161.
(4) Levy, H. L. *Clin. Chem.* **1998**, *44*, 2401−2402.
(5) Chace, D. H. *Chem. Rev.* **2001**, *101*, 445−477.
(6) Plumb, R. S.; Stumpf, C. L.; Gorenstein, M. V.; et al. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1991−1996.
(7) Plumb, R. S.; Stumpf, C. L.; Granger, J. H.; Castro-Perez, J.; Haselden, J. N.; Dear, G. J. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2632−2638.
(8) Saghatelian, A.; Trauger, S. A.; Want, E. J.; Hawkins, E. G.; Siuzdak, G.; Cravatt, B. F. *Biochemistry* **2004**, *43*, 14332−14339.
(9) Fernie, A. R.; Trethewey, R. N.; Krotzky, A. J.; Willmitzer, L. *Nat. Rev. Mol. Cell. Biol.* **2004**, *5*, 763−769.
(10) Griffin, J. L.; Shockcor, J. P. *Nat. Rev. Cancer* **2004**, *4*, 551−561.
(11) Shockcor, J. P.; Holmes, E. *Curr. Top. Med. Chem.* **2002**, *2*, 35−51.
(12) Chace, D. H.; Kalas, T. A.; Naylor, E. W. *Clin. Chem.* **2003**, *49*, 1797−1817.
(13) Roessner, U.; Luedemann, A.; Brust, D.; et al. *Plant Cell* **2001**, *13*, 11−29.
(14) Taylor, J.; King, R. D.; Altmann, T.; Fiehn, O. *Bioinformatics* **2002**, *18* (Suppl 2), S241−S248.
(15) Hall, R.; Beale, M.; Fiehn, O.; Hardy, N.; Sumner, L.; Bino, R. *Plant Cell* **2002**, *14*, 1437−1440.
(16) Fiehn, O.; Kopka, J.; Dormann, P.; Altmann, T.; Trethewey, R. N.; Willmitzer, L. *Nat. Biotechnol.* **2000**, *18*, 1157−1161.
(17) Want, E. J.; Cravatt, B. F.; Siuzdak, G. *ChemBioChem.*, in press.
(18) Siuzdak, G. *The Expanding Role of Mass Spectrometry in Biotechnology*; MCC Press: San Diego, CA, 2003.
(19) Leinonen, A.; Kuuranne, T.; Kostiainen, R. *J. Mass Spectrom.* **2002**, *37*, 693−698.
(20) Gelpi, E. *J. Chromatogr., A* **1995**, *703*, 59−80.

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in complex biofluids, reducing ionization suppression by decreasing the number of competing analytes entering the mass spectrometer ion source at any one time. Currently, numerous endogenous metabolites, such as amino acids, organic acids, and sugars are measured routinely, and today over 35 diseases, including phenylketonuria, hyperphenylalaninemia, and maple syrup urine disease in newborns, are monitored directly using electrospray ionization mass spectrometry (ESI-MS) analysis of metabolites.[4,21−24]

The metabolite profile[25] of a biofluid can allow for the prediction or detection of disease states.[12] However, to perform large-scale studies of total metabolite profiles, the development of an efficacious extraction technique is crucial for comprehensive and reliable statistical analysis. Although metabolite profiling is becoming established in plant biotechnology,[16] mammalian biofluids pose significant methodological challenges, as well as ethical and logistical ones.[17] The most commonly analyzed biofluids are plasma, serum, urine, and cerebrospinal fluid (CSF) but could include whole blood, milk, seminal fluid, and saliva. While urine analysis is useful for monitoring drug metabolites and disease states,[26] dramatic variations in urine output can occur between individuals reducing its utilities for large-scale studies. Acquiring samples of CSF is invasive and therefore undesirable for metabolite profiling studies where multiple samples are required. Analysis of serum or plasma appears to be the most popular in metabolite profiling studies and has the advantage of being less invasive than CSF collection. Additionally, plasma houses a complex metabolite mixture that reflects global changes resulting from system-wide catabolism and anabolism.

Inherent in serum metabolite analysis is that proteins can dominate the LC analysis and cause signal suppression of less abundant compounds. Therefore, the applicability of an extraction method may be determined by the number of metabolites recovered as well as the efficiency of protein removal. Deproteinization can be achieved using organic solvents such as methanol and acetonitrile, lowering pH with acid, or denaturation using heat. For example, Polson and colleagues[27] examined protein precipitation efficiencies of various organic solvents and acids, and Souverain et al.[28] evaluated the extraction efficiencies of six drug metabolites spiked into serum using acetonitrile and two organic acids. Other groups have also examined protein precipitation techniques using organic solvents and acids.[29−33] However, a comprehensive analysis of all detectable endogenous metabolites is absent from the literature.

The variation observed between extraction methods is attributed to factors including (a) differences in type and amount of protein precipitated from serum, (b) extent of metabolites binding to proteins, (c) degree of protein unfolding and denaturation, resulting in the release or capture of metabolites, and (d) solubility of the metabolites in the reagent used. Our goal was to develop an efficient methodology for metabolite profiling by LC/ESI-MS through a comparative analysis of metabolite extraction and deproteinization techniques, as well as to determine the number of unique metabolites that could be observed through all the approaches. Here, 14 protein precipitation methods[27,29−32,34] were evaluated based on (1) the number of reproducible serum metabolite features detected, where a metabolite feature is defined as a mass spectral peak in the mass region of 100−1000 with a signal-to-noise ratio exceeding 10:1 (see Experimental Section), (2) the type of metabolites extracted, based on hydrophobicity, (3) the similarity of metabolite features between different extraction methods, (4) the amount and type of protein remaining following sample preparation, and (5) method reproducibility. Important in metabolite profiling is the identification of potential biomarkers. Here we present the characterization of a family of lysophosphatidylcholines from serum.

## EXPERIMENTAL SECTION

**Materials.** Human serum (male, H-1388) and 1-palmitoyl-*sn*-glycero-3-phosphocholine (L5254) were obtained from Sigma Chemical Co. (St. Louis, MO). All organic solvents used for serum metabolite extraction were Optima grade from Fisher Scientific (Los Angeles, CA), and all acids were obtained from Sigma. All solvents for the LC/MS studies were HPLC grade (J. T. Baker, Phillipsburg, NJ). For the protein concentration studies, the Quick Start Bradford assay reagent was obtained from BioRad (Hercules, CA) and bovine serum albumin from Sigma. For the protein identification studies, tris(2-carboxyethyl)phosphine (TCEP), iodoacetamide (IAA), and hydrochloric acid were from Sigma, RapiGest was from Waters (Milford, MA), and porcine trypsin from Promega (Madison, WI).

**Serum Metabolite Extraction.** Fourteen serum metabolite extraction methods were compared using LC/MS. These methods involved (1) four organic solvents, methanol,[27] ethanol,[27] acetonitrile,[27,32] and acetone,[31] as well as combinations of methanol and acetone or acetonitrile in ratios of 30:70, 50:50, and 70:30; (2) acid precipitation using trichloroacetic acid (TCA),[32] perchloric acid (PCA),[31] or sulfosalicylic acid (SSA);[33] and (3) heat denaturation.[32]

**Solvent and Acid Extraction.** The starting volume of serum was 50 $\mu$L. All ratios of solvent and acid to serum were 2:1 (v/v) except for perchloric acid (1:1) and TCA (1:10); the final acid concentrations were 3% for SSA, 10% for TCA, and 3% for perchloric acid. After brief vortexing, serum samples were incubated for 20 min, either at 4 °C (acids) or at −20 °C (organic solvents). Supernatants were collected after centrifugation at 13200*g* for 10 min, dried, and resuspended in 50 $\mu$L of 95:5 (v/v) water/acetonitrile. Solvent control samples (without human serum) were


(21) Chace, D. H.; Millington, D. S.; Terada, N.; Kahler, S. G.; Roe, C. R.; Hofman, L. F. *Clin. Chem.* **1993**, *39*, 66−71.
(22) Chace, D. H.; DiPerna, J. C.; Naylor, E. W. *Acta Paediatr. Suppl.* **1999**, *88*, 45−47.
(23) Chace, D. H.; Kalas, T. A.; Naylor, E. W. *Annu. Rev. Genomics Hum. Genet.* **2002**, *3*, 17−45.
(24) Naylor, E. W.; Chace, D. H. *J. Child Neurol.* **1999**, *14* (Suppl 1), S4−8.
(25) Villas-Boas, S. G.; Rasmussen, S.; Lane, G. A. *Trends Biotechnol.*, in press.
(26) Chan, E. C.; Ho, P. C. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1959−1964.
(27) Polson, C.; Sarkar, P.; Incledon, B.; Raguvaran, V.; Grant, R. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2003**, *785*, 263−275.
(28) Souverain, S.; Rudaz, S.; Veuthey, J. L. *J. Pharm. Biomed. Anal.* **2004**, *35*, 913−920.
(29) van Oss, C. J. *J. Protein Chem.* **1989**, *8*, 661−668.
(30) Sivaraman, T.; Kumar, T. K.; Jayaraman, G.; Yu, C. *J. Protein Chem.* **1997**, *16*, 291−297.
(31) Daykin, C. A.; Foxall, P. J.; Connor, S. C.; Lindon, J. C.; Nicholson, J. K. *Anal. Biochem.* **2002**, *304*, 220−230.
(32) Vanholder, R.; Hoefliger, N.; De Smet, R.; Ringoir, S. *Kidney Int.* **1992**, *41*, 1707−1712.
(33) Khan, K.; Blaak, E.; Elia, M. *Clin. Chem.* **1991**, *37*, 728−733.
(34) Creighton, T. E. *Proteins: structures and molecular principles*; W. H. Freeman: New York, 1983.

prepared for each extraction method, to ensure that the features detected did not arise from the reagents used.

**Protein Removal by Heat.** Nanopure water was added to 50 $\mu$L of serum in a ratio of 2:1 and vortexed for 10 s. The sample was heated in a water bath at 90 °C for 30 min, then incubated at 4 °C for 20 min, and centrifuged, and supernatant was transferred, dried, and reconstituted in 95:5 (v/v) water/acetonitrile.

**LC/MS Metabolite Analyses.** Reversed-phase chromatography and mass detection of the serum metabolite extracts was performed on an Agilent 1100 LC/MSD SL system. Triplicate runs of duplicate extractions from each of the 14 methods (totaling 84 samples) were analyzed randomly, with a blank run between each sample to prevent carryover. For each run, 10 $\mu$L of serum metabolite extract was injected onto the same $C_{18}$ column (Symmetry Column, 2.1 × 100 mm, 3.5 $\mu$m; Waters) and eluted at a flow rate of 250 $\mu$L/min under gradient conditions of 5−90% B over 60 min. Mobile phase A consisted of water/acetonitrile/formic acid (95:5:0.1, v/v/v), and B consisted of acetonitrile/formic acid (100:0.1, v/v). Mass spectral data from 100 to 1000 $m/z$ were collected in the positive ionization mode.

**LC/MS Data Analysis.** LC/MS data was processed using XCMS software, which can be downloaded freely as an R package[35] from the Metlin Metabolite Database (http://metlin-.scripps.edu) and has been tested and approved recently by the Bioconductor bioinformatics project (http://www.bioconductor-.org). The software and algorithms of XCMS will be described fully in a forthcoming paper. The poster presented at the 53rd meeting of the American Society of Mass Spectrometry describing XCMS is provided in the Supporting Information (Figure S3) while key technical points of the data analysis are discussed here. In summary, raw LC/MS extracted ion chromatograms (EIC), every 0.1 $m/z$, were denoised with matched filtration, using the second-derivative Gaussian as the model peak shape.[36,37] A metabolite feature was defined as a mass spectral peak in the mass region of 100−1000 with a signal-to-noise ratio exceeding 10:1. To quantitate each feature, the area under the original EIC was integrated between the zero-crossing points of the filtered EIC. Isotopic and adduct peaks were treated as separate metabolite features, thus contributing to the total number of metabolite features.

Features were aligned and matched across samples by (1) slicing the feature list from all 84 samples (6 replicates of 14 methods) into sections using a 0.25 $m/z$ window, (2) binning and smoothing each slice into a feature density vector along retention time,[38] and (3) Identifying peaks in the vector and then grouping together features falling under each peak.

Feature distribution plots were produced using Feature Distribution steps 1 and 2 of feature alignment and matching, without slicing. Retention time varied 5−25 s during the LC/MS runs. Matching of such deviations was found to be within the limits of the feature-alignment algorithm.

A "reproducible feature" was defined as a metabolite feature that was detected in at least five out of six LC/MS runs for a given extraction method. To examine similarities between methods, pairwise comparisons were performed to give the number of identical reproducible features shared between every two methods. To display these similarities, a dendrogram was produced by applying a hierarchical clustering algorithm to the data.[39]

**Protein Concentration Estimation.** The Bradford assay was employed to estimate the amount of protein remaining in each reconstituted serum metabolite extract and was repeated four times for each extraction method. Extracted metabolite samples were directly mixed with the Bradford reagent in a ratio of 1:50 (v/v), while untreated serum was first diluted with water prior to the addition of Bradford reagent. A calibration curve was generated using known concentrations of a standard protein (bovine serum albumin).

**Protein Digestion.** Dried serum metabolite extracts were reconstituted in 50 $\mu$L of ammonium bicarbonate buffer (100 mM, pH 7.5) with added RapiGest (2%). All samples were incubated at room temperature for 60 min before reduction and alkylation, where 1 mM TCEP was added and incubated at 37 °C for 30 min, followed by the addition of 5 mM IAA and incubation at 37 °C in darkness for 30 min. Porcine trypsin was added at a ratio of 1:50 (w/w, trypsin/total protein, as determined from the Bradford assay). The trypsin enzyme digest was incubated at 37 °C overnight followed by a second trypsin digestion under the same conditions. Each sample was then acidified by the addition of 6 M HCl to pH 2 to inactivate the trypsin and incubated at 37 °C for 60 min, followed by 4 °C for 60 min to precipitate the insoluble hydrolysis products of RapiGest. Precipitates were removed by centrifugation at 13200$g$ for 10 min, and supernatants were retained for proteomic analysis.

**LC/MS/MS Proteomics Analysis.** An Agilent 1100 LC/MSD Trap system coupled directly to an Agilent 1100 nanopump and a microautosampler was used for tandem reversed-phase LC/MS analysis for peptide and protein identification. The mobile phases consisted of buffer A, water/formic acid (100:0.1, v/v), and buffer B, acetonitrile/formic acid (100:0.1, v/v). Eight microliters of each trypsin digested serum metabolite extract was injected into a fused-silica column (100-$\mu$m i.d., 15 cm long) with a tip pulled to a diameter of less than 5 $\mu$m and packed with $C_{18}$ stationary phase (Zorbax SB-C18, Agilent Technologies). A flow rate of 250 nL/min was used with gradient conditions of 5% B to 70% B over 100 min. A wash run (no injection) was performed between each sample to prevent carryover. MS/MS data were searched against the NCBInr database using Spectrum Mill MS Proteomics Workbench (Agilent Technologies).

**Fourier Transform Mass Spectrometry (FTMS).** The high-accuracy measurements were performed in the positive ion mode using a Bruker (Billerica, MA) APEX III (7.0 T) FTMS instrument equipped with an Apollo electrospray source. The collected LC fractions were mixed with a collection of small molecule standards and directly infused at 3 $\mu$L/min using a Harvard Apparatus (Holliston, MA) syringe pump. Pneumatic assist at a backing pressure of 60 psi was used along with an optimized flow rate of heated countercurrent drying gas (300 °C). Ion accumulation was performed using SideKick without pulsed gas trapping. Data acquisition times of ~1 min were used, yielding a resolving power

(35) R Development Core Team. *R: A language and environment for statistical computing*; R Foundation for Statistical Computing: Vienna, Austria, 2004.
(36) Danielsson, R.; Bylund, D.; Markides, K. E. *Anal. Chim. Acta* **2002**, *454*, 167−184.
(37) Andreev, V. P.; Rejtar, T.; Chen, H. S.; Moskovets, E. V.; Ivanov, A. R.; Karger, B. L. *Anal. Chem.* **2003**, *75*, 6314−6326.
(38) Silverman, B. W. *Density Estimation*; Chapman and Hall: London, 1986.
(39) Murtagh, F. Multidimensional Clustering Algorithms. In *COMPSTAT Lectures 4*; Physica-Verlag: Wuerzburg, 1985.

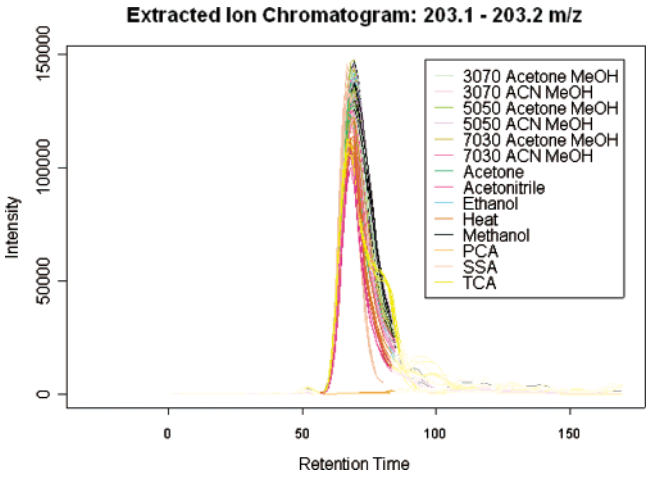



**Figure 1.** EIC generated by XCMS for enhanced metabolite feature visualization. The title of each EIC shows the *m*/*z* range of the feature being plotted, where the *x* and *y* axes show retention time (in seconds) and intensity, respectively. The color of each EIC can be chosen so that different sample groups can be distinguished.

of 90 000 at $m/z$ 496 in broadband in the $m/z$ range of 200−2200. Calculated molecular masses for ions generated by a mixture of small-molecule standards were used to internally calibrate the data.

**Tandem MS Experiments.** MS/MS experiments were performed in the positive ion mode using a Micromass (Manchester, U.K.) Q-TOF Micro instrument equipped with a Z-spray electrospray source and a lock mass sprayer. The source temperature was set to 110 °C with a cone gas flow of 150 L/h, a desolvation gas temperature of 365 °C, and a nebulization gas flow rate of 350 L/h. The capillary voltage was set at 3.2 kV, and the cone voltage at 30 V. The collision energy was set at 30−35 V. Samples were directly infused at 4 $\mu$L/min using a Harvard Apparatus syringe pump. MS/MS data were collected in the centroid mode over a scan range of $m/z$ 100−600 for acquisition times of 2 min.

## RESULTS AND DISCUSSION

The metabolite feature data presented here were generated entirely using XCMS, an in-house-developed data analysis software package (see Supporting Information Figure S3, and Experimental Section). A metabolite feature was defined as a mass spectral peak in the mass region of 100−1000 with a signal-to-noise ratio exceeding 10:1. XCMS enables the accurate comparison of sample groups through peak alignment and peak matching, employing stringent filtering criteria. Retention time, $m/z$, and intensity values are recorded for each metabolite feature detected. Using this software, between 1000 and 2000 reproducible metabolite features were detected for each extraction method. Isotopic and adduct peaks were treated as separate metabolite features. By compiling the unique features detected in all 14 methods, a value of over 10 000 was attained. Individual features can be plotted as EICs to allow for visual comparison of sample groups, in this case the extraction methods. An example of a well-aligned metabolite feature detected from all 84 runs is shown in Figure 1. This EIC is representative of our overall observation that methanol is the best metabolite extraction method. However, different aspects of the metabolite feature analysis need to be considered, and these are discussed in the following sections.

**Method Reproducibility.** Organic solvents were the most efficient and reproducible for both metabolite recovery and protein precipitation. Visually, the resulting LC/MS chromatograms showed great similarity (Figure 2), with the exception of a peak at ~600 s in the chromatogram of acetonitrile, ethanol, and heat-treated serum, which after deconvolution was determined to be a 6-kDa protein. Preparation using acid or heat alone was not desirable for metabolite profiling, with less peaks observed as well as incomplete protein removal.

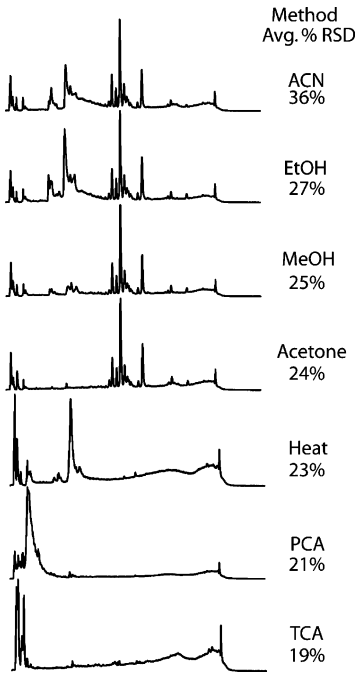



**Figure 2.** LC/MS chromatograms of serum samples after treatment with (from top to bottom) acetonitrile (ACN), ethanol (EtOH), methanol (MeOH), acetone, heat (90 °C), PCA, and TCA. Individual metabolite feature intensity RSD was obtained from six replicates, where Avg. % RSD reports the average value for all detected features within each method. The chromatograms of the solvent mixtures (30:70, 50:50, and 70:30 mixtures of either acetone/methanol or acetonitrile.methanol) are similar to those of methanol extracted samples, with average RSD ranging from 20 to 30%. The chromatogram of sulfosalicylic acid treated serum is very similar to that of TCA, with average RSD of 20%. Additional data are provided in the Supporting Information (Table S1, S2, and Figures S1, S2).

The average percent relative standard deviation (RSD) for the intensity of all detected metabolite features from each method is illustrated in Figure 2. Individual metabolite feature intensity RSD was obtained from six replicates, where Avg. % RSD reports the average value for all detected features within each method. Overall, acetonitrile-extracted samples showed the most variation in feature intensity with an average RSD of >35%, at least 8% higher than all methods containing no acetonitrile. It is important to emphasize that as this is an average of several thousand metabolite features, individual metabolite RSD values can vary greatly within each method. For example, RSD values ranged from 2 to 130% within the acetonitrile-extracted serum samples, and a graphical illustration of intensity variation of over 40 features is provided in the Supporting Information (Table S1 and Figure S1).The acids and heat treatments all showed RSDs of <25%; however, fewer metabolites are detected after acid treatment (with total number of features being 60% of the organic solvents, see Number of Reproducible Features section). The RSD values for

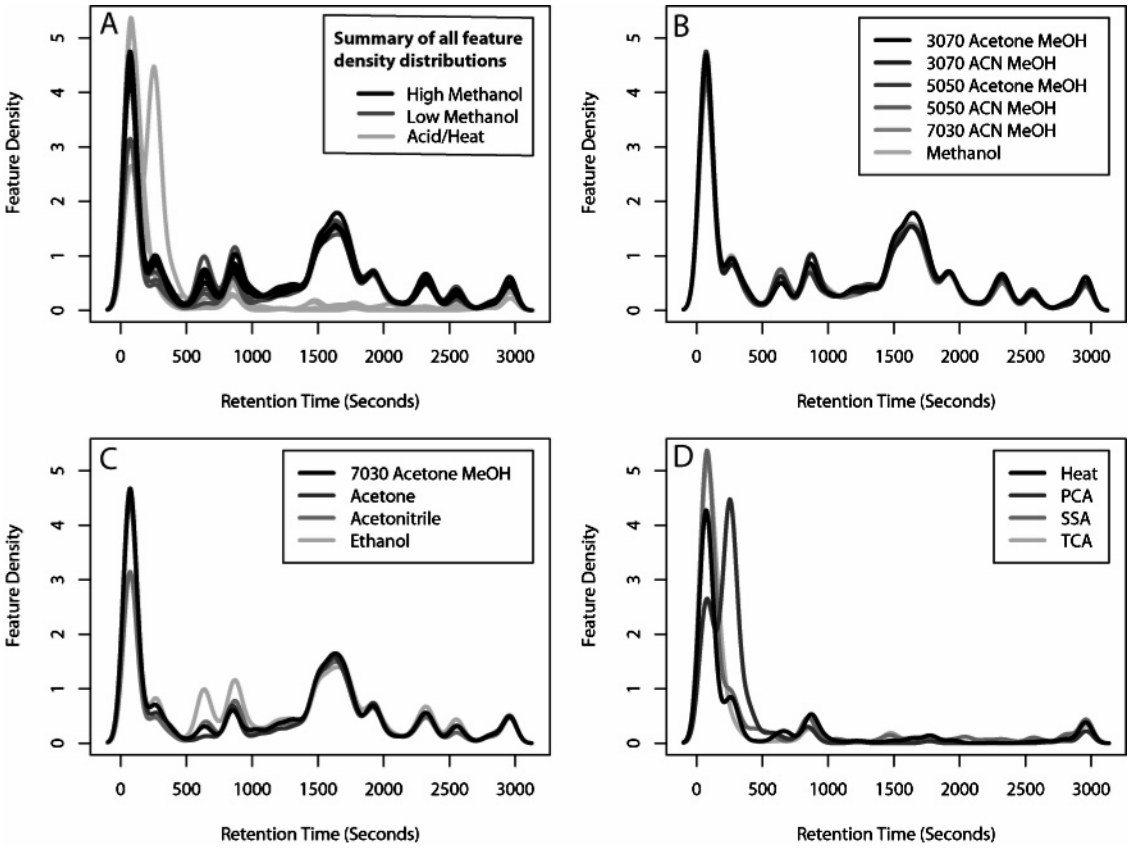



**Figure 3.** Feature density distribution. (A) Overview of all 14 extraction methods grouped into three classes: high methanol, low methanol, and acid/heat. (B) High-methanol group. All showed comparable distribution of features across chromatographic time. This group corresponds to group I in the dendrogram of Figure 4. (C) low-methanol group. These showed lower feature density during the 0−1000-s range. This group corresponds to groups II and III in Figure 4. (D) Acid/heat group. All showed a significantly lower number of features after 500 s, with perchloric acid showing a highly elevated number of features at 250 s. This group corresponds to group IV in Figure 4. Feature density is shown as a relative value. Abbreviations as given in text.

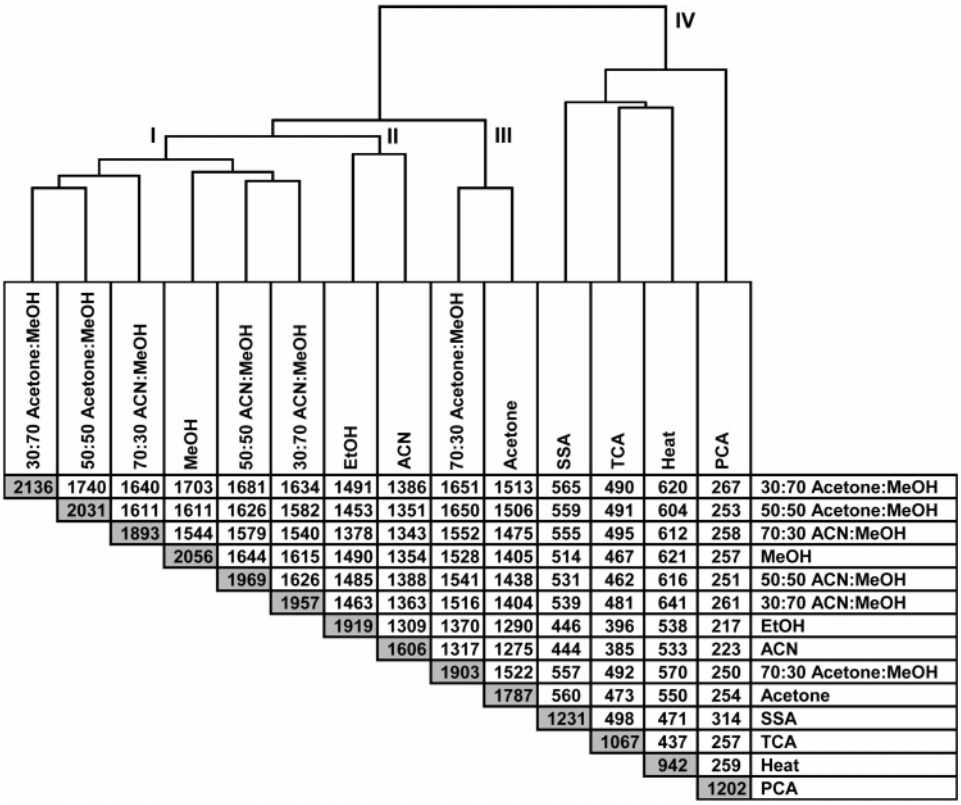
all features are tabulated in the Supporting Information (Table S2 and Figure S2).

**Feature Distributions.** The goal in metabolite profiling is to maximize the number and type of metabolite features obtained. Therefore, to investigate extraction efficiency with regard to the hydrophobicity of the metabolite features, we examined their distribution along chromatographic time (Figure 3). It is important to distinguish these feature density plots from the LC/MS chromatograms plotted along retention time in Figure 2. Whereas Figure 2 illustrates the total intensity of ions detected at each time point, the feature density plots show the relative number of unique features detected at each time point, irrespective of feature intensity.

Upon examination of the feature distributions, the methods were divided into three groups: B, C, and D (Figure 3A), illustrated separately in Figure 3B−D. Group B, termed "high methanol", comprises all methanol-containing solvents, with the exception of 70:30 acetone/methanol (Figure 3B). Methods in group B showed a very similar distribution of features with consistently high peak density throughout the plot. Group C, termed "low methanol", consists of 70:30 acetone/methanol, acetone, acetonitrile, and ethanol (Figure 3C). Methods in group C had distributions with a lower feature density at several specific time points in the first 1000 s. Methods 70:30 acetone/methanol, acetone, and acetonitrile all showed lower density at 250, 650, and 850 s, while ethanol showed significantly lower density at 50 s. Interestingly, 70:30 acetonitrile/methanol, placed in the high-methanol group, did not show the same characteristic lower feature density as 70:30 acetone/methanol. At 70% concentration with methanol, perhaps acetone is a more dominant part of the mixture than 70% acetonitrile with methanol. Group D, termed "acid/heat", showed much stronger bias toward hydrophilic molecules, with significantly lower feature density after 500 s (Figure 3D). Perchloric acid was unique among all the other methods in showing a lower feature density at 50 s and significantly higher feature density at 250 s. These feature distributions (Figure 3A−D) show strong correlation with the clustering described in the Clustering of Extraction Methods section (Figure 4).

**Number of Reproducible Features.** The number of reproducible features detected from each extraction method is shown in the shaded diagonal boxes of Figure 4. A "reproducible feature" is defined as a metabolite feature that was detected in at least five out of six LC/MS runs for a given method. Overall, 100% methanol or methanol-containing solvent mixtures resulted in the highest number of reproducible features detected, with the three best methods in this respect being (1) 30:70 acetone/methanol, (2) 100% methanol, and (3) 50:50 acetone/methanol, each with over 2000 reproducible features.

Within the organic solvents, acetonitrile proved to be the least effective, producing ~1600 reproducible features (Figure 4). The addition of methanol to acetonitrile increased the number of

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2136 | 1740 | 1640 | 1703 | 1681 | 1634 | 1491 | 1386 | 1651 | 1513 | 565 | 490 | 620 | 267 | 30:70 Acetone:MeOH |
| | 2031 | 1611 | 1611 | 1626 | 1582 | 1453 | 1351 | 1650 | 1506 | 559 | 491 | 604 | 253 | 50:50 Acetone:MeOH |
| | | 1893 | 1544 | 1579 | 1540 | 1378 | 1343 | 1552 | 1475 | 555 | 495 | 612 | 258 | 70:30 ACN:MeOH |
| | | | 2056 | 1644 | 1615 | 1490 | 1354 | 1528 | 1405 | 514 | 467 | 621 | 257 | MeOH |
| | | | | 1969 | 1626 | 1485 | 1388 | 1541 | 1438 | 531 | 462 | 616 | 251 | 50:50 ACN:MeOH |
| | | | | | 1957 | 1463 | 1363 | 1516 | 1404 | 539 | 481 | 641 | 261 | 30:70 ACN:MeOH |
| | | | | | | 1919 | 1309 | 1370 | 1290 | 446 | 396 | 538 | 217 | EtOH |
| | | | | | | | 1606 | 1317 | 1275 | 444 | 385 | 533 | 223 | ACN |
| | | | | | | | | 1903 | 1522 | 557 | 492 | 570 | 250 | 70:30 Acetone:MeOH |
| | | | | | | | | | 1787 | 560 | 473 | 550 | 254 | Acetone |
| | | | | | | | | | | 1231 | 498 | 471 | 314 | SSA |
| | | | | | | | | | | | 1067 | 437 | 257 | TCA |
| | | | | | | | | | | | | 942 | 259 | Heat |
| | | | | | | | | | | | | | 1202 | PCA |

**Figure 4.** Comparison of reproducible features shared in common between metabolite extraction methods. The dendrogram was produced by applying a hierarchical clustering algorithm, to show similarities between the methods. The length of the vertical lines is a measure of similarity, with shorter lines demonstrating more common features. This produced four groups, I−IV. Within the constraints established by the dendrogram, the table is sorted by the number of reproducible features. The diagonal shaded boxes contain the total number of reproducible features for each method, defined as a feature that was detected in at least five out of six LC/MS runs for a given extraction method. The remaining boxes contain the number of identical features shown as a pairwise comparison between methods. Abbreviations as given in text.

detected reproducible features by up to 25% (from ~1600 to ~1900), although varying the percentage of methanol between 30 and 70% did not have a significant effect. The addition of methanol to acetone also increased the number of reproducible features; however, in contrast to acetonitrile, increasing ratios of methanol to acetone resulted in a stepwise increase (~100 features per increment of methanol addition). There is an increase of 100 reproducible features when comparing acetone, ethanol, and methanol, with methanol giving the higher number.

Acid and heat treatment were the least effective approaches for metabolite extraction, producing significantly fewer reproducible features (~950 to ~1250) (Figure 4). The incomplete removal of the acid from these samples may have affected the quality of mass spectral data collected, thus requiring a further cleanup step to achieve better results.[27] Heat resulted in the lowest number of observed reproducible features (~950), perhaps due to enhanced binding of the metabolites to the denatured protein or destruction of the metabolites themselves.

The number of identical reproducible features between methods was also considered. Pairwise comparisons of all 14 extraction methods are shown in Figure 4. The organic solvents all shared a relatively high number of features (~1300 to ~1750). Heat and two of the acids, SSA and TCA, shared a significantly lower number of features with the other methods (~400 to ~650) as well as among themselves (~500). Perchloric acid, however, shared the fewest number of features with all the other methods (<300).

**Clustering of Extraction Methods.** Hierarchical clustering[39] of the extraction methods was performed to produce a dendrogram illustrating which methods produced the most similar reproducible features (Figure 4). The length of the vertical lines in the dendrogram is a measure of similarity, with shorter lines demonstrating more common features. This approach produced three evident classes of organic solvent methods (I, II, III). The first six organic solvent methods, each containing methanol, all clustered together with only small differences (I). The two methods with the highest proportion of acetone (100 and 70%) clustered quite closely together (III). Ethanol and acetonitrile also clustered together (II), perhaps due in part to these solvents being less effective at removing protein from serum. Heat, SSA, and TCA also clustered together (IV), whereas perchloric acid proved to be the most dissimilar to any method.

**Protein Analysis.** The residual serum protein concentration was estimated for each method using the Bradford assay; the results are expressed as a percentage of the protein concentration before treatment (Figure 5). Each value is expressed as the average of four replicates. Acetonitrile contained the largest amount of residual protein (~6%), a percentage similar to that reported by Polson and colleagues (~4%),[27] although this group rated acetonitrile more effective at protein removal than methanol and ethanol. Using the Bradford assay, they reported residual protein values of methanol and ethanol as ~10 and ~12%, respectively, whereas we observed 2 and 4%. In fact, all methanol-containing extraction methods produced residual protein of 2%

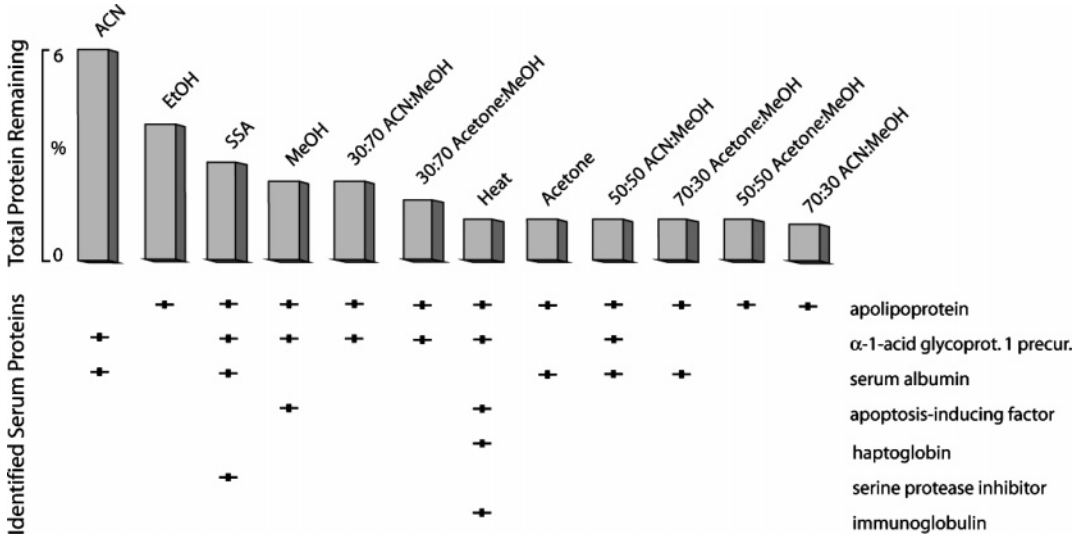



**Figure 5.** Residual protein in the serum metabolite extracts. (Top) Results from Bradford assay analysis of serum extracts reported as a percentage of the protein concentration before treatment. (Bottom) Protein identities determined from LC/MS/MS data on peptide fragments obtained for trypsin digests of each extract. Proteins were identified by searching against the NCBInr database using Spectrum Mill MS Proteomics Workbench (Agilent Technologies). The presence of a protein is indicated by a + sign. TCA and PCA negatively affected the quantitative analysis using the Bradford assay and so were omitted from the table. Abbreviations as given in text.

or less. Based on our findings, 100% methanol or methanol-containing solvents were more effective at protein removal compared to acetonitrile and ethanol. Despite heat and SSA treatment resulting in less than 3% residual protein, the number of reproducible features was less than 1200 in both cases. The strong acids (TCA and PCA) interfered with the Bradford reagent binding mechanism, producing falsely negative results, and were therefore omitted from this analysis. It should be noted that while a standard protein (bovine serum albumin) is used as the Bradford assay standard, the actual protein concentration may vary due to the fact that a protein mixture is being analyzed, and the Bradford dye reagent binds primarily to basic and aromatic amino acids.[40]

To expand the protein analysis, the serum extracts were digested with trypsin and the resulting peptides were analyzed by nano-LC/MS/MS in order to identify the residual proteins. The major proteins identified are shown in Figure 5. Apolipoprotein was found in over 90% of all the serum metabolite extracts and serum albumin, the most abundant soluble serum protein,[41] was also identified in half of the samples. This is in agreement with studies where apolipoprotein is known for its solubility in organic solvents and is even robust enough to remain intact after heat treatment.[42−44] The Bradford assay results indicated erroneously that no protein remained in the TCA- and perchloric acid-treated samples, but upon LC/MS/MS analysis, at least five proteins were identified, with the most abundant being apolipoprotein and serum albumin.

Despite the acetonitrile method showing the highest measured protein content in the Bradford assay (Figure 5), only two proteins, serum albumin and α-1-acid glycoprotein precursor, were identified. It is likely that there were other proteins in the sample that were not detected due to ionization suppression by the peptides generated from these two highly abundant proteins. It was observed that, when using acetonitrile, the precipitated protein pellet was less compact than with other solvents, making it difficult to obtain a clear supernatant and thus contributing to the retention of some precipitated proteins in the serum extract.

**Monitoring Individual Metabolite Features.** In addition to evaluating the methods based on the number and type of metabolite features, and their protein removal efficiencies, individual metabolites should be examined, as different methods may enhance the extraction of particular groups of molecules. Using XCMS, sample groups can be compared visually; four representative EICs are shown in Figure 6.Two of the EICs, Figure 6A and B, compared all 14 extraction methods, each represented by a different color. The second pair of EICs, Figure 6C and D, compared 100% methanol (black) with acetone-containing methods (blue). As discussed earlier, organic solvents extract many more serum metabolites than acids and heat and also share more reproducible features, as illustrated in Figure 6A. Within the organic solvents, 100% acetonitrile (darker purple) was the least effective at metabolite extraction. The wide range of extraction efficiencies for an individual feature is demonstrated in Figure 6B, where heat and methanol performed the best. From the clustering data, acetone exhibited properties very different from methanol, as illustrated in Figure 6C and D, where methanol was more efficient at extracting a particular feature in the former and acetone-containing solvents were more efficient in the latter. By plotting features this way, it can be seen that different extraction methods can recover different metabolite features from serum. In fact, it was calculated that a total number of 10 000 unique metabolite features were detected as a result of using all 14 extraction methods and performing only positive mode LC/MS analyses at normal flow rate (250 $\mu$L/min).

(40) Compton, S. J.; Jones, C. G. *Anal. Biochem.* **1985**, *151*, 369−374.
(41) Peters, T. *All about albumin: biochemistry, genetics, and medical applications*; Academic Press: San Diego, CA, 1996.
(42) Chertov, O.; Biragyn, A.; Kwak, L. W.; et al. *Proteomics* **2004**, *4*, 1195−1203.
(43) Farwig, Z. N.; Campbell, A. V.; Macfarlane, R. D. *Anal. Chem.* **2003**, *75*, 3823−3830.
(44) Xiong, Z. *J. Chromatogr., B: Biomed. Sci. Appl.* **1997**, *702*, 216−220.

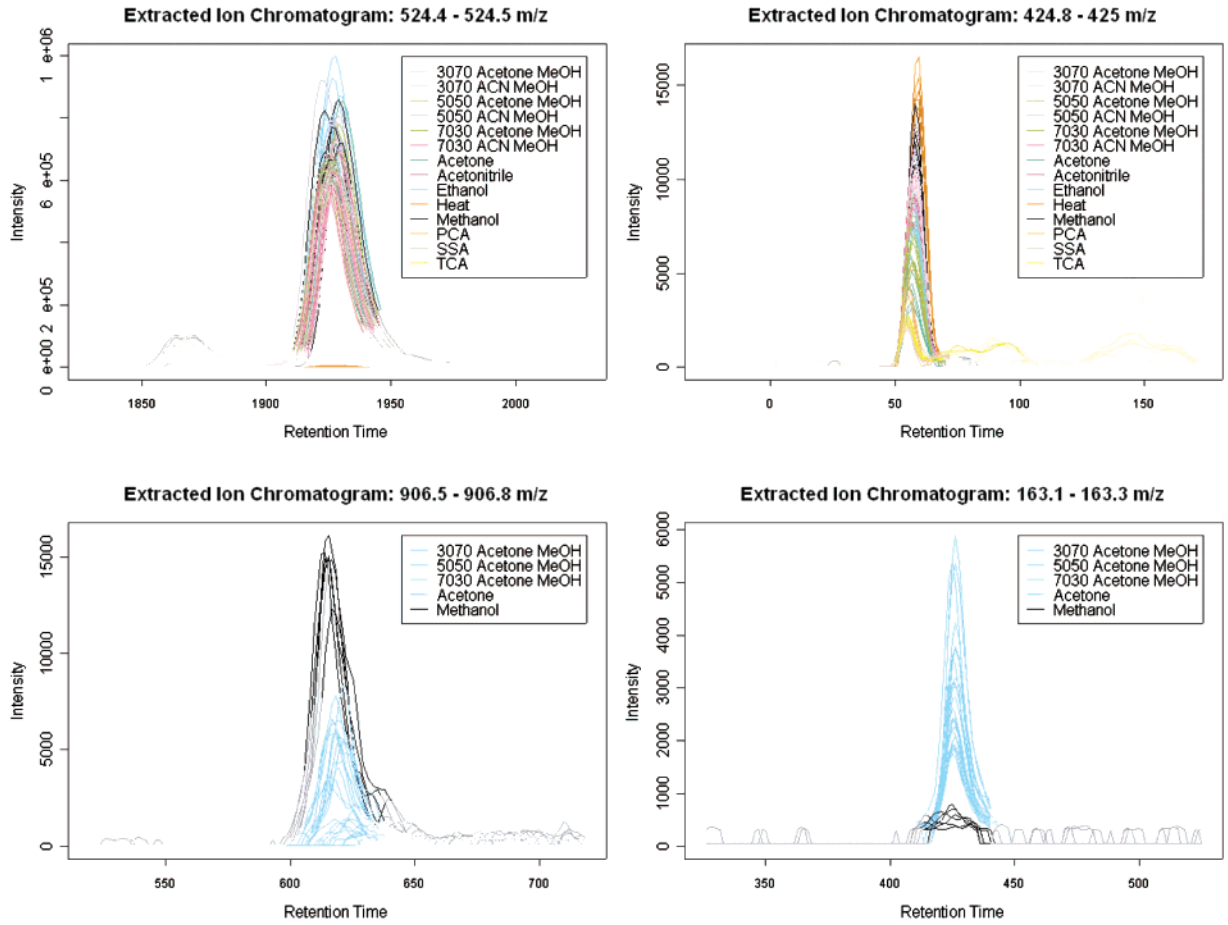



**Figure 6.** Representative EICs generated by XCMS for enhanced metabolite feature visualization. The title of each EIC shows the *m*/*z* range of the feature being plotted, where the *x* and *y* axes show retention time (in seconds), and intensity, respectively. The color of each EIC can be chosen so that different sample groups can be distinguished.

As discussed in the Method Reproducibility section, acetonitrile had the highest average RSD (36%), which reflects an overall low reproducibility of metabolite extraction. In fact, as this value is an average of several thousand metabolite features, individual metabolite RSD values can vary greatly within each method. The RSD values for the same metabolite feature also vary dramatically between methods (Figure 7). In general, the organic solvents were more effective than acids or heat at recovering metabolites from serum. Methanol was found to be a reproducible extraction solvent, with a relatively low average RSD (25%). Further plots illustrating these findings are available in the Supporting Information (Tables S1, S2 and Figures S1, S2).

**Identification of Endogenous Metabolites.** Once metabolite features of interest have been selected, precise identification is required. To this end, we have identified four metabolites, all belonging to the family of lysophosphatidylcholines, by employing FTMS and MS/MS of fractions collected from LC/MS analyses of human serum. Here, the chemical characterization of one such metabolite, 1-palmitoylglycerophosphatidylcholine, which we found to be the most intense feature ($m/z$ 496) in human serum using organic solvent extraction, is illustrated in Figure 8. Analysis of this $m/z$ 496 feature by ESI-FTMS provided a relative error of 0.10 parts per million (ppm), corresponding to a molecular formula of $C_{24}H_{51}NO_7P$. The calculated isotope pattern for $C_{24}H_{51}NO_7P$ (blue) overlaid well with the experimental spectrum (red). The inset of Figure 8 shows the MS/MS data of commercially purchased 1-palmitoylglycerophosphatidylcholine, which was identical to the MS/MS of $m/z$ 496 from serum LC/MS analysis. Highlighted in the MS/MS spectrum are prominent fragments at $m/z$ 104 and 184 and a loss of water at $m/z$ 478. Data obtained from ESI-FTMS and MS/MS analyses enabled the identification of three other lysophosphatidylcholines (Figure 9).

## CONCLUSIONS

Growing interest in metabolite profiling has fueled a need for straightforward and efficient metabolite extraction methods. Thus, a key aspect to our clinical metabolite profiling analyses has been to develop and optimize metabolite extraction and protein removal methods. Many current approaches to metabolite extraction, particularly in drug metabolism research or the study of a particular disease, focus on one or a few specific metabolites. While some methods involve protein precipitation with a simple solvent system such as methanol or acetonitrile, in many cases, the approach is tailored to particular metabolites. Knowledge of the types of metabolites being monitored enables the use of internal standards to optimize extraction efficiency.[45] Importantly, these "tailored" approaches cannot be applied directly to untargeted, or "global" metabolite profiling, due to the large dynamic range and vast variation in chemical structure of the thousands of metabolites. Clearly a comprehensive approach is needed. To

(45) Flaherty, J. M.; Connolly, P. D.; Decker, E. R.; et al. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2005**, *819*, 329−338.

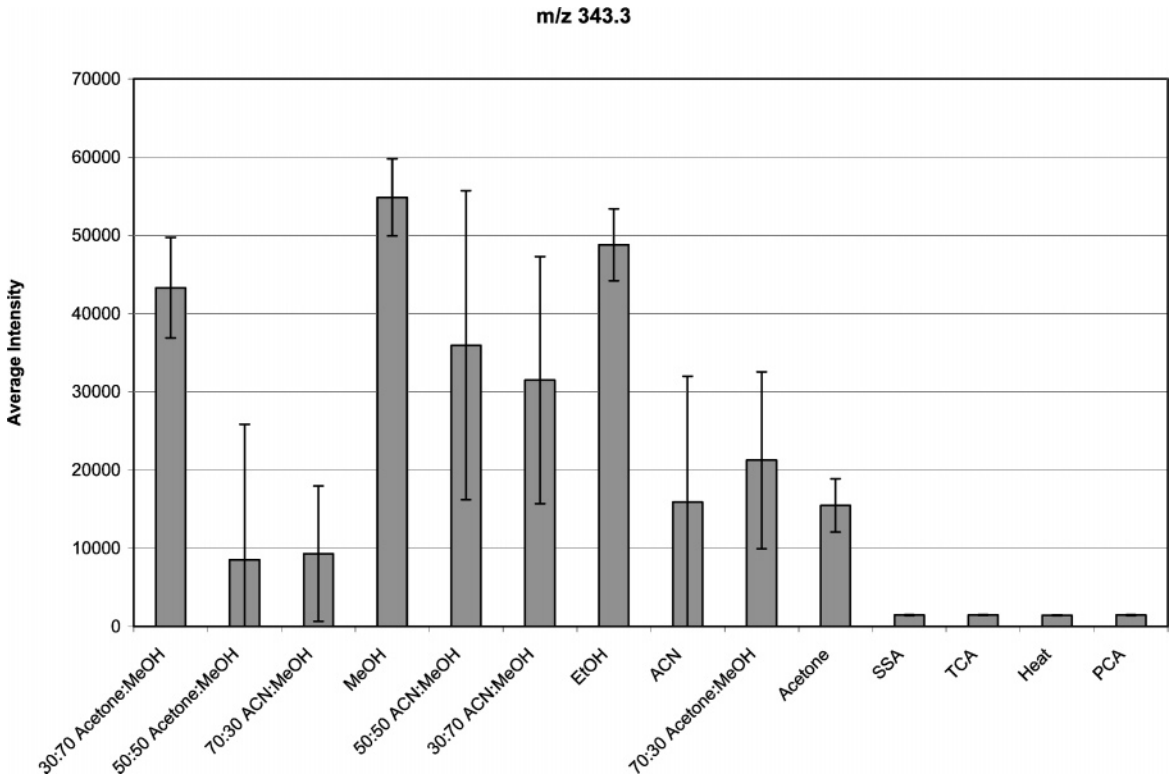



**Figure 7.** Average intensity of a single feature plotted for each extraction method. The error bars illustrate the standard deviation between the six replicates. The order of the extraction methods in this figure is identical to that in Figure 3. Additional feature intensity plots are available in the Supporting Information (Figure S1).

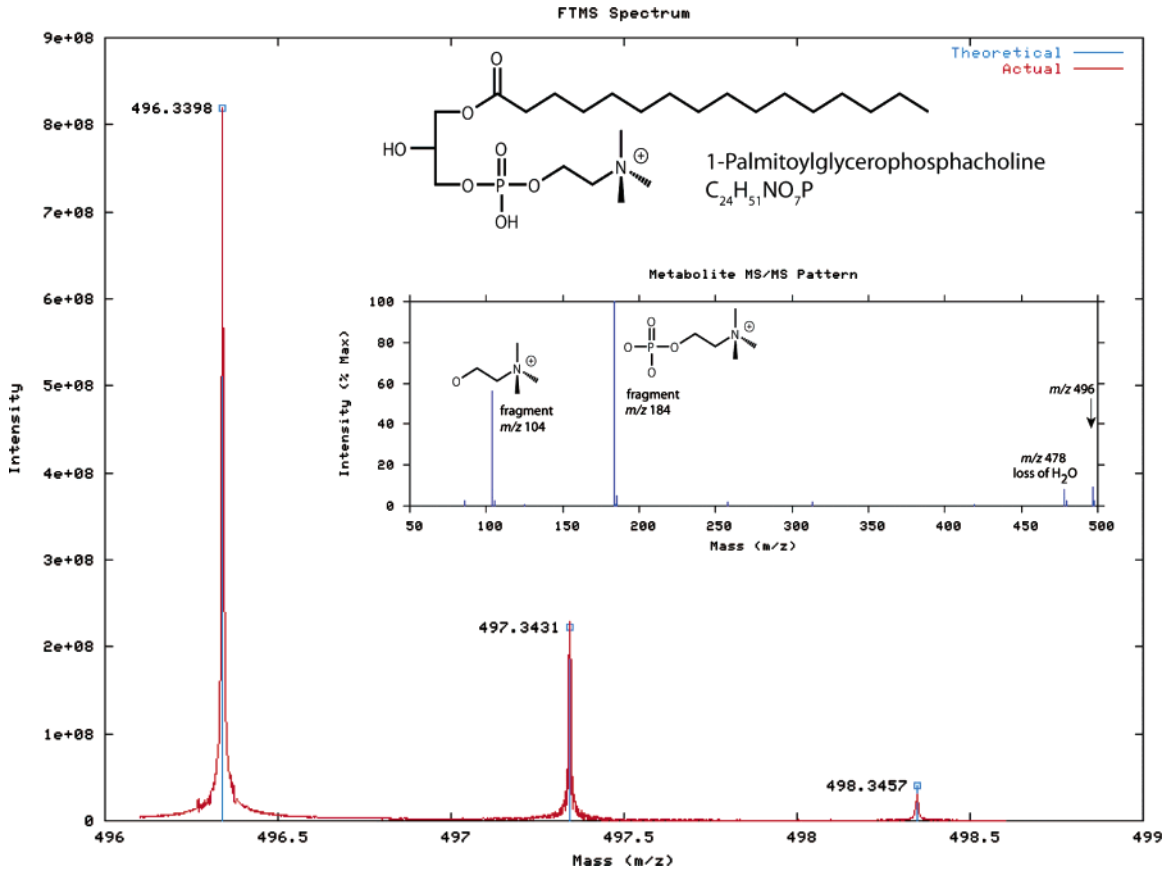
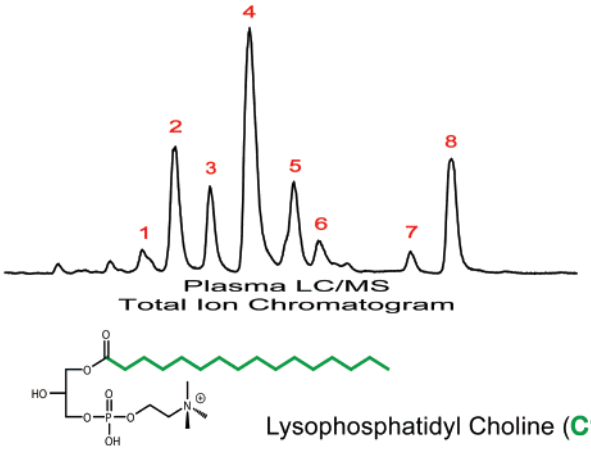



**Figure 8.** FTMS and MS/MS data for 1-palmitoylglycerophosphatidylcholine (*m*/*z* 496), the most intense feature observed in human serum after organic solvent extraction. Analysis of this *m*/*z* 496 metabolite by ESI-FTMS (Bruker APEX III 7.0-T instrument) provided an exact mass of 496.3398, corresponding to a molecular formula of $C_{24}H_{51}NO_7P$. The theoretical isotope pattern for $C_{24}H_{51}NO_7P$ (blue) overlaid well with the experimental spectrum (red). Inset is the MS/MS data of 1-palmitoylglycerophosphatidylcholine obtained on a Micromass Q-TOF instrument. Highlighted are prominent fragments at *m*/*z* 104 and 184 and a loss of water at *m*/*z* 478.




| Fraction | m/z | Carbon-chain | Double bonds | Relative error (ppm) |
|---|---|---|---|---|
| 1, 2 | 520 | C18 | 2 | 1.1 |
| 3, 4 | 496 | C16 | 0 | 0.10 |
| 5, 6 | 522 | C18 | 1 | 0.98 |
| 7, 8 | 524 | C18 | 0 | 0.57 |

**Figure 9.** Identification of the eight most intense ions from human serum extracted with organic solvents. A truncated LC/MS chromatogram with retention time spanning 1200−2000 s is shown with the fractions labeled (top). The structure of 1-palmitoylglycerophosphatidylcholine (*m*/*z* 496), an example lysophosphatidylcholine, is shown with the variable carbon chain in green. The most intense ions from each of the eight fractions, determined to be various forms of lysophosphatidylcholines, are tabulated (bottom). The carbon chain column lists the number of carbons in the green part of example lysophosphatidylcholine. The Double bonds column lists values corresponding to the number of double bonds in the variable carbon chain. Relative error in units of ppm calculated between FTMS data from experimental analysis and theoretical data calculated based on the elemental composition of the four lysophosphatidylcholines are listed in the far right column.

this end, we have presented our findings from investigating the efficacy of commonly used protein precipitation and metabolite extraction methods.

We have shown that it is important in metabolite profiling to obtain a broad range of metabolites, making acid and heat treatments the least effective. These methods produced significantly fewer features, with little similarity to those obtained using organic solvents. Organic solvents proved to be the most effective for the detection of the largest number and broadest range of serum metabolite features, as well as for protein removal efficiency. Of these, acetonitrile removed the least protein and resulted in the fewest features being detected. However, any of the methods in group I (Figure 4) would be suitable for metabolite profiling; importantly, all are 100% methanol and methanol-containing solvent mixtures. There is no discernible difference between the top three methods from this group, 30:70 acetone/methanol, 100% methanol, and 50:50 acetone/methanol, based on the number of reproducible features and protein removal efficiency.

Based on our observations, 100% methanol offers low protein interference, a comprehensive metabolite profile with the most straightforward sample preparation, and reproducible results. This approach is also relatively inexpensive, making it ideal for large-scale metabolite profiling studies. Therefore, 100% methanol extraction is the most robust approach for global serum metabolite profiling studies.

Using a combination of approaches in the positive ionization mode, over 10 000 metabolite features were detected using normal flow ESI-LC/MS (250 $\mu$L/min). Most intriguing about these studies is that other approaches would reveal significantly even more metabolites. Preliminary observations from negative ESI data showed thousands of additional metabolite features. Alternative ionization sources such as atmospheric pressure chemical ionization and atmospheric pressure photoionization would enable the detection of molecules that are unobservable with ESI. In addition, the application of nano-LC/MS (<200 nL/min) and high-resolution separation techniques[46] could further improve dynamic range to produce an even more comprehensive metabolite profile.

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## SUPPORTING INFORMATION AVAILABLE

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

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(46) Patel, K. D.; Jerkovich, A. D.; Link, J. C.; Jorgenson, J. W. *Anal. Chem.* **2004**, *76*, 5777−5786.