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Expanding Coverage of the Metabolome for Global Metabolite Profiling

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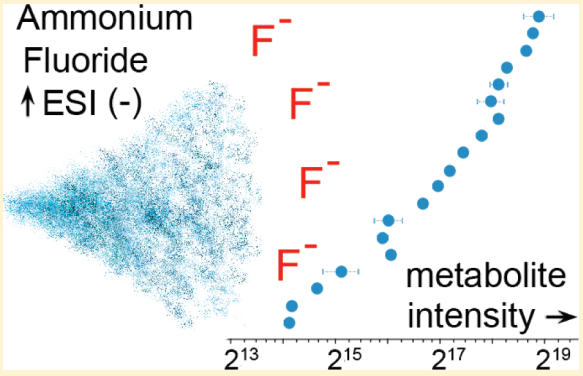
ABSTRACT: Mass spectrometry-based metabolomics is the comprehensive study of naturally occurring small molecules collectively known as the metabolome. Given the vast structural diversity and chemical properties of endogenous metabolites, biological extraction and chromatography methods bias the number, property, and concentration of metabolites detected by mass spectrometry and creates a challenge for global untargeted studies. In this work, we used *Escherichia coli* bacterial cells to explore the influence of solvent polarity, temperature, and pH in extracting polar and nonpolar metabolites simultaneously. In addition, we explored chromatographic conditions involving different stationary and mobile phases that optimize the separation and ionization of endogenous metabolite extracts as well as a mixture of synthetic standards. Our results reveal that hot polar solvents are the most efficient in extracting both hydrophilic and hydrophobic metabolites simultaneously. In addition, ammonium fluoride in the mobile phase substantially improved ionization efficiency in negative electrospray ionization mode by an average increase in signal intensity of 5.7 and over a 2-fold increase in the total number of features detected. The improvement in sensitivity with ammonium fluoride resulted in 3.5 times as many metabolite hits in databases compared to ammonium acetate or formic acid enriched mobile phases and allowed for the identification of unique metabolites involved in fundamental cellular pathways.



Metabolomics is a rapidly growing field focused on the profiling and quantification of small, naturally occurring compounds that collectively constitute the so-called metabolome. Electrospray ionization mass spectrometry coupled to liquid chromatography (LC−ESI MS) provides the most comprehensive technology for metabolomics studies.[1,2] Yet, two different mass spectrometry-based approaches have been described in the field: targeted and untargeted metabolomics. In general, targeting a specific metabolite or a small group of distinct metabolites is associated with hypothesis-driven studies[3] and involves optimization of chromatographic conditions (i.e., retention times) and selected reaction monitoring (SRM) transitions with pure standards.[4] Recent studies showed that up to 50−100 metabolites can be quantified using this approach.[5,6] Untargeted metabolomics studies, in contrast, are designed to simultaneously profile the largest number of compounds possible and therefore have the capacity to implicate previously unexplored biochemical pathways.[7,8] It is essential for the untargeted approach to maximize ionization efficiency of metabolites over a broad mass range (e.g., m/z 80−1000), since this determines the number and intensity (abundance) of the features to be analyzed. A feature is defined as a molecular entity with a unique m/z and retention time value. The number of features can be used as a general metric for the comprehensiveness of a global metabolomics study and thereby reflect overall coverage of the metabolome. The intensity of each feature is also important in that a certain threshold is needed for accurate relative quantification and further identification by tandem mass spectrometry (MS/MS). The intensity of the precursor ion dictates the signal-to-noise ratio of the tandem MS fragment ions, which is of critical importance in untargeted metabolomics studies because metabolites are identified by comparing the entire fragmentation pattern of a naturally occurring compound to that of a pure standard.

Given the large number of molecules with chemical and structural diversity constituting the metabolome, the method used to biologically extract metabolites and separate them by using liquid chromatography is fundamentally related to the number of features detected by MS with a sufficient signal-intensity threshold. The basic philosophy of an untargeted metabolomics approach is to detect as many metabolites as possible to maximize the opportunity of identifying compounds that are dysregulated in a particular biological condition. Therefore, studies that comprehensively examine optimal extraction

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methods and LC−MS conditions to detect the largest number of metabolites simultaneously are important. The isolation of metabolites from tissues, cells, or biofluids requires that proteins are precipitated and that polar and nonpolar metabolites are dissolved in solution without degradation. Further, the ideal chromatographic conditions should retain and separate the complex mixture of extracted metabolites using a mobile phase (solvent) that promotes ionization of the largest number of analytes.

In the present study, we used *Escherichia coli* as a model organism to optimize metabolite extraction and chromatography conditions coupled to ESI-MS. Specifically, we applied seven extraction methods involving different aqueous/organic solvents, temperature, pH, and molecular weight filters to *E. Coli* cultures. Additionally, we explored the separation of 36 model compounds of varying polarity from a standard mixture using different reverse-phase columns containing unique stationary phases. Finally, we investigated the influence of the chromatographic mobile phase to enhance ionization efficiency of complex mixtures of metabolites in negative ionization mode, which is generally associated with the detection of fewer features relative to positive ionization mode. Overall, our results indicate that polar solvents (e.g., water, ethanol/water) in combination with high temperature are more efficient in extracting both hydrophobic and hydrophilic metabolites compared to less polar solvents such as acetone or methanol. The choice of the chromatographic mobile-phase conditions in negative ionization mode proved to be strikingly significant, and we report that the addition of ammonium fluoride substantially increased the absolute intensity of nearly all compounds analyzed up to 22-fold while also increasing the total number of features in *E. Coli* extracts by 2.50-fold compared to mobile phases enriched with ammonium acetate or formic acid.

EXPERIMENTAL SECTION

Materials. All pure standards were purchased from Sigma Aldrich (St. Louis), except Peptide T (GenScript, Piscataway) and LysoPC (Cayman Chemical, Ann Arbor). Peptides Phe-Gly-Phe-Gly and Thymopoietin II fragment 32-36 were custom synthesized. Ammonium acetate, ammonium fluoride, formic acid, and EDTA were purchased from Sigma Aldrich (St. Louis). LC−MS grade methanol, acetonitrile, and water were purchased from J.T. Baker (Phillipsburg). M9 minimal salts (5×) and casamino acids were purchased from Difco (Franklin Lakes), and glucose, $MgCl_2$, $CaCl_2$, and meta-phosphoric acid (MPA) were purchsed from Sigma Aldrich (St. Louis). The Microcon YM-3 (3,000 NMWL) devices and HPF Millex filters (hydrophilic PTFE, 0.20 μm) were purchased from Millipore (Billerica, MA).

Growth of *Escherichia coli*. The *E. Coli* strain MC4100 (F-, araD139, Δ(arg F-lac)U169, ptsF25, relA1, flb5301, rpsL 150.λ-) was grown overnight at 37 °C in minimal media containing M9 salts (×1), 2% casamino acids, 0.2% glucose, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$.

Metabolite Extraction Methods. An overnight batch culture of *E. Coli* (25 mL) was divided into seven aliquots (1.5 mL each), and the supernatant was removed by centrifugation (5 min at 3 000 rpm, 4 °C). Metabolites were extracted by using one of the following methods.

Hot MeOH. A volume of 200 μL of hot (80 °C) methanol (100%) was added to the *E. Coli* pellet, vortexed for 30 s, and incubated 1−2 min at 80 °C (oven). A heating block or other methods can be used to control the temperature. The solution was centrifuged for 15 min at 13 000 rpm (4 °C), and the supernatant was transferred to a new tube. The same process was repeated with the precipitate using 100 μL of hot methanol. Finally, the supernatants (∼300 μL total) were pooled in an HPLC vial.

Hot EtOH/Ammonium Acetate. A volume of 400 μL of hot (80 °C) 60% ethanol/40% water in 5 mM ammonium acetate, 1 mM EDTA (pH 7.2) was added to the *E. Coli* pellet, vortexed for 30 s, and incubated 1−2 min at 80 °C (oven). The solution was centrifuged for 15 min at 13 000 rpm (4 °C), and the supernatant was transferred to a new tube. The same process was repeated twice, and the resulting supernatants were pooled in a 1.5 mL tube. The solution was desiccated with a vacuum concentrator (SpeedVac) at room temperature and redissolved in 300 μL of 5% ethanol/water 5 mM ammonium acetate (pH 7.2). Finally, the sample was centrifuged again for 10 min at 13 000 rpm (4 °C), and the supernatant was transferred to an HPLC vial.

Cold EtOH/Ammonium Acetate. A volume of 300 μL of cold (4 °C) 60% ethanol/40% water in 5 mM ammonium acetate, 1 mM EDTA (pH 7.2) was added to the *E. Coli* pellet, vortexed for 30 s, and the sample was incubated 1 min in liquid nitrogen. The sample was thawed at room temperature and incubated in liquid nitrogen two more times. Next, the sample was incubated 1 h at −20 °C followed by a 15 min centrifugation at 13 000 rpm. The resultant supernatant (∼300 μL) was transferred to an HPLC vial.

Boiling Water. A volume of 300 μL of LC−MS-grade water in 1 mM HEPES and 1 mM EDTA (pH 7.2) was added to the *E. Coli* pellet, vortexed for 30 s, and the sample was incubated 1−2 min in boiling water. Next, the sample was incubated for 1 min in liquid nitrogen and thawed at room temperature. The incubation in liquid nitrogen was repeated. Finally, the sample was incubated 1 h at −20 °C, followed by 15 min centrifugation at 13 000 rpm (4 °C). The resultant supernatant was transferred to an HPLC vial.

Acetone/MeOH. A volume of 400 μL of cold (−20 °C) acetone was added to the *E. Coli* pellet, vortexed for 30 s, and the sample incubated 1 min in liquid nitrogen. The sample was thawed at room temperature and incubated in liquid nitrogen two more times. After 1 h at −20 °C, the sample was centrifuged at 13 000 rpm for 15 min. The resultant supernatant was transferred to a separate vial, and the precipitate was mixed with 200 μL of cold methanol/water/formic acid (86.5:12.5:1.0). The sample was vortexed for 30 s and then sonicated for 10 min (4 °C) before leaving the sample 1 h at −20 °C. Next, the sample was centrifuged 15 min at 13 000 rpm (4 °C), and the supernatant was pooled with the previous. The solution was dried with a vacuum concentrator (SpeedVac) at room temperature and redissolved in 300 μL of 95% acetonitrile/5% water. The final solution was then centrifuged for 10 min at 13 000 rpm and the supernatant transferred to an HPLC vial.

MPA. A volume of 300 μL of cold (4 °C) solution of 5% meta-phosphoric acid (MPA), 1 mM EDTA, 0.1% formic acid was added to the *E. Coli* pellet, vortexed for 30 s, and the sample was incubated 1 min in liquid nitrogen. The sample was thawed at room temperature and incubated in liquid nitrogen two more times. Next, the sample was centrifuged 15 min at 13 000 rpm (4 °C), and the resultant supernatant (∼300 μL) was transferred to an HPLC vial. It should be noted that the MPA buffer was

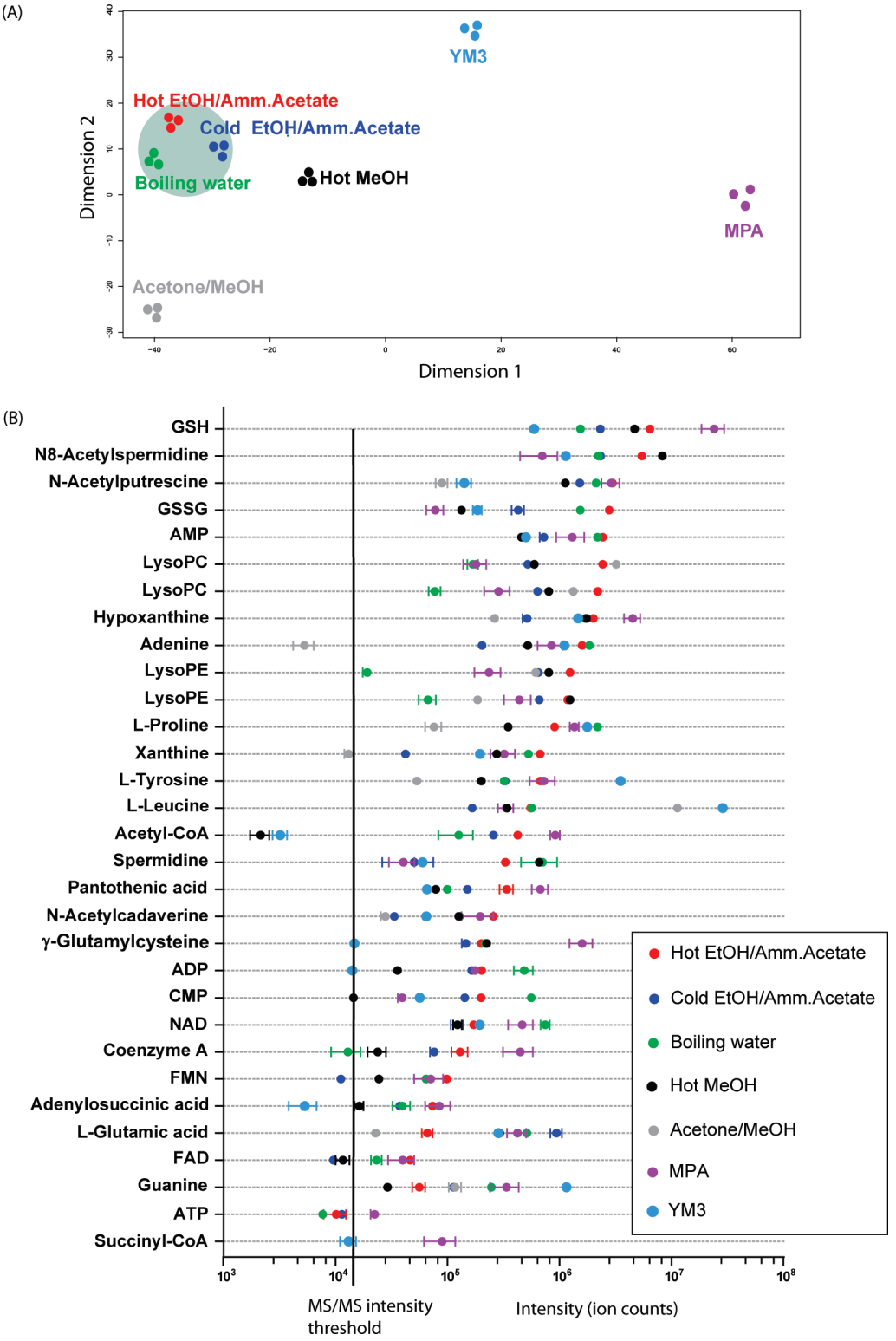

Figure 1. (A) Two-dimensional representation of the XCMS matrix of retention time, m/z, and feature intensity values using a multidimensional scaling (MDS) plot. Data points for extraction methods producing similar features are closer to one another than data points for extraction methods producing more dissimilar features. (B) Quantification of 31 identified metabolites extracted from *E. Coli* using seven different methods and analyzed with a C18 column (Cogent Bidentate) in positive ionization mode. Data points and error bars represent mean intensity values and standard deviation for three replicates. The black solid vertical line indicates the intensity threshold required for tandem mass spectrometry, as established from our experimental conditions. Parent ions below this threshold produced fragment ions with low signal-to-noise ratio (i.e., at background noise level). The intensity scale of the *X* axis is log 10. LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine.

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filtered with an HPF Millex filter (Millipore) before mixing with the *E. Coli* pellet.

YM3. A volume of 300 μL of cold (4 °C) LC−MS-grade water in 1 mM HEPES and 1 mM EDTA (pH 7.2) was added to the *E. Coli* pellet, vortexed for 30 s, and the sample was incubated for 1 min in liquid nitrogen. The sample was thawed at room temperature and bath sonicated for 5 min in cold water. This process was repeated two more times. Next, the sample was centrifuged 10 min at 13 000 rpm (4 °C), and the resultant supernatant was transferred to a microcon centrifugal filter unit with a YM-3 membrane (3 kDa nominal molecular weight cutoff (NMWCO)). The solution was spun down 1 h (4 °C), and the retentate was recovered in an HPLC vial. It should be noted that the membrane was spin-rinsed with deionized water 5 times to remove trace amounts of glycerin before applying the sample.

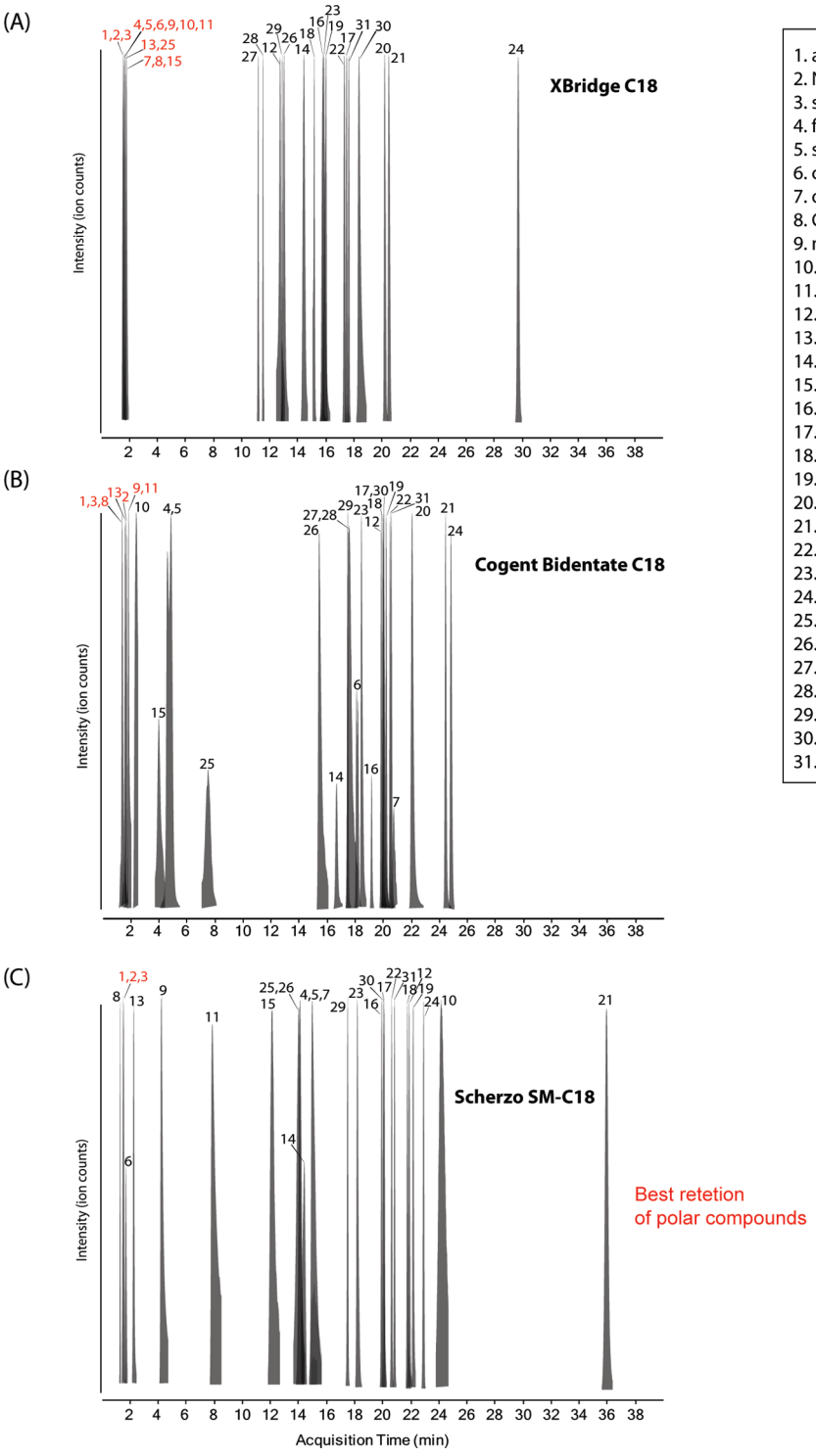
Standard Mix 1 and 2. Each compound was dissolved in 50% methanol/water and prepared at a final concentration of 10 μM and 0.1 μM for LC−MS analysis.

LC−MS and MS/MS Analysis. Analyses were performed using an HPLC system (1200 series, Agilent Technologies) coupled to a 6538 UHD Accurate-Mass Q-TOF (Agilent Technologies) operated in positive (ESI+) or negative (ESI−) electrospray ionization mode. Vials containing extracted metabolites using one of the seven methods described above or the standard mixture were kept at −20 °C prior to LC−MS analysis. *E. Coli* extractions and standard mixtures were separated using a Cogent Bidentate C18: 4 μm, 100 Å, 150 mm × 2.1 mm i.d. (catalog no. 40018-15P-2), a Waters XBridge C18, 3.5 μm, 135 Å, 150 mm × 1.0 mm i.d. (part no. 186003128), or an Imtakt Scherzo SM-C18, 3 μm, 13 nm, 150 mm × 2 mm i.d. (product no. SM025) column. When the instrument was operated in positive ionization mode, regardless of the column used, the solvent system was A = 0.1% formic acid in water, and B = 0.1% formic acid in acetonitrile. When the instrument was operated in negative ionization mode, we used one of the following pairs of solvent systems: A = 0.1% formic acid in water, B = 0.1% formic acid in acetonitrile; A2 = 1 mM ammonium fluoride in water, B2 = acetonitrile; A3 = 5 mM ammonium acetate in water, B3 = 5 mM ammonium acetate in 90% acetonitrile; A4 = 1 mM ammonium acetate in water, B4 = 200 mM ammonium acetate in 50% acetonitrile. The linear gradient elution used started at 100% A (time 0−5 min) and finished at 100% B (35−40 min). The injection volume was 8 μL. ESI conditions were gas temperature 325 °C, drying gas 11 L/min, nebulizer 30 psig, fragmentor 120 V, and skimmer 65 V. The instrument was set to acquire over the m/z range 80−1000 with an acquisition rate of 1.3 spectra/s. MS/MS was performed in targeted mode, and the instrument was set to acquire over the m/z range 50−1000, with a default iso width (the width half-maximum of the quadrupole mass bandpass used during MS/MS precursor isolation) of 4 m/z. The collision energy was fixed at 20 V.

Data Processing. LC−MS data from the *E. Coli* extractions (ESI+ and ESI− modes) were processed by using XCMS software[9] (version 1.24.1) to detect and align features. Each metabolite extraction method was compared using the same column (Cogent Bidentate C18) and ionization mode (ESI+). XCMS analysis of these data provided a matrix containing the retention time, m/z value, and intensity of each feature for every extraction method discussed above. Each row in the matrix represented a feature. It is important to note that while the retention time and m/z values for each feature were consistent among extraction methods, the intensities of the features varied. By using the statistical software R, each row of feature intensities was normalized such that the highest value was 1. A two-dimensional representation of this matrix was calculated using multidimensional scaling (MDS) as implemented within the software R. In negative ionization mode, experimental blanks were run in triplicate to remove "background" features arising from the mobile phases (e.g., ammonium fluoride and ammonium acetate). "Background" features detected in each of the three blanks were removed from the *E. Coli* data run with the same mobile phase. Standards were manually quantified by extracting ion chromatograms and integrating peak intensities with Qualitative Analysis of MassHunter Workstation (Agilent Technologies).

RESULTS

Influence of the Extraction Method on the Analysis of Polar and Nonpolar Metabolites. *E. Coli* pellets obtained from the same batch culture were used to extract metabolites with seven different protocols (see the Experimental Section). The selected protocols represent examples in which different fundamental conditions for metabolite solubility and stability are varied such as solvent polarity, temperature, pH, and molecular weight cutoff filtering. In brief, in method Hot MeOH, metabolites were extracted using hot 100% methanol. Method Hot EtOH/Ammonium Acetate was modified from Buescher et al.,[10] with metabolites extracted using hot ethanol/water buffered at pH 7.2. In both methods, the solvents were preheated and mixed with the *E. Coli* pellet at 80 °C for a short period (∼1−2 min) to avoid thermal degradation or methyl/ethyl ester formation. Method Cold EtOH/Ammonium Acetate used the same solvent as method Hot EtOH/Ammonium Acetate, but the solvent was precooled in ice and the extraction was performed at low temperature. Method BoilingWater was modified from Bhattacharya et al.,[11] with metabolites extracted using a polar solvent (100% H_2O) buffered at pH 7.2 and incubated for a short time in boiling water. In method Acetone/MeOH, metabolites were extracted at low temperature using a precooled nonpolar solvent (100% acetone). Method MPA was modified from Rellan-Alvarez et al.,[12] with metabolites extracted using strong acidic/aqueous conditions (meta-phosphoric acid in water) at low temperature. Finally, in method YM3, metabolites were extracted using a cold aqueous solvent buffered at pH 7.2 and isolated using a centrifugal filter unit with a cutoff of 3 kDa. All extractions were run as triplicates under the same LC−MS conditions using the Cogent Bidentate reverse-phase C18 column in positive ionization mode (see the Experimental Section). Each data set was visualized using a multidimensional scaling plot (Figure 1A) to show similarities in the results. With the use of feature intensities from the XCMS matrix, the data were scaled such that similar methods are near each other and dissimilar methods are farther apart from each other. The multidimensional scaling plot shows short distances between the methods Hot EtOH/Ammonium Acetate, Cold EtOH/Ammonium Acetate, and BoilingWater, highlighting that the number and intensities of features detected by XCMS in each method are similar. Hot MeOH is also relatively similar to the previous methods. Three extraction protocols, however, are strikingly different from the others: Acetone/MeOH, MPA, and YM3. We deduced that Acetone/MeOH is the most nonpolar of the seven extractions, and YM3 is the most polar.



1. alanine
2. N,N-dimethylglycine
3. serine
4. fumaric acid
5. succinic acid
6. cysteine
7. oxaloacetic acid
8. Gly-Gly
9. malic acid
10. alpha-ketoglutarate
11. Citric acid
12. 2-methylhippuric acid
13. gamma-D-glutamylglycine
14. salbutomal
15. AMP
16. riboflavin
17. Phe-Gly-Phe-Gly
18. s-(p-nitrobenzyl)glutathione
19. s-(p-azidophenacyl)glutathione
20. Taurocholic acid
21. O-beta-D-glucuronosyl-naphthol AS-BI
22. Leucine enkephalin
23. Arg-Pro-Pro-Gly-Phe (Bradykinin fragment 1-5)
24. Reserpine
25. GSSG
26. Arg-Lys-Asp-Val-Try (Thymopoietin II fragment 32-36)
27. Coenzyme A
28. Acetyl-CoA
29. Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr (peptide T)
30. Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe (bradykinin fragment 1-8)
31. Tyr-Tyr-Tyr-Tyr-Tyr-Tyr

Figure 2. Extracted ion chromatograms of 31 standard compounds separated by reverse-phase chromatography using a (A) XBridge C18, (B) Cogent Bidentate C18, and (C) Scherzo SM-C18 column.

The mean intensity of 31 specific metabolites identified in *E. Coli* by using MS/MS data are shown in Figure 1B. Metabolites characterized by different polarities and chemical functional groups were extracted with different efficiencies based on the method used. The intensity of a feature as determined by its integrated LC−MS peak area has important implications in that metabolite identification requires MS/MS analysis. Tandem MS in Q-TOF analyzers requires ion isolation, and without sufficient signal-to-noise, produces ambiguous and unreliable fragment ions. On the basis of our experimental conditions, we established a threshold for which the intensity of detected metabolites did not allow for reliable MS/MS fragmentation (see black line in Figure 1B). Our results indicate that the Acetone/MeOH protocol is the least efficient extraction to profile hydrophilic and hydrophobic metabolites simultaneously. Although this method provided increased detection of 4 phospholipids, only

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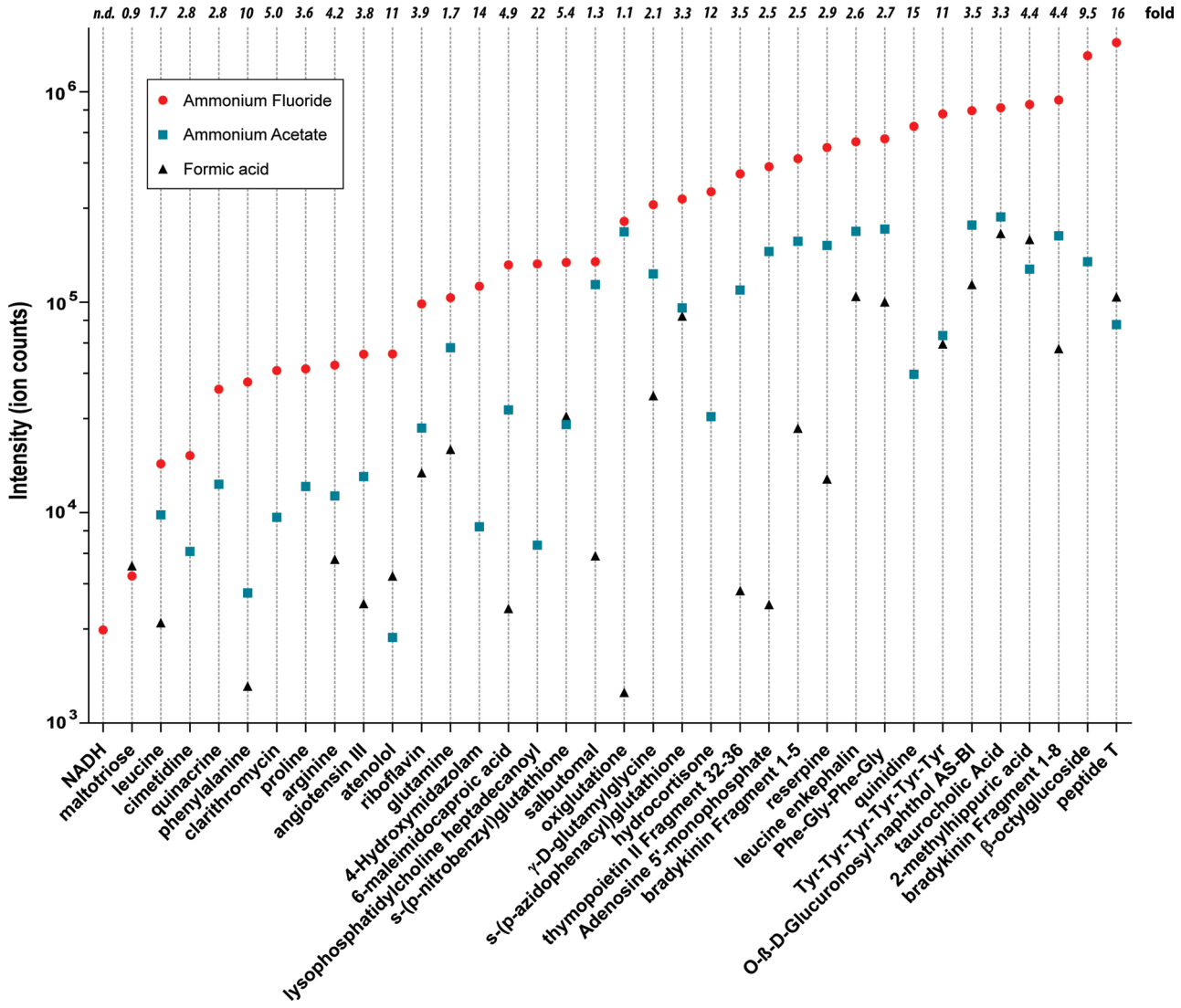

Figure 3. Quantification of 36 standard compounds analyzed in negative ionization mode (ESI−) by using three different additives in the mobile phase: 1 mM ammonium fluoride, 5 mM ammonium acetate, or 0.1% formic acid. Compounds were separated by reverse-phase chromatography by using an XBridge C18 column. Fold values indicate the difference in intensity between ammonium fluoride and the closest mobile phase. The intensity scale of the *X* axis is log 10.

14 out of the 31 metabolites shown were detected. In contrast, method YM3 did not efficiently extract the 4 phospholipids or other structurally unrelated metabolites such as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and coenzyme A (CoA). In addition, the intensity of metabolites such as acetyl-CoA, succinyl-CoA, γ-glutamylcysteine, adenylsuccinic acid, and ATP/ADP was below or slightly above the MS/MS threshold level. Using method Hot MeOH we detected 29 out of the 31 metabolites shown, with the exceptions being succinyl-CoA and ATP. It should be noted, however, that the intensity of 4 metabolites (acetyl-CoA, FAD, CMP, and adenylsuccinic acid) was below or slightly above the MS/MS threshold. With the use of method MPA, all 31 metabolites were detected with intensities above the established MS/MS threshold, but we observed an unexpected high analytical variability for most of the compounds (see error bars in Figure 1B). Methods Hot EtOH/Ammonium Acetate, Cold EtOH/Ammonium Acetate, and BoilingWater appear to be the most efficient extraction protocols examined in this study, and the similarity of the results from these three methods is consistent with the clustering displayed in Figure 1A (see blue area). With the exception of succinyl-CoA, the rest of the 31 metabolites were detected with high reproducibility in all three extractions. A total of 19 of the metabolites showed higher intensity with Hot EtOH/Ammonium Acetate, as compared to 9 metabolites with BoilingWater, and only 2 with Cold EtOH/Ammonium Acetate. Importantly, none of the 31 metabolites formed methyl or ethyl esters due to the short incubation time in boiling water or hot ethanol. Overall, we interpret our results in decreasing order of efficiency in extracting both polar and nonpolar metabolites simultaneously as follows: Hot EtOH/Ammonium Acetate < BoilingWater < Cold EtOH/Ammonium Acetate < MPA < Hot MeOH < Acetone/MeOH = YM3.

Exploring Different C18 Stationary-Phases for Metabolite Profiling. Reliable quantification of thousands of chemically diverse features requires optimization of chromatographic separation to reduce ion-suppression effects. Traditionally, untargeted metabolomics analyses have been performed using reverse-phase (RP) C18 columns because they generally result in the

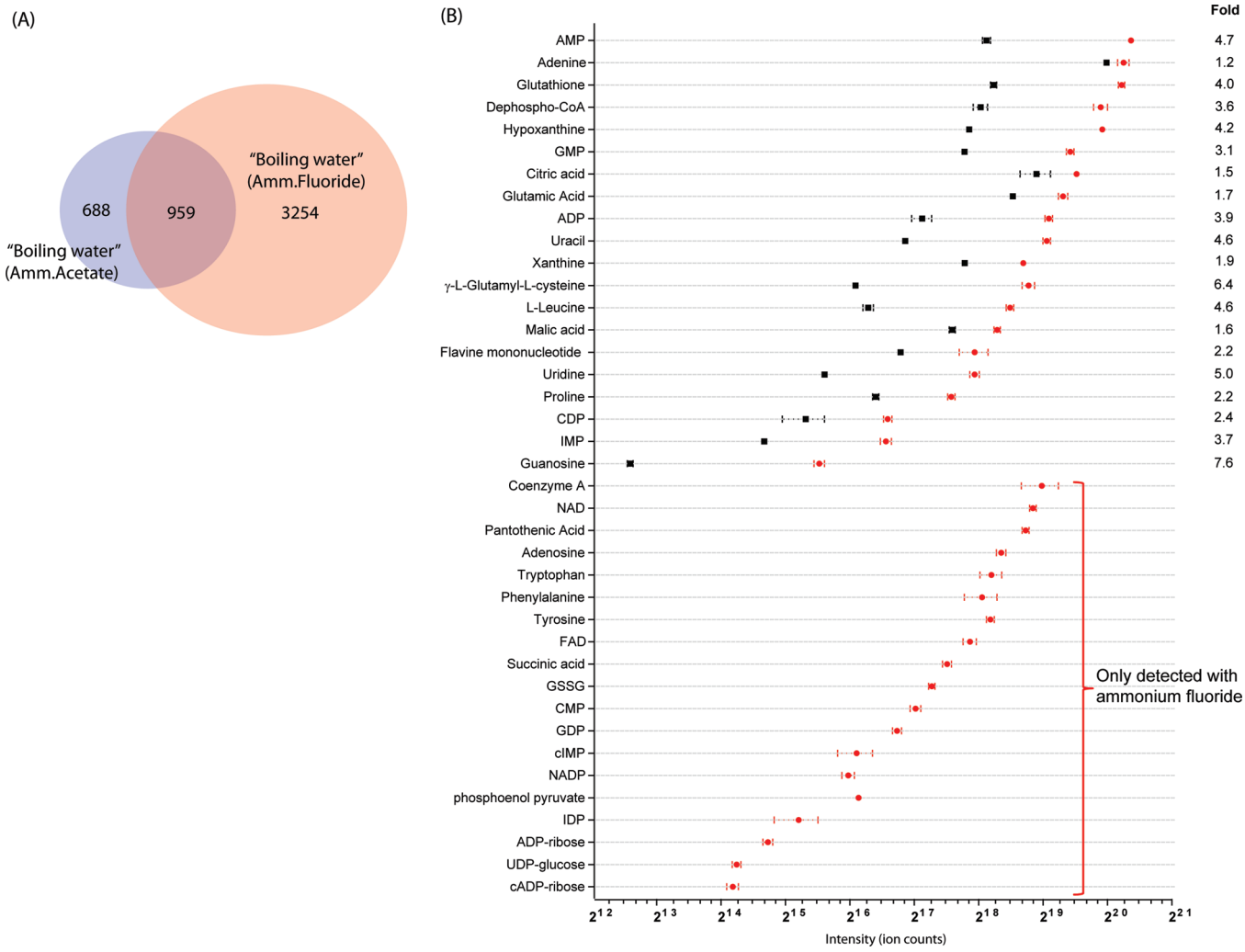

Figure 4. (A) Venn diagram representing the total number of features from LC−MS data of *E. Coli* samples extracted using the method Boiling Water and analyzed using ammonium acetate or ammonium fluoride enriched mobile phases. (B) Quantification of 39 metabolites from *E. Coli* analyzed using ammonium acetate and ammonium fluoride enriched mobile phases. The intensity scale of the *X* axis is log 2. Metabolites were separated by reverse-phase chromatography by using an XBridge C18 column and detected in negative ionization mode (ESI−). Identification is based on accurate mass and MS/MS data. Fold values indicate the difference in intensity between ammonium fluoride and ammonium acetate. Examples of unique metabolites detected with ammonium fluoride are also represented. Data points and error bars represent mean intensity values and standard deviation for three replicates.

detection of more features. RP C18 columns, however, are limited in their capacity to retain hydrophilic compounds and consequently result in ion suppression for polar metabolites in the dead volume, thereby limiting MS coverage of the metabolome. Recent developments in RP C18 technology offer opportunities to improve retention of polar molecules and therefore increase metabolite coverage in metabolomics analysis. We analyzed the ability of three different RP C18 columns with unique properties to retain polar model compounds: (i) the XBridge C18 column characterized by broad pH range stability (pH 1−11), (ii) the Cogent Bidentate C18 column characterized by silicon-hydride (Si−H) groups instead of the common silanol group (Si−OH),[13,14] and (iii) the multimodal Scherzo SM-C18 column containing cation and anion ligands that allow for reverse-phase separation in addition to anion and cation exchange. We analyzed a standard mixture of 31 model compounds characterized by different polarities (e.g., amino acids, tricarboxylic acids, vitamins, peptides, and xenobiotics, see Figure 2 for the full list of compounds in Standard Mix 1) using each column with the same gradient and mobile phase (A = water, 0.1% FA; B = acetonitrile, 0.1% FA). With the XBridge column, 14 out of the 31 compounds (45%) coeluted within the first 2 min (Figure 2A). Similar results were obtained with traditional C18 columns (e.g., Zorbax, Atlantis T3) (data not shown). The Cogent column retained more compounds, with only 7 of them (22%) coeluting within the first 2 min (Figure 2B). With the Scherzo column, we observed a remarkable improvement in the retention of polar compounds with only three compounds (10%) coeluting within the first 2 min (Figure 2C). Overall, all three columns showed good performance in separating the rest of the compounds, although taurocholic acid, coenzyme A (CoA), and acetyl-CoA did not elute from the Scherzo column due to strong ion exchange interactions with the stationary phase. Increasing the ionic strength of the mobile phase by adding 5 mM ammonium acetate resulted in elution of taurocholic acid. Further increasing the ionic strength of the mobile phase with

200 mM ammonium acetate resulted in the elution of CoA and acetyl-CoA.

Optimizing the Mobile Phase in Negative Ionization Mode for Global Metabolite Profiling. In electrospray ionization, the composition of the solvent (i.e., mobile phase in LC−ESI MS) influences the gas-phase acid−base processes required for the ionization of the compounds to be analyzed. Global profiling of metabolites in positive ionization mode generally produces more features compared to negative mode, most likely due to higher efficiency of protonation relative to deprotonation. Protonation is facilitated by the addition of an acid to the mobile phase, such as formic acid, acetic acid, or TFA. In negative ionization mode, deprotonation in the gas phase is typically promoted by the addition of ammonium salts such as ammonium formate or ammonium acetate. Given the strong basicity of the fluoride anion (F^-) in the gas phase,[15] we examined the effect of adding ammonium fluoride to the mobile phase in negative-mode analysis. A previous report showed that fluoride anions in the electrospray solvent resulted in increased deprotonation, $[M - H]^-$, of neutral steroids with higher abundances than other anions tested.[16] Using Standard Mix 2 containing 36 compounds at 10 μM (see Figure 3 for full list of compounds), three different mobile phases were compared with the same reverse-phase C18 column (i.e., XBridge) and MS conditions in negative ionization mode: (i) 1 mM ammonium fluoride, (ii) 5 mM ammonium acetate, and (iii) 0.1% formic acid. Mobile phases enriched with ammonium fluoride and ammonium acetate were maintained at pH$\sim$ 7, ruling out the possibility that differences in ionization efficiency are related to the pH of the solution.

Figure 3 shows the intensity values and fold change of each compound, revealing that ammonium fluoride is a superior additive to increase ionization efficiency in negative ionization mode. On average, ammonium fluoride increased the intensity of the compounds analyzed by 5.7-fold, including a 15-, 16-, and 22-fold increase for quinidine, peptide T, and lysophosphatidylcholine, respectively. Notably, when the same 36 compounds were prepared at 0.1 μM, 20 compounds (55%) were not detected with 0.1% formic acid and 18 (50%) were not detected with ammonium acetate. Only 6 compounds (16%) were not detected with ammonium fluoride, and all remaining compounds showed significantly higher intensity values with ammonium fluoride compared to ammonium acetate and formic acid (data not shown). It is worth noting that lysophosphatidylcholine was detected as $[M - H]^-$ at 10 μM in negative ionization mode using ammonium fluoride; however, no signal of this compound was observed at 0.1 μM. Lysophosphatidylcholine, however, was detected at 0.1 μM in positive ionization mode using 0.1% formic acid.

The total number of features and their absolute intensity were also compared from *E. Coli* extractions analyzed by using ammonium fluoride and ammonium acetate enriched mobile phases. *E. Coli* samples in addition to blank samples were run as triplicates with each mobile phase, and features consistently present in all three blanks were subtracted from the *E. Coli* data as "background". Then, only features present in the 3 *E. Coli* replicates with intensity values above 5 000 ion counts were considered for quantification purposes. The Venn diagram in Figure 4A shows a total of 4 213 features obtained with the method BoilingWater using an ammonium fluoride enriched mobile phase and 1 647 features from the method BoilingWater using an ammonium acetate enriched mobile phase, which is a 2.5-fold increase. Importantly, 77% of the features analyzed by using ammonium fluoride additive were not detected when using an ammonium acetate additive. A similar distribution of unique features was also found when the method BoilingWater and the method Hot MeOH enriched with the same mobile phases were compared. A total of 1 008 features were detected using Hot MeOH with an ammonium acetate enriched mobile phase, and only 24% of these features overlapped with BoilingWater using the same mobile phase (data not shown). The increased sensitivity achieved by using an ammonium fluoride enriched mobile phase was also reflected in the number of putative metabolite identifications made on the basis of accurate mass from the METLIN database. With the use of ammonium fluoride, 722 features out of 4 213 (17.1%) matched with known compounds in METLIN (error < 5 ppm, $[M - H]^-$). With the use of ammonium acetate, 211 out of the 1647 (12.8%) matched with known compounds in METLIN. The difference reflects nearly a 3.5-fold increase in database hits. It is noteworthy that 81% of the METLIN database hits using ammonium fluoride were not present in the ammonium acetate analysis. Features with high intensity, presumably corresponding to abundant metabolites, were typically detected with both enriched mobile phases, whereas less abundant metabolites were often uniquely detected in ammonium fluoride enriched analyses. To confirm this observation, the mean intensity value was determined for various metabolites structurally identified based on accurate mass, retention time, and MS/MS data (Figure 4B). The results were consistent with our previous data using Standard Mix 2 containing 36 compounds. All metabolites identified showed higher intensity using an ammonium fluoride enriched mobile phase, with important cellular metabolites being detected only in the presence of ammonium fluoride.

DISCUSSION

Global metabolite profiling of biological samples is a challenging task due to the chemical and structural diversity of naturally occurring compounds ranging from polar metabolites such as amino acids and nucleotides to nonpolar molecules such as steroids and membrane lipids. As a result of this diversity, methods used for metabolite extraction and metabolite separation significantly influence the number and intensity of compounds detected by ESI-MS analysis. The choice of extraction and chromatography methods biases the chemical distribution of metabolites detected and is therefore problematic for untargeted metabolomics investigations aimed at accomplishing unbiased profiling. The obvious complexity in performing untargeted studies is that the metabolites of potential interest are unknown and therefore extraction and chromatography methods cannot be tailored toward a specific chemical class of compounds. In this context, it is unclear what evaluation criteria should be used to assess the quality of untargeted metabolomics extraction and chromatography methods. The approach we have developed here involves using our metabolomics software XCMS to analyze the number and intensity of features identified in each of different extraction protocols and LC−MS conditions. We interpret methods leading to the identification of more features of greater intensity to be better suited for untargeted studies.

Decades of research has provided an extensive library of detailed extraction and chromatography methods for analysis of unique classes of compounds, and it is not our intent to comprehensively survey all of them here. Rather, our study is aimed at providing an overview of easy and rapid extraction and

reverse phase (RP) LC−MS protocols for the metabolomics scientist to use as a general guideline. Optimization of metabolite extraction has been recently pursued using a two-stage approach or biphasic mixtures.[17] Although these methods may reduce complexity in the number of metabolites to be separated and ionized and may ultimately extend metabolite coverage, we have not explored them because they are generally more time-consuming and prone to analytical error. Other studies have shown that many metabolites can be extracted by a broad spectrum of solvent mixtures,[18−20] suggesting that there is no specific extraction protocol that should be used in metabolomics. Our data corroborate this point but highlight that a number of considerations should be taken into account when carrying out global metabolite profiling studies. For example, highly polar solvents such as water or 100% nonpolar organic solvents such as acetone/methanol are not particularly suitable to extract both hydrophilic and hydrophobic metabolites simultaneously. In particular, solvents with intermediate polarity such as a mixture of ethanol/water are more appropriate for this goal. We also report that incubation of the sample with the solvent at high temperatures (80−100 °C) for short periods of time (1−2 min) is more efficient than incubation of the sample at cold temperatures (−20 °C). Water may have a polarity similar to some organic solvents when boiled,[21] which might explain the similarity of the method Boiling Water with extraction protocols using high percentages of ethanol such as the methods Hot EtOH/Ammonium Acetate and Cold EtOH/Ammonium Acetate. The general utility of the described extraction protocols to other cell types, which have heavily weighted distributions of nonpolar metabolites (e.g., adipose tissue), possess increased tensile properties (e.g., muscle tissue), or contain functionalities that are not stable to the conditions employed, requires further investigation.

Much attention has been dedicated recently to different chromatographic approaches to improve separation and analysis of naturally occurring compounds. Hydrophilic interaction chromatography (HILIC)-like stationary phases[22] or the use of ion pairing agents with reverse-phase columns[23−25] improves the retention of polar metabolites; however, each of these approaches has drawbacks for processing global metabolite profiling data. HILIC columns do not retain hydrophobic compounds well and are generally associated with broader peak shapes. The use of ion pairing agents (e.g., tetrabutylammonium (TBA)) has led to inconsistent results, lengthy equilibration, and general incompatibility with mass spectrometry and typically requires a dedicated negative-mode LC stack as some ion pairing agents are difficult to remove from the instrument lines and cause contamination affecting analysis in the positive-ionization mode. In contrast, we demonstrate that the stationary phases utilized in our study show unique advantages for global metabolite profiling. We found the flexibility of the Scherzo column to be particularly effective, as it combines a nonpolar stationary phase with anion and cation ligands for ion exchange separation. Perhaps the major drawback is the strong ionic interaction of this stationary phase with some metabolites such as coenzyme A. This issue can be resolved by increasing the ionic strength of the mobile phase with 200 mM ammonium acetate; however, it is important to mention that percentages of acetonitrile higher than 50 led to precipitation of the ammonium salt, and low percentages of acetonitrile likely affects the elution of highly hydrophobic compounds from the column.

Finally, a striking result from our work is the substantial improvement of ionization efficiency in negative ionization mode by using an ammonium fluoride enriched mobile phase. To the best of our knowledge, this approach has not been described before for metabolomics studies. The use of an ammonium fluoride enriched mobile phase in negative ionization mode is not intended to replace positive ionization mode analysis in metabolomics studies. Ammonium fluoride is stable in different solvents (e.g., water, acetonitrile, methanol) and led to reproducible results. In addition, no contamination of the HPLC lines was observed after using ammonium fluoride. Importantly, the highest sensitivity was achieved when using 1 mM ammonium fluoride, and concentrations of 5 mM or higher introduced significant background in the mass spectra. The source of background was attributed to the impurities of the ammonium fluoride stock solution and to the interaction at pH 7.0 of fluoride anions with the silica of the stationary phase of most columns tested. The XBridge column, however, is stable within a wide pH range and performed excellent with 1 mM ammonium fluoride, generating mass spectra with low background. When a polymeric (PSDVB) reverse-phase column was tested with ammonium fluoride, such as the Hamilton PRP-1, the background was almost absent (similar to ammonium acetate) with no effect on sensitivity. Overall, the consistency of our data with standard compounds and biological samples suggests that ammonium fluoride should be used as a standard additive to mobile phases in negative ionization mode for global metabolomics studies.

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