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Trace phosphate improves ZIC-pHILIC peak shape, sensitivity, and coverage for untargeted metabolomics

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Abstract

Existing hydrophilic interaction liquid chromatography (HILIC) methods, considered individually, each exhibit poor chromatographic performance for a substantial fraction of polar metabolites. In addition to limiting metabolome coverage, such deficiencies also complicate automated data processing. Here we show that some of these analytical challenges can be addressed for the ZIC-pHILIC, a zwitterionic stationary phase commonly used in metabolomics, with the addition of trace levels of phosphate. Specifically, micromolar phosphate extended metabolome coverage by hundreds of *credentialed* features, improved peak shapes, and reduced peak-detection errors during informatic processing. Although the addition of high levels of phosphate (millimolar) as a HILIC mobile phase buffer has been explored previously, such concentrations interfere with mass spectrometric (MS) detection. We show that using phosphate as a trace additive at micromolar concentrations improves analysis by electrospray MS, increasing signal for a diverse set of polar standards. Given the small amount of phosphate needed, comparable chromatographic improvements were also achieved by direct addition of phosphate to the sample during reconstitution. Our results suggest that defects in ZIC-pHILIC performance are predominantly driven by electrostatic interactions, which can be modulated by phosphate. These findings

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AUTHOR CONTRIBUTIONS

The authors declare the following competing financial interest(s): G.J.P. is a scientific advisory board member for Cambridge Isotope Laboratories and a recipient of the 2017 Agilent Early Career Professor Award. The remaining authors have no competing interests.

SUPPORTING INFORMATION

(Table S1) List of chemical standards and associated suppliers; (Table S2) log *P* values, neutral charge states, and isomeric indication of chemical standards; (Table S3) chromatographic conditions tested for method optimization; (Figure S1) heatmap of standards' chromatographic performance in the presence of ammonium phosphate; (Figure S2) effects of sample solvent composition on polar compound solubility; (Figure S3) extracted ion chromatograms of chemical standards in the absence and presence of ammonium phosphate; (Figure S4) heatmap of chemical standard isomeric resolution in the presence of ammonium phosphate; (Figure S5) extracted ion chromatogram comparing phosphate's effect in mobile phase versus sample solvent; (Figure S6) bar chart of chemical standards' overall chromatographic performance with ammonium phosphate in sample solvent; (Figure S7) box plot of phosphate's effects on standards' ionization efficiencies; (Figure S8) extracted ion chromatogram of a metabolite that is *credentialed* only in the presence of phosphate; (Figure S9) heatmap of abundant metabolites detected in *E. coli* extracts in the absence and presence of ammonium phosphate; (Figure S10) extracted ion chromatogram of a standard's chromatographic performance after preloading phosphate onto the column; and (Figure S11) bar chart of standard peak height as a function of mobile phase salt concentrations

constitute both a methodological improvement for untargeted metabolomics and an advance in our understanding of the mechanisms limiting HILIC coverage.

TOC Graphic

Keywords

bioinformatics; coverage; credentialing; electrostatic interactions; hydrophilic interaction liquid chromatography; peak detection; phosphate; untargeted metabolomics

INTRODUCTION

The most commonly used technique for profiling the polar metabolome is hydrophilic interaction liquid chromatography (HILIC) coupled with mass spectrometry (MS).^(1,2) Notably, however, no existing HILIC method reliably separates the entire polar metabolome.⁽³⁾ For any given HILIC method, a significant fraction of polar compounds are not retained, exhibit poor chromatographic peak shapes, or go undetected due to inappropriate or incomplete elution.^(4–6) When the objective of an experiment is to analyze only a targeted set of chemically similar polar molecules, a HILIC method can typically be selected to provide high-quality chromatographic data for the analytes of interest.^(7,8) For untargeted metabolomics, on the other hand, it is currently not possible to obtain high-quality data for the entire polar metabolome with a single HILIC method.

A potential solution to this problem in untargeted metabolomics is to analyze each sample with multiple HILIC methods in successive experiments, but this introduces several challenges.^(5,6,9) First, limitations in resources and sample availability reduce the practicality of multimethod approaches. Second, each additional method confers diminishing improvements in comprehensive coverage. Accordingly, most published metabolite profiling studies only use a single HILIC method, sacrificing coverage for analytical efficiency.^(3–5,10,11) More importantly, using multiple HILIC methods does not solve the informatic challenges posed by low-quality chromatographic peaks.^(12–14) Given the complexity of untargeted metabolomic data sets, which commonly contain thousands of chromatographic peaks with unique retention times and mass-to-charge values (so-called “features”), researchers rely on software programs for automated peak detection.^(15–18) Inconsistent or non-Gaussian chromatographic peak shapes lead to improper selection of feature bounds and thereby compromise quantitative analysis of the data.^(18,19) The application of multiple HILIC methods in successive experiments does not remove low-quality peaks from the

analysis and instead only increases the amount of data that cannot be reliably processed with existing software solutions.⁽³⁾ Although computational strategies are being developed to compensate for the effects of poor chromatography, low-quality peak shapes still limit data interpretation.^(20–22) A more effective solution for untargeted metabolomics is therefore to improve the overall quality of peak shapes for a given HILIC method, which was the goal of the current work.

One strategy to improve peak quality and coverage in HILIC is to modulate electrostatic interactions by adding buffer salts to the mobile phase. Electrostatic interactions (i.e., hydrogen bonding and ion exchange) are thought to be a predominant mechanism governing HILIC retention, in addition to liquid–liquid partitioning.^(12,23) Unlike liquid–liquid interactions, however, electrostatic interactions are less energetically homogeneous for each analyte, suggesting that they may be responsible for asymmetric elution profiles.^(24–27) In reversed-phase (RP) separations, for example, some electrostatic interactions are known to distort peak shape and have been suppressed by adding micromolar concentrations of phosphate.^(28,35) In contrast to RP stationary phases where the abundance of electrostatic sites are low, electrostatic sites in HILIC stationary phases are abundant.⁽¹⁴⁾ As such, only high concentrations of phosphate (millimolar) as a mobile phase buffer have been evaluated in HILIC, and the effect of trace concentrations (micromolar) remains largely unexplored. Although millimolar levels of phosphate do indeed improve chromatographic behavior, they are incompatible with MS and thus have been primarily limited to ultraviolet–visible (UV) spectroscopy workflows with insufficient molecular resolution for untargeted metabolomics.^(29,30) In this work, we sought to determine whether micromolar concentrations of phosphate that are compatible with MS-based metabolomics are adequate to improve HILIC peak behavior by selectively shielding some electrostatic sites.

Here we evaluated the use of trace phosphate in HILIC/MS analysis with the SeQuant ZIC-pHILIC column, a zwitterionic stationary phase that is frequently used in untargeted metabolomic analyses of the polar metabolome.^(5,9,31,32) Strikingly, the addition of micromolar concentrations of phosphate to the mobile phase improved coverage, peak shape, and MS signal intensity for a set of 65 physiochemically diverse polar standards. We also benchmarked our analyses at the metabolome level by evaluating *credentialed* signals from *Escherichia coli* samples. Not only did trace phosphate extend metabolome coverage by hundreds of *credentialed* features, it also increased MS signal intensities and improved the overall accuracy of automated peak detection. Of note, we found that trace phosphate had equal benefits when added to the sample solvent itself rather than the mobile phase. Considered collectively, our results suggest that shielding some electrostatic interactions between analytes and the ZIC-pHILIC stationary phase with only trace levels of phosphate is sufficient to improve peak shape for a significant number of polar metabolites. Beyond their impact in advancing untargeted metabolomics, these results also provide insight into the general mechanisms limiting HILIC performance.

EXPERIMENTAL SECTION

Materials

LC/MS-grade, Burdick & Jackson brand water, acetonitrile, and methanol were purchased from Honeywell (Muskegon, MI). LC/MS-grade eluent ammonium acetate, ammonium formate, and ammonium carbonate were purchased from Sigma-Aldrich (St. Louis, MO). TraceSELECT Fluka brand ammonium phosphate monobasic was purchased from Honeywell (Muskegon, MI). Dried metabolic extracts of *credentialed E. coli* were purchased from Cambridge Isotope Laboratories (MSK-CRED-DD-KIT). A list of the full names and suppliers of the 65 chemical standards we used to benchmark experiments is included in Table S1.

Credentialed Metabolite Sample Preparation—*E. coli* cultures were extracted and vacuum concentrated as previously described.⁽³³⁾ For LC/MS analysis, the dried extracts were resuspended in acetonitrile:water (2:1) with and without 20 mM ammonium phosphate in the aqueous fraction.

LC/MS Analysis

For initial method comparisons and optimizations, all commercial standards were diluted to 20 μ M in acetonitrile:water (2:1). For some experiments, 5, 10, 20, or 40 mM ammonium phosphate was dissolved in the aqueous fraction of the reconstitution solvent as specified.

We used an ultrahigh performance LC/MS (UHPLC/MS) system with the SeQuant ZIC-pHILIC column (EMD Millipore, Burlington, MA). For high chromatographic resolution when analyzing our biological samples, we used a 150 $\times$ 2.1 mm, 5 μ m column. When analyzing our standards, we used a 50 $\times$ 2.1 mm, 5 μ m column for higher throughput. Metabolite analysis was performed on an Agilent 6545 Q-TOF interfaced with an Agilent 1290 Infinity II LC system. Mobile phase solvents were composed of A = 20 mM ammonium acetate in water:acetonitrile (95:5) and B = 100% acetonitrile. For some experiments, the composition of the A solvent was altered to contain 5, 10, or 40 mM ammonium acetate as specified. When the ammonium acetate concentration was 40 mM, the gradient started at 85% B instead of 90% B to ensure the solubility of ammonium acetate in the presence of organic solvent. For select experiments, 5 μ M ammonium phosphate was added to the aqueous mobile phase solvent. The column compartment was maintained at 45 $^{\circ}$ C during all experiments. The column was equilibrated with 20 column volumes of starting mobile phase between injections to provide high retention time reproducibility. For the 150 mm column, we ran with the following linear gradient at 200 μ L/min: 0–0.5 min: 90% B, 0.5–30 min: 90–30% B, 30–31 min: 30% B. For the 50 mm column, we ran with the following linear gradient at 275 μ L/min: 0–0.5 min: 90% B, 0.5–18 min: 90–0% B, 18–20 min: 0% B. When using the 50 mm column, we also found that metabolites could be reliably analyzed with a shorter gradient at the same flow rate: 0–0.5 min: 90% B, 0.5–10 min: 90–50% B, 10–12 min: 50–37% B, 12–12.5 min: 37% B. Injection volumes were 2 μ L for all experiments.

The MS settings were kept consistent regardless of the chromatographic separation being tested. Mass range was set from 50 to 1500 m/z . MS parameters were as follows: gas, 200 °C at 4 L/min; nebulizer, 44 psi at 2000 V; sheath gas, 300 °C at 12 L/min, capillary, 3000 V; fragmentor, 100 V; skimmer, 65 V; and scan rate, 3 scans/second. The MS was operated in both positive and negative ionization mode for all samples analyzed.

High Salt LC/MS Analysis

The 200 mM ammonium acetate ramp and shock experiments were performed on the UHPLC/MS system as detailed above. A ZIC-pHILIC column was coupled to MS detection for these experiments. For the salt-ramp experiment, solvents were composed of A = 200 mM ammonium acetate in water:acetonitrile (95:5) and B = 5 mM ammonium acetate in water:acetonitrile (20:80). The linear gradient for the ramp experiment was as follows: 0–2 min: 87% B, 2–20 min: 87–0% B, 20–23 min: 0% B. For the salt-shock experiment, all four lines of the UHPLC system were needed to switch from low salt to high salt midgradient. The pair of solvents comprising the low-salt portion of the method were composed of A1 = 15 mM ammonium acetate in water:acetonitrile (95:5) and B1 = 100% acetonitrile, while the solvents for the high-salt portion of the method were composed of A2 = 200 mM ammonium acetate in water:acetonitrile (95:5) and B2 = 55 mM ammonium acetate in water:acetonitrile (20:80). The linear gradient for the salt portion of the method was as follows: 0–2 min: 92% B1, 2–5 min: 92–79% B1, 5–7 min: 79% B1. Upon completion of this portion of the gradient, the solvent lines were switched to A2/B2 and the following linear gradient was applied: 7–7.5 min: 89% B2, 7.5–10 min: 89–60% B2, 10–14 min: 60–0% B2. All other conditions, including column compartment settings, injection volumes, and MS settings were kept the same as detailed above.

Data Analysis

All experiments were performed in replicates of three ($n = 3$) per sample group. Compounds that ionized in both positive and negative ESI modes were analyzed in the mode that produced a more abundant peak, shown for each compound in Figure S1. Chromatographic performance of standard compounds in each experiment was evaluated by classification of peaks into one of five categories. “Optimal” peaks were continuous peaks with a peak asymmetry factor (A_s) < 2 and a full width at half-maximum (fwhm) < 20 s. “Suboptimal” peaks were continuous or nearly continuous peaks with A_s < 6 and/or $45 > \text{fwhm} > 20$, with the additional requirement that the MassHunter Qualitative Analysis software could reliably integrate them. We note that acceptable peaks, as mentioned throughout the Results section, include both the “optimal” and “suboptimal” categories. “Quantitatively unreliable” peaks were peaks with $A_s > 6$ and/or $\text{fwhm} > 20$ and/or multiple sub-peaks. “Non-retained” peaks were peaks with a retention factor $k < 1$. Finally, an “undetected” peak occurred when a compound failed to produce a mass trace that exceeded a signal-to-noise threshold of 8. Under this classification system, isomer peaks were considered individually and resolution evaluated separately (see Results).

The analysis of raw *credentialing* data was done using the latest version of the *credentialing* software, which is freely available on our laboratory Web site at <http://pattilab.wustl.edu/software/credential/credential.php>.⁽³³⁾ The MassHunter Qualitative Analysis software

(Agilent Technologies) was used for some data analyses. Peak picking was accomplished by using the centWave algorithm within the XCMS software package.(19)

RESULTS

Ideally, untargeted metabolomic methods would be benchmarked by comparing the number of metabolites detected to the total number of metabolites in the comprehensive metabolome. Unfortunately, such an approach is currently impractical because the comprehensive metabolome is poorly defined and many signals in a typical untargeted metabolomic data set cannot be readily identified.(3,34) Thus, as an alternative to compare various methods involving the ZIC-pHILIC column, we applied two independent strategies. First, as described below, we selected 65 polar standards with a range of electrostatic and structural properties that are representative of the physiochemical diversity of the polar metabolome (Table S2). Second, we evaluated a complex metabolic extract from *E. coli* by using the *credentialing* technology. The latter facilitates filtering of artifacts and contaminants from untargeted metabolomic data sets so that the number of true metabolites detected can be more accurately estimated.

Selection of 65 Physiochemically Diverse Polar Standards

Compounds were chosen so that the distribution of the logarithm of the partition coefficient ($\log P$) values would be complementary to those within the analytical range of current RP separations used to analyze semipolar and nonpolar metabolites.(35) The $\log P$ values of our standards ranged from -6 to 4 , as calculated by Advanced Chemistry Development Laboratories. This $\log P$ range includes highly polar metabolites like cyanocobalamin and ATP on the negative end of the spectrum, as well as amphipathic molecules like palmitoyl-CoA on the positive end. The standards were also selected to have an assortment of charges. The overall charge of standards was represented by their neutral charge state (i.e., charge at neutral pH) as calculated by ChemAxon, ranging between -4 and $+4$ across the set. Compounds on the negative end of this scale contained multiple negatively charged moieties like carboxyls and phosphates (e.g., citrate), whereas those on the positive end contained several amines (e.g., spermine). Effort was made to include compounds with multiple charges in multiple steric arrangements. Furthermore, some compounds were comprised of a mix of negatively and positively charged moieties in addition to hydrophobic (e.g., phenyl ring, alkyl chain) functional groups. While the majority of our standards were endogenous molecules (e.g., amino acids, central carbon metabolites), several exogenous compounds were selected to examine the effects of multivalency, aromatic complexity, and steric hindrance (e.g., kanamycin). Inclusion of such exogenous compounds was also intended to avoid biasing the standard set toward well-studied metabolites. Finally, we included six sets of isomers to assay chromatographic resolving power. A list of the full names, $\log P$ values, and neutral charge states for the 65 standards can be found in Table S2.

Trace Phosphate Improves Coverage, Peak Shape, and MS Signal of Polar Standards when Using ZIC-pHILIC

Before testing the effects of phosphate, we first optimized our ZIC-pHILIC method for peak quality and coverage by using our standard set. We dissolved our standards in a sample

solvent of 2:1 acetonitrile:water. We found that higher proportions of acetonitrile caused significant reduction in the solubility of important, highly charged metabolites such as ADP and citrate/isocitrate (Figure S2). Furthermore, we observed that changing the sample solvent composition to 4:1 acetonitrile:water did not improve peak shape. Next, we applied a combination of mobile phase modifications totaling 12 conditions as shown in the Table S3. For a description of the criteria used to evaluate chromatographic performance, see “Data Analysis” in the Experimental Section. Ultimately, we found that 20 mM ammonium acetate at neutral pH produced the highest coverage and peak quality with our standard set. Under the other less optimal chromatographic conditions, we observed relative coverage trade-offs of various magnitudes, in accordance with previous work.[\(5,31\)](#)

After determining the optimal gradient conditions, we evaluated the chromatographic effects of adding 5 μ M ammonium phosphate to the aqueous fraction of the mobile phase. Although micromolar concentrations of phosphate have been reported to improve RP separations, it was unclear whether such low levels of phosphate would have substantive effects for HILIC separations considering the much higher abundance of electrostatic interactions relative to RP. Strikingly, however, trace phosphate improved overall coverage and peak quality, increasing the percentage of standards detected with optimal peak shapes from 52% to 88% (Figure 1A, Figure S1). Trace phosphate had a measurable effect on the peak shape of 36 polar standards, 24 of which showed improved chromatographic classification (Figure S1, S3). The main chromatographic changes included peak narrowing, reduced tailing, and less multipeak elution behavior (see EICs in Figure S3). Retention times were highly consistent, varying less than 3 s across 100 injections on a single column. Narrowed peaks led to an increase in average peak height (Figure 1B) and complete isomer resolution (Figure S4). Interestingly, the average peak area also increased with phosphate, suggesting that the increase in average peak height was not only due to a decrease in peak width (Figure 1C). Although phosphate had different effects on different analytes, these changes were highly reproducible. The variance in peak size between injections of the same sample was negligible, both with and without phosphate in the mobile phase.

With 5 μ M phosphate in the mobile phase, we calculated that 40 nmol of phosphate was introduced to the column during each sample run. Considering that our injection volume was 2 μ L for each gradient analysis, we calculated that the same amount of phosphate could be injected per sample by including it in the sample solvent at 20 mM instead of at micromolar levels in the mobile phase. Notably, adding phosphate to the sample solvent produced the same or slightly better chromatographic improvements as seen with its addition to the mobile phase (Figure S5–S6). To further explore this phenomenon, we performed a dose-dependence experiment with increasing concentrations of phosphate in the sample solvent. In addition to observing a comparable increase in MS signal as measured during the mobile phase analysis, we saw a decrease in peak width up to sample phosphate concentrations of 10 mM (Figure 1, panels D and E). We repeated a dose-dependence experiment with metabolic extracts from *E. coli* and observed a similar plateau of peak narrowing (Figure 1F).

It is intriguing to consider the potential effects of trace phosphate on electrospray ionization (ESI) efficiency. While trace phosphate improves chromatographic performance, it could

simultaneously have negative impacts on ionization. Indeed, historically, high levels of phosphate have been associated with ion suppression.⁽³⁶⁾ Interestingly, even though peak widths did not decrease significantly with the addition of 10 to 20 mM phosphate to the sample, peak heights showed a dose-dependent increase through 20 mM of phosphate (Figure 1, panels D and E). We point out that 10–20 mM phosphate added to the sample approximates ~5 μ M phosphate added to the mobile phase and suggests that trace phosphate may actually boost ESI efficiency. To directly test the effects of trace levels of phosphate on ESI efficiency, we measured the MS signal of our standards with 5 μ M ammonium phosphate in the mobile phase via direct infusion. On average, 5 μ M ammonium phosphate increased ionization by 6% for negatively ionizing compounds and 16% for positively ionizing compounds (Figure S7). We note that these average differences are relatively small compared to the changes observed in Figure 1 (panels B and C) when MS detection is coupled with HILIC separation, indicating that most of the improvements we observe due to trace phosphate are a result of chromatographic effects.

Trace Phosphate Reduces Errors during Automated Peak Detection

LC/MS-based untargeted metabolomics produces large data sets with thousands of signals and therefore generally requires software for efficient analysis.^(3,37) A major obstacle in the analysis of data sets exhibiting poor chromatography is that asymmetric peaks frequently result in informatic errors during automated detection of features.^(19–21) When peak shapes are non-Gaussian, for example, different regions of the same elution profile are often classified as unique features. A related problem is that the beginning and end of an elution profile are incorrectly defined, creating integration region variance. In some cases, peaks may fail to be detected by software all together. Inaccurate peak detection is particularly problematic in untargeted metabolomics because these errors are propagated throughout all subsequent stages of data processing such as correspondence determination and peak quantitation.^(15,18,20) When features are inaccurately integrated, statistical comparisons between sample groups are compromised. Since many researchers use statistical data alone as a filter to prioritize features for further investigation,⁽³⁴⁾ poor chromatography can lead researchers to incorrect conclusions and cause important metabolic differences to be missed. At best, it increases analysis time to have to reprocess data manually.

Accordingly, we considered the number of peak-detection errors during automated data processing as an important metric to benchmark HILIC/MS methods. Given the improvement that micromolar phosphate had on chromatographic peak quality, we sought to evaluate whether it also reduced errors during informatic analysis. We applied the centWave peak detection algorithm, which is widely implemented in commonly used software programs such as XCMS and MZmine 2,^(16,19,38) to assess the effects of trace phosphate when processing HILIC/MS data. To isolate the effects of peak quality on bioinformatic performance, we only considered features at each standard's $[M - H]^-$ or $[M + H]^+$ mass-to-charge value for negative mode or positive mode, respectively. For each standard, only the more abundant ion ($[M - H]^-$ or $[M + H]^+$) was considered in the analysis. As expected, poor quality peaks in the no-phosphate condition were detected as multiple features, whereas peaks in the phosphate condition behaved more ideally and were generally detected by centWave as a single feature. As a representative example, we show the extracted ion

chromatogram of isocitrate in Figure 2 (panels A and B). Without adding phosphate, improper peak detection artificially inflated the number of features detected to 43 above the actual number of standards. When trace phosphate was added, on the other hand, the number of features approximated the number of standards measured (Figure 2C).

Using Trace Phosphate with the ZIC-pHILIC Column Yields Broader Coverage of the Polar Metabolome

Having identified that trace phosphate improves ZIC-pHILIC analysis for a set of select standards, we next sought to assess its utility for profiling the entire polar metabolome. Since it is impractical to assign chemical structures to every feature in an untargeted metabolomic data set, we used the *credentialing* technology to better benchmark metabolite coverage.⁽³³⁾ *Credentialed* samples are mixtures of two separate *E. coli* cultures, one grown on a natural-abundance carbon source and the other grown on a uniformly ¹³C-labeled carbon source. Cells from each of the cultures are mixed in two specific ratios (1:1 and 1:2 in this study) to be extracted and analyzed with LC/MS. The data are then inspected for pairs of coeluting peaks that satisfy the following conditions: (i) peak intensities correspond to the mixing ratios, and (ii) the difference in accurate masses between the natural-abundance and uniformly ¹³C-labeled peaks corresponds to an integer number of carbons. Peaks matching these criteria are deemed to be *credentialed* (i.e., they originate from the *E. coli* samples). Features that do not match the above criteria result from artifacts (e.g., informatic errors) or contaminants (e.g., carry over from a previous experiment) and are removed. In our experiences, the total number of features in an untargeted metabolomic data set is not well correlated with the total number of metabolites detected because artifacts and contaminants represent a significant contribution that varies from experiment to experiment.⁽³³⁾ *Credentialing* helps minimize this problem to better compare metabolite coverage between methods.

We analyzed *credentialed E. coli* extracts from Cambridge Isotope Laboratories on the ZIC-pHILIC column with and without phosphate in the injection solvent. We also compared our results to previously published *credentialing* data acquired by using a Luna aminopropyl method, which is often used for global profiling of the polar metabolome.^(4,10,33) Relative to data published from the Luna aminopropyl column, we saw an 11.5% increase in the number of *credentialed* features on the ZIC-pHILIC column without the use of phosphate (Table 1). When the ZIC-pHILIC analysis included phosphate, we saw an additional 12.4% increase in *credentialed* features, which totaled to an approximate 25% increase in *credentialed* features relative to the Luna aminopropyl method. We wish to point out that the number of *credentialed* features in Table 1 excludes both phosphate adducts and multimers. Improvements in MS sensitivity, which extend coverage to lower concentration metabolites, may be one cause for the increase in *credentialed* features. The increase in *credentialed* features when using phosphate may also result from improved chromatographic behavior that allowed previously filtered peaks to be more reliably quantitated, thereby allowing them to pass the *credentialing* criteria listed above. We confirmed this to be the case for at least some compounds (Figure S8). Although it is impractical to chemically identify all *credentialed* features, we identified a select subset and compared their peak shapes between chromatographic methods. Figure S9 shows the peak shape of 10 *credentialed* features

identified to be nucleotides and intermediates in central carbon metabolism. Superior results were obtained when using the ZIC-pHILIC with phosphate, especially in comparison to the commonly used Luna aminopropyl method.

Phosphate Improves Chromatography by Shielding Electrostatic Sites on the ZIC-pHILIC Stationary Phase

After observing the chromatographic benefits of trace phosphate with the ZIC-pHILIC column, we sought to better understand its mode of action. We first tested whether phosphate interacts directly with analytes in the mobile phase via ion-pairing interactions, in which case phosphate would be expected to coelute with compounds whose peaks it affected. We created a new standard solution with high concentrations of four standards (4 mM each) and phosphate (10 mM). We selected four standards whose elution profile was strongly affected by phosphate, and we used high concentrations to increase the likelihood of detecting interactions. Under gradient elution, two compounds eluted before phosphate and two compounds eluted after phosphate. No coelution or co-ionization was observed, as would be expected for an ion-pairing interaction (Figure 3A). Next, we tested whether injecting 2 μ L of a 20 mM phosphate solution 30 s before the standard sample was injected, instead of including phosphate in the sample solvent or mobile phase, would alter phosphate's ability to improve peak shape. The effects of preloaded phosphate were not notably different than when phosphate was added directly to the mobile phase or the sample solvent itself, further suggesting that the observed chromatographic improvements were not due to phosphate interacting with analytes during bulk contact in solution (Figure S10).

We then aimed to determine whether trace phosphate improves chromatography by shielding electrostatic interactions between analytes and the stationary phase. Our strategy was to compare the effects of trace phosphate to those of ammonium acetate, a salt commonly used as a mobile phase buffer. Ammonium acetate is known to shield electrostatic interactions between analytes and the stationary phase as its concentration in the mobile phase increases. (39) We tested a range of ammonium acetate concentrations in the mobile phase, from 5 to 200 mM. As salt levels increased, we observed graded improvements in peak shape across our standard set (Figure 3B and Figure 4). Interestingly, analytes whose peak shapes were more affected by phosphate (so as to change their chromatographic classification) required higher levels of ammonium acetate to achieve optimal peak shapes. These data suggest that these analytes have stronger electrostatic interactions with the stationary phase (Figure S1 and Figure 4). Previously, it has been shown that compounds whose retention times shift in response to increased mobile phase buffer concentration are subject to electrostatic retention mechanisms, while compounds whose retention times do not shift are retained by partitioning mechanisms only. (13) Even though the chromatographic gradient was kept constant, all standards affected by phosphate showed retention time shifts when ammonium acetate was increased from 5 to 20 mM (Figure 3C). Collectively, these data suggest that phosphate produces its effects by modifying electrostatic interactions between analytes and the ZIC-pHILIC stationary phase.

We next sought to determine the extent to which electrostatic interactions are responsible for poor peak shape and coverage when using ZIC-pHILIC. Many standards failed to retain at

salt concentrations of 40 mM or higher because the solubility limits of ammonium acetate required us to lower the organic starting percentage of our gradient. Decreased retention complicated our ability to isolate electrostatic interactions as the cause of poor peak shape. Higher salt concentrations (i.e., more shielding of electrostatic interactions) always improved peaks for compounds with poor peak shape, but only if they were successfully retained. Thus, we also performed a 200 mM “shock” experiment where we maintained the starting portion of the original chromatographic gradient by using a second aqueous channel to introduce 200 mM ammonium acetate after 7 min. Such a two-part gradient could shield most electrostatic interactions without any loss in initial retention. Strikingly, we found that this “shock” experiment produced optimal peak shapes for nearly all standards evaluated (62/65, Figure 3B and Figure 4). While three standards with high neutral pH charges of +4 did not exhibit optimal peak shapes with the 200 mM shock, they still showed significant improvements compared to lower salt conditions (Figure 4). This result shows that removing electrostatic interactions is sufficient to eliminate nearly all peak shape and coverage defects seen with the ZIC-pHILIC stationary phase. Nevertheless, we wish to emphasize that although 200 mM ammonium acetate provided excellent chromatographic results that are conceptually interesting, the method is practically limited. As expected, such a high concentration of salt destroyed our LC column within three runs, clogged our MS source, and decreased MS sensitivity due to ion suppression (Figure S11).

Consistent with our conclusion that trace phosphate improves chromatographic peak shape by shielding electrostatic interactions between analytes and the ZIC-pHILIC stationary phase, we found that the addition of trace phosphate to both 200 mM ammonium acetate gradients (“ramp” and shock”) produced no further effect on peak shape (Figure 3D). These data indicate that high concentrations of ammonium acetate and trace levels of phosphate improve chromatography by the same chemical mechanism.

DISCUSSION

Phosphate has been used as a mobile phase buffer at millimolar levels for decades.^(27,29,30) At such concentrations, phosphate both buffers mobile phase pH and shields electrostatic interactions.^(14,40) Unfortunately, however, such high concentrations of phosphate are unsuitable for MS-based metabolomic workflows.⁽³⁶⁾ Here we show that, when using the ZIC-pHILIC stationary phase, low concentrations of phosphate in the mobile phase (micromolar) are sufficient to improve HILIC peak quality and extend metabolome coverage. At these trace concentrations, we have observed no signs of buildup, source contamination, or any other instrumentation failure after a year of experiments. Even after long-term use, trace phosphate continues to improve chromatographic peak shape and improve ESI efficiency. We note that the amount of phosphate per sample used in our analyses (~40 nmol) is still significantly lower than the amount of salts such as Na⁺ and K⁺ typically present in plasma samples (98 and 79 nmol, respectively, after extraction dilution).⁽⁴¹⁾ In contrast, the ~40 nmol of phosphate we add to samples is a substantial increase relative to endogenous phosphate levels in extracted biological samples, based on previously reported values and extraction dilutions.^(42–44) Indeed, for biological samples in which exogenous phosphate has been omitted, the MS signal corresponding to phosphate decreases by more than 5-fold.

Our results indicate that phosphate improves chromatographic peak shape by modulating electrostatic interactions between analytes and the ZIC-pHILIC stationary phase, but determining the exact nature of these interactions will require further investigation. It seems likely that trace phosphate is selective for high-activity electrostatic sites having an outsized effect on peak quality. First, the concentration of phosphate needed to produce measurable chromatographic benefits is considerably lower than that of other mobile phase buffers. Additionally, phosphate has a high charge density and selectively improves the peak shapes of compounds whose elution profile is negatively affected by strong electrostatic interactions.

One possibility is that phosphate interacts with low-abundance electrostatic sites arising from flaws in the manufacturing of the stationary phase. For some of the compounds affected by trace phosphate, we have observed that the performance of the ZIC-pHILIC stationary phase varies slightly between individual columns and over time. When the column's baseline performance is poorer, adding micromolar concentrations of phosphate to the mobile phase produces greater peak-shape improvements. Manufacturing irregularities or column-conditioning effects may alter the abundance and/or accessibility of stationary-phase sites that phosphate acts upon. A second related possibility is that low concentrations of phosphate block trace metals (e.g., iron, copper, aluminum, etc.) within the stationary phase matrix from coordinating with analytes and thereby influencing chromatographic peak shape.⁽⁴⁵⁾ Trace metals may be introduced by column manufacturers or may result from other sources within the chromatographic system. For example, the ultrahigh purity water typically used in LC/MS can leech metals from stainless steel.⁽⁴⁶⁾ Interestingly, changing the flow path of our system from steel to PEEK did not alter the chromatographic influence of phosphate. While it is possible that metal impurities were introduced to the stationary phase prior to switching the flow path from steel to PEEK, the results suggest that any trace metal impurities present are associated with the column itself rather than elsewhere in the LC system.

Beyond the methodological significance of trace phosphate, our results provide insight into the fundamental mechanisms limiting HILIC performance at the comprehensive metabolome level. The observation that a 200 mM ammonium acetate “shock” gradient produced high-quality peaks for nearly all of the standards we evaluated indicates that electrostatic interactions are the predominant cause of poor elution behavior in metabolomic analyses when using our ZIC-pHILIC method. Although the retention mechanisms of most HILIC stationary phases are similar, additional experiments will be required to determine whether electrostatic interactions are similarly the primary cause of poor chromatographic performance when using other HILIC columns. From an application perspective, the use of 200 mM ammonium acetate proved to be practically limited because the high levels of salt destroyed our LC column, clogged the source of our MS, and decreased MS sensitivity due to ion suppression. The use of trace phosphate to modulate electrostatic interactions instead of millimolar ammonium acetate, on the other hand, yielded significant chromatographic improvements, and other strategies to shield electrostatic interactions may represent a potential focus of future HILIC research for untargeted metabolomics. From a broader perspective, untargeted metabolomics is unique compared to most other applications of HILIC in that it compels a holistic understanding of chromatographic mechanisms for a

large set of physiochemically diverse compounds. Thus, we believe that the data presented herein provide not only an improved method for untargeted metabolomics but also reveal mechanistic insight related to HILIC performance that may further advance comprehensive metabolite profiling.

CONCLUSIONS

Although ZIC-pHILIC is a commonly used stationary phase for performing untargeted metabolomics, existing methods produce low-quality peaks for a significant fraction of polar metabolites.^(5,9,31,32) Here we report that the addition of trace phosphate improves both polar metabolome coverage and chromatographic peak behavior when using the ZIC-pHILIC column. Chromatographic peak behavior is particularly important to the fidelity of data analysis when using metabolomic software packages for automated processing. Asymmetric peaks lead to errors in feature detection and inconsistent integration of signals from sample to sample, ultimately compromising the quantitative reliability of the results. We determined that electrostatic interactions are the primary cause of poor peak behavior for the ZIC-pHILIC stationary phase, but that these can be modulated by phosphate. Given the low concentration needed, we found that micromolar phosphate could be added to the mobile phase or millimolar phosphate could be added to the sample solvent itself. Both produced similar chromatographic improvements. Notably, the concentration of phosphate we used here is 3–4 orders of magnitude smaller than that which has been applied previously in HILIC-UV spectroscopy applications. Such low concentrations of phosphate are compatible with MS, having no negative impact on our instrumentation after a year of use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Effects of trace phosphate (in the mobile phase or sample solvent) on ZIC-pHILIC coverage, peak shape, and performance. (A) Overall peak quality and coverage for a set of standards analyzed with and without 5 μ M ammonium phosphate in the mobile phase (see Data Analysis in the Experimental Section for explanations of peak classifications). (B) Box plot of relative peak height for detected standards with and without 5 μ M ammonium phosphate in the mobile phase. (C) Box plot of relative peak area of detected standards with and without 5 μ M ammonium phosphate in the mobile phase. (D) Relative peak height of detected standards as a function of increasing ammonium phosphate concentration in the sample solvent. (E) Dose-dependence curve comparing average peak width of standards as a function of increasing phosphate concentrations in the sample solvent. (F) Dose-dependence curve of average peak widths of 10 metabolites extracted from *E. coli* that were also in our standard set as a function of increasing phosphate concentrations in the sample solvent. Data are normalized to the no-phosphate condition for each experiment. Error bars represent 95% confidence intervals. *p-value 0.05, **p-value 0.01, ***p-value 0.001, n.s. = not significant.

Figure 2.

Effects of trace phosphate on automated peak detection using centWave as implemented in XCMS. (A) Extracted ion chromatogram of an isocitrate standard from XCMS. Isocitrate was analyzed without phosphate present. XCMS detected isocitrate as three distinct features, as indicated by *. (B) Extracted ion chromatogram of an isocitrate standard from XCMS, as analyzed with trace phosphate (20 mM in sample solvent) present. XCMS detected isocitrate as a single feature, as indicated by *. (C) Aggregate feature numbers detected in a standard set with and without phosphate used in LC/MS analysis. Only data from the 59 standards that could be detected when using the ZIC-PHILIC stationary phase with trace phosphate are included in the plot. The mass channels for the standards were used as input values for peak detection.

Figure 3.

Examining the effects of phosphate when using ZIC-pHILIC. (A) Extracted ion chromatograms of epinephrine, cidofovir, ATP, isocitrate, and phosphate. Four standards (each at 4 mM) were coinjected with phosphate (10 mM) to determine whether phosphate coeluted/co-ionized with them. Broad peak widths and tailing are due to extreme column overloading. Positive and negative ionization data are superimposed on the top EIC. (B) Effects of varying mobile phase buffer concentrations on standard coverage and peak shape. Detailed descriptions of the 200 mM ramp and shock conditions can be found in the Experimental Section. (C) Plot of retention time ratios between two gradients that only vary by the amount of salt in the mobile phase. Compounds are grouped by those whose chromatographic behavior was and was not affected by phosphate. (D) Plot of average peak widths of standards analyzed using the 200 mM ramp method with and without phosphate in the mobile phase. Only compounds that showed chromatographic changes with phosphate were included in the analysis. Only compounds that retained past the void volume were included in the analysis. n.s. = not significant.

Standard	Neutral Charge State	5 mM	10 mM	20 mM	40 mM	200 mM Ramp	200 mM Shock	Standard	Neutral Charge State	5 mM	10 mM	20 mM	40 mM	200 mM Ramp	200 mM Shock
Geneticin	4							Uracil	0						
Kanamycin	4							Uridine	0						
Spermine	4							Vidarabine	0						
Streptomycin	3							1,3-dicaffeoylquinic acid*	-1						
Adipic acid dihydrazide*	2							Biotin	-1						
Putrescine	2							Butyrate	-1						
Acetylcholine*	1							Cidofovir*	-1						
Arginine*	1							Glutathione	-1						
Butylamine*	1							p-Aminohippuric acid	-1						
Choline	1							Pyruvate	-1						
Cyclohexylammonium*	1							Sodium-p-toluene-sulfinate	-1						
Dopamine*	1							Taurocholic acid*	-1						
Epinephrine*	1							2-deoxyglucose 6-phosphate*	-2						
Ethanolamine*	1							2-hydroxyglutarate (2-HG)*	-2						
Guanidine*	1							ADP*	-2						
Histamine*	1							α -Ketoglutarate*	-2						
Lysine*	1							AMP*	-2						
Trimethyphenylammonium	1							Chelidamic acid	-2						
Phenethylamine*	1							Myoinositol 4-monophosphate*	-2						
Piperazine*	1							Folic acid	-2						
Serotonin*	1							Fucose 1-phosphate*	-2						
2-deoxyglucose (2-DG)	0							Fumaric acid*	-2						
2'-deoxyguanosine	0							Iris 7- <i>ws</i> carboxylic acid	-2						
3'-deoxyguanosine	0							L-Ascorbic acid 2-sulfate	-2						
Adenine	0							Neryl pyrophosphate*	-2						
Adenosine	0							2-phosphoglycerate (2-PG)*	-3						
Cyanocobalamin	0							3-phosphoglycerate (3-PG)*	-3						
GABA*	0							ATP*	-3						
Galactose	0							Citrate*	-3						
Glucose	0							EDTA*	-3						
Guanine*	0							Isocitrate*	-3						
Toluene	0							Phosphoenolpyruvate (PEP)*	-3						
								Palmitoyl-CoA*	-4						

Figure 4.

Heatmap of standard chromatographic performance on ZIC-pHILIC as a function of mobile phase salt concentration. Detailed descriptions of the 200 mM ramp and shock conditions can be found in the Experimental Section. Optimal (green) peaks appeared well above baseline noise with narrow, symmetrical shape. Suboptimal (yellow) peaks were deemed quantitatively reliable but showed slight peak tailing, band broadening, or asymmetry. Orange represents quantitatively unreliable peaks with peak distortion in the form of significant band broadening, asymmetry, peak splitting, or jaggedness. A purple color was given to peaks that did not retain on the column past the void volume. Red designates

standards that were not detected significantly above baseline or went undetected entirely, presumably because they did not elute from the column. * indicates compounds whose peak shape were affected by phosphate.

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Table 1.Comparison of *Credentialed* Features between Methods

method	no. of signals ^a	no. credentialed
Luna Aminopropyl	32010 ^b	1475 ^b
ZIC-pHILIC (no phosphate)	30493	1644
ZIC-pHILIC (with phosphate)	30741	1848

^a Only features with peak areas greater than 5000 ion counts were considered.^b Previously published data.(33)