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A sample extraction and chromatographic strategy for increasing LC/MS detection coverage of the erythrocyte metabolome☆

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ABSTRACT

Reproducible and comprehensive sample extraction and detection of metabolites with a broad range of physico-chemical properties from biological matrices can be a highly challenging process. A single LC/MS separation method was developed for a 2.1 mm × 100 mm, 1.8 μm ZORBAX SB-Aq column that was used to separate human erythrocyte metabolites extracted under sample extraction solvent conditions where the pH was neutral or had been adjusted to either, pH 2, 6 or 9. Internal standards were included and evaluated for tracking sample extraction efficiency. Through the combination of electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) techniques in both positive (+) and negative (−) ion modes, a total of 2370 features (compounds and associated compound related components: isotopes, adducts and dimers) were detected across all pHs. Broader coverage of the detected metabolome was achieved by observing that (1) performing extractions at pH 2 and 9, leads to a combined 92% increase in detected features over pH 7 alone; and (2) including APCI in the analysis results in a 34% increase in detected features, across all pHs, than the total number detected by ESI. A significant dependency of extraction solvent pH on the recovery of heme and other compounds was observed in erythrocytes and underscores the need for a comprehensive sample extraction strategy and LC/MS analysis in metabolomics profiling experiments.

1. Introduction

Metabolomics is the comparative analysis of metabolites, the intermediate or final products of cellular metabolism, found in sets of similar biological samples. It is a small molecule analysis problem (molecular weight <1500), often of highly complex samples, in which the chemical identity is often unknown. The metabolome of an organism refers to the qualitative and quantitative set of all small molecules that participate in general metabolic reactions for cell growth and normal maintenance [1]. A more complete characterization of the number and composition of metabolites contained within the metabolome would lead to a better understanding of disease biology and molecular circuitry of cells. Indeed, metabolomics is increasingly being used as a tool in a wide variety of applications, such as in functional genomics [2,3], drug discovery [4], nutrition [5,6], cancer [7–9], chemical biology [10] and systems biology [11–15].

Due to differences in chemical mass, polarity and charge contributions to chemical heterogeneity, and a very large dynamic range of concentration ranges, many components of the metabolome remain undetected [16]. A successful analytical strategy for untargeted metabolomics workflows ideally should be rapid, robust and follow an extraction and separation protocol that gives adequate consideration to variables such as the nature of extraction solvent, quenching of metabolic turnover and inclusion of internal standards that helps gauge the success of the extraction procedure. The sample storage temperature, protein precipitation methods and processing-time considerations are also important. The extraction solvent pH however is often overlooked. The principle behind sample pH adjustment in the extraction solvent is to bring analytes to a state where they can be extracted from one of the matrices, maximizing selectivity at a particular pH with minimal loss of recovery [17]. The resulting pool of extracted metabolites at the different pHs would therefore be expected to be larger than if the extraction were done at neutral pH alone.

LC/MS is increasingly becoming the platform of choice for metabolomics. This is mainly due to its increased sensitivity, specificity and wide dynamic range. Several different ionization methods and mass spectrometry platforms have been used in metabolomics: ESI [18–21], is most commonly used in conjunction with LC/MS due

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to its soft ionization, good quantitative capability and high sensitivity [22]. APCI [23] may induce little fragmentation, less background noise and provides a greater dynamic range [24].

Recently, a dual ionization mode mass spectrometry-based metabolomics strategy was described for human serum extracts [19], where increased coverage of detected metabolites using ESI, APCI and a multimode ion source in both polarities was achieved. Although applications of mass spectral analyses of targeted small molecules in erythrocytes using LC/ESI-MS [25], GC/MS [25,26] and targeted analysis of drugs and metabolites in biological fluids [27–30] has previously been described, much less is known of the metabolomic profile of erythrocytes.

The goal of this study was to develop a more comprehensive strategy for extraction and metabolomic profiling analysis of erythrocytes that includes appropriate internal standards. We demonstrate our approach on a single binary solvent system coupled to ESI (+/−) and APCI (+/−) ionization modes. It has the advantage of the same RTs for metabolites detected by the different ionization methods. Finally, we determine the total number of metabolites detected at each pH, their distribution between the different detection techniques and the number of metabolites that were detected by a specific combination of approaches. We also discuss the importance of this analytical approach using examples of extracted compounds.

2. Experimental

2.1. Reagents and chemicals

All chemicals and solvents were of analytical or HPLC grade. Acetonitrile and methanol were obtained from Burdick and Jackson. A 10-mg/mL stock solution of 1-naphthylamine (Sigma–Aldrich, St. Louis, MO, USA) and 2-(methylthio)benzothiazole (Acros organics, Morris Plains, NJ, USA) was prepared in 100% methanol and diluted into the extraction solvent at a ratio of 1:1000.

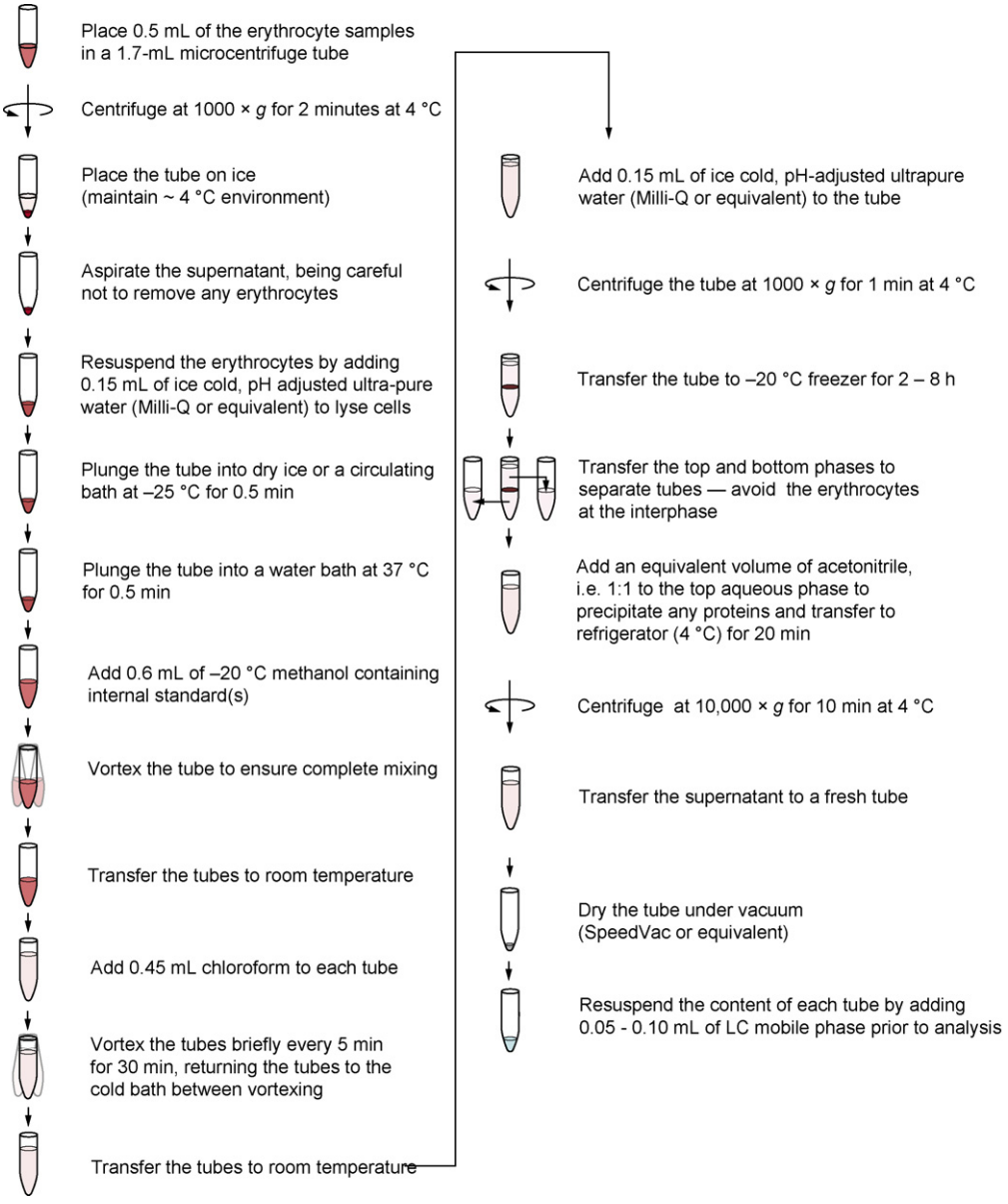

Fig. 1. Workflow for liquid–liquid extraction of metabolites from erythrocytes using solvents adjusted to varying pHs.

2.2. Sample processing

A circulating bath (RTE-7, Thermo Fisher Scientific, San Jose, CA, USA) containing ethylene glycol was operated at −25 °C. Alternatively, a dry ice/isopropanol bath can also be used to chill samples. The following stock water solutions were prepared using ultra-pure, low resistance, 18 Ωm Milli-Q (Millipore, MA, USA) water in 50 mL glass vials or Erlenmeyer flasks on ice at the following pHs: (A) Milli-Q water, (i.e. ∼pH 7), (B) 1% formic acid, pH 2 (C) 20 mM ammonium acetate, pH 6 (provides buffering capacity while extracting erythrocytes), and (D) 2% ammonium hydroxide, pH 9. The final pH of working stock solutions was checked with pH strips. Human erythrocytes (Stanford Blood Center) were donated from four anonymous individuals. Four vacutainer tubes, each of approximately 4 mL of blood, were collected from each donor. The collection tubes were pre-treated with a 3.2% solution of sodium citrate anti-coagulant and stored refrigerated prior to use. An equivalent volume of erythrocytes from each of the four donor tubes was pooled into one 15 mL Falcon tube that became the stock from which sample aliquots were withdrawn. Triplicate erythrocyte samples were analyzed. For each sample, 0.5 mL from the pooled erythrocyte stock was transferred into a microcentrifuge tube and processed (see Fig. 1 for details of the protocol). Finally, the dried samples were re-suspended in 0.1 mL of 50% methanol in water, 0.2% acetic acid and transferred to glass autosampler vials for LC/MS analysis.

2.3. LC/MS

A 1200 Rapid Resolution system (Agilent Technologies, Santa Clara, CA, USA) containing a binary pump and degasser, well-plate autosampler with thermostat, thermostatted column compartment, and an Agilent 6520 Q-TOF mass spectrometer equipped with either a dual ESI or APCI source was used to analyze the samples. An Agilent ZORBAX SB-Aq column 2.1 mm × 100 mm, 1.8 μm was used to separate the erythrocyte extracts. An Agilent ZORBAX C-8, 2.1 mm × 30 mm, 3.5 μm guard column was placed in series in front of the analytical column. This prevented plugging of the analytical column, which would otherwise have resulted in increasing system pressure, by an as yet unidentified component of the erythrocyte extract. LC parameters: autosampler temperature, 4 °C; injection volume, 4 μL; column temperature, 65 °C; and flow rate of 0.5 mL/min. The LC solvent and timetable were identical for all four ionization modes. A 2–98% linear gradient of solvent A (0.2% acetic acid in water) to B (0.2% acetic acid in methanol) was employed over 16 min followed by a solvent B hold of 2 min and a 5-min post-time.

Mass spectra for metabolite extracted erythrocyte samples were acquired in extended dynamic range, TOF only mode. Data were acquired using the following settings: ESI capillary voltage was set at 4000 V (+) ion mode and 3500 V (−) ion mode and fragmentor at 170 V. The liquid nebulizer was set to 45 psig and the nitrogen drying gas was set to a flow rate of 12 L/min. Drying gas temperature was maintained at 250 °C. APCI capillary voltage was set at 4000 V (both ion modes), corona current was set to 4 μA and fragmentor at 170 V. The liquid nebulizer was set to 60 psig and the nitrogen drying gas was set to a flow rate of 5 L/min. Drying gas temperature was maintained at 250 °C. The vaporizer temperature was maintained at 350 °C. Data was stored in centroid mode. Internal reference ions were used to continuously maintain mass accuracy. Data was acquired at a rate of 2.5 spectra/s with a stored mass range of *m/z* 50–1000. Data was collected using Agilent MassHunter Workstation Data acquisition software.

2.4. Data processing

LC/MS data files were processed by Agilent MassHunter Qualitative Analysis software. Feature finding was achieved using the feature extraction and correlation algorithms, which locates the groups of co-variant ions in each chromatogram. Each of these groups represented a unique feature. Processing of raw data files resulted in features which are defined as time-aligned ions (isotopes, adducts and dimers) summarized to the calculated neutral mass, possessing an abundance and a RT. The algorithm located all compounds and compound-related components (i.e. features) in a spectrum. In positive-ion mode this included adducts (H^+, Na^+ and K^+), isotopes and dimers; and in negative ion mode it included adducts ($-H^+$, and $+CH_3COO^-$), isotopes or dimers. These related ions were treated as a single compound or feature. Finally, the calculated neutral mass and RT and summed ion abundances were stored in a file for subsequent comparative analysis in Agilent Mass Profiler or Agilent GeneSpring-MS software. All missing values were set to 0.001 to permit generation of log ratios. Mass, RT and abundances and their standard errors were displayed so that differences between pairs of groups, each containing analytical triplicate samples could be seen. Pre-analysis filters such as allowed charge state (+1), number of ions detected (≥1), masses to exclude (from blank runs), RT alignment parameters (intercept of 0.15 min and slope of 0.20%/min and mass tolerance parameters (intercept of 2.0 mDa and slope of 5.0 ppm) were entered into the program. For global comparisons of all the data, the files were processed as a single, collective group in GeneSpring-MS software. The RT and mass tolerances values were assigned (as above) prior to global alignment. In order to find metabolites that were extracted under a specific pH and detection strategy, the data was filtered so that only masses appearing in triplicate samples in at least one condition were accepted. Minimum absolute abundances were determined empirically and set at 5000 for ESI (+/−) and 1500 for APCI (+/−). The filtered mass sets were subsequently used to find masses that were shared or unique to the different conditions using Boolean commands in GeneSpring-MS software. The results were displayed in Venn diagrams. Further data reduction was accomplished in Microsoft Excel (Microsoft, Redmond, WA, USA).

2.5. Tentative compound identifications

The Agilent METLIN Personal metabolite database was used to make tentative identifications from the mass lists created in either GeneSpring-MS or in Agilent Mass Profiler software. This database includes masses, chemical formulas, and structure information for over 15,000 metabolomic-related compounds. All features were searched against the METLIN database and an empirical formula was calculated by an automated Molecular Formula Generator (MFG) algorithm that uses isotopic pattern matching to score the formula to the observed spectra. The results of the database and MFG result were compared and used to generate a putative metabolite compound match and formula(s).

3. Results and discussion

3.1. Sample extraction and processing at different solvent pHs

In liquid–liquid extractions (LLE), various drugs and metabolites are typically partitioned between polar, aqueous solutions and non-polar organic solvents such as chloroform to form a simple, robust two-phase system. We used a modification of a methanol/water/chloroform solvent extraction system [31,32] in a three-step extraction protocol: (1) lysis of erythrocytes

Table 1
Influence of the sample extraction solvent pH on RTs by using a single methanol/acetic acid LC solvent system

Source mode/polarity	Extraction solvent pH				
	pH 2	pH 6	pH 7	pH 9	All pHs
ESI (+)	0.013	0.013	0.018	0.010	0.011
ESI (−)	0.053	0.024	0.030	0.020	0.023
APCI (+)	0.021	0.014	0.016	0.013	0.013
APCI (−)	0.027	ND	ND	ND	ND

Average standard deviation of RTs (in min) for all metabolites detected between 1 and 18 min. ND: not determined due to insufficient data.

under hypotonic, solvent adjusted pH conditions, (2) quenching of metabolism by the addition of a biphasic solvent mixture containing −25 °C methanol [21,33], that facilitates denaturation of proteins, uncoupling of protein/metabolite interactions and dissolving of metabolites under acidic, neutral or basic conditions, and (3) extraction of metabolites by driving their separation into polar and non-polar phases. Fig. 1 shows the workflow for erythrocyte metabolite extraction using a LLE protocol for different samples where the solvent pH was adjusted to pH 2, 6 and 9, or was not changed (i.e. pH 7). It was important to keep the volumes of erythrocytes, solvents and their ratios exact to achieve biphasic separation. Due to residual protein carryover we also included a protein precipitation step with acetonitrile that greatly improved resolubilization into the LC re-suspension solvent after the supernatant had been dried down. Formic acid and ammonium hydroxide are volatile and therefore good choices for adjusting the pH of the aqueous phase.

3.2. A single LC method compatible with downstream MS ionization modes

One of the goals of the present analytical strategy was to employ a single LC method that would greatly simplify the construction of a RT and accurate mass library for untargeted analysis of compounds using multiple detection modes. Methanol was used instead of acetonitrile as the eluting solvent because of the improved APCI ionization efficiency for some analytes. Acetic acid was used instead of formic acid as a mobile phase modifier because it results in improved negative ion sensitivity for acidic compounds. Our preliminary work with standards had shown equivalent separation and sensitivity using methanol/acetic acid compared to acetonitrile/formic acid mobile phase (data not shown). To examine the suitability of the LC method, we examined the average standard deviation (S.D.) of the RTs of the analytes. Table 1 shows the calculated average S.D.s of RTs for analytes between 1 and 18 min for the various conditions. By only analyzing data that was collected after 1-min void volume variability on data quality was avoided. The average SD ranged from 0.010 to 0.053 min. The number of analytes in this time frame varied for each condition. It was as low as 35 analytes for ESI (−)/all pHs and as high as 585 analytes for ESI (+)/pH 6 (data not shown). Although the S.D.s of RTs for pH 2 extracts was observed to be larger compared to other pHs, and the S.D. for ESI (−) was significantly greater than ESI (+), the differences were in tenths of a second and therefore negligible. These results indicate that regardless of whether one is operating in ESI (+/−) or APCI (+) modes, the chromatography is sufficiently reproducible that the RT window for compound tracking of ±0.15 min can be easily supported using this analytical system. The smallest possible variation in RT is desirable as RT is used as one of the physical parameters to both track metabolites in an untargeted mass profiling experiment and identify metabolites in a RT and accurate mass database. Interestingly, samples extracted between pH 6 and 9 did not ionize sufficiently by APCI (−) to be detected after 1 min.

3.3. Internal standards

One of the purposes for using an internal standard in metabolomics experiments is to track sample extraction performance. To be a useful internal standard the compound should co-extract with other analytes at different solvent pHs, be inexpensive, ionize by ESI and APCI and ionize in positive and negative ion modes. Two internal standards, 2-(methylthio)benzothiazole and 1-naphthylamine were added to each sample at the extraction step (see Fig. 1). They were used to demonstrate the effect of the extraction protocol on the relative extraction efficiency of a known compound. 2-(Methylthio)benzothiazole is a weakly polar non-ionic compound, whereas 1-napthylamine is basic. Therefore, one would expect an increase in extraction efficiency into the aqueous phase under acidic pH. Table 2 shows the recovery of each of these compounds as the pH is varied. 2-(Methylthio)benzothiazole is a good example of a compound that is ionized by APCI but not by ESI. Its recovery was significantly dependent on pH and salt conditions, reaching a minimum in the ammonium acetate buffered, pH 6 condition, and maximum recovery at pH 2. The pH 6 result is most likely a salting out effect as the weakly polar internal standard has greater solubility in the chloroform phase at this pH and salt level. The lack of additional salt in the pH 7 and 9 extracts would be expected to increase its solubility in those samples, which is consistent with what was observed. Moreover, under strongly acidic conditions, the aromatic nitrogen is partially protonated and this increases the distribution coefficient in favor of the aqueous phase, and hence increased solubility. This molecule is a good example of the need for including different internal standards as the behavior of some compounds can be dramatically affected by seemingly minor changes in conditions. 1-Napthylamine had good solubility in all extracts tested but the pH 2 condition resulted in a 2–3-fold increase in recovery as compared to the other tested pHs. It is also sufficiently volatile that it ionizes by APCI, and therefore, it is a suitable internal standard for further investigation in positive-ion mode. When the data for this paper was generated an acceptable acidic internal standard was not identified. However, we are now in the process of evaluating 9-anthracene carboxylic acid and

Table 2
Comparison of the Mean ion intensities for internal standards spiked into samples during extraction

Samples ($N=3$) for each pH	1-Naphthylamine (m/z 144.082 $[M+H]^+$)		2-(Methylthio)benzothiazole (m/z 182.009 $[M+H]^+$)	
	ESI (+) ($\times 10^6$)	APCI (+) ($\times 10^6$)	ESI (+)	APCI (+)
2	6.0	4.8	ND	7.0×10^6
6	2.0	2.2	ND	4.9×10^4
7	2.8	2.5	ND	5.4×10^5
9	2.2	2.1	ND	6.5×10^5

The internal standards were evaluated for their utility in positive-ion mode only. Not detected (ND). No signal was detected in negative-ion mode. Abundances are the average of three replicates.

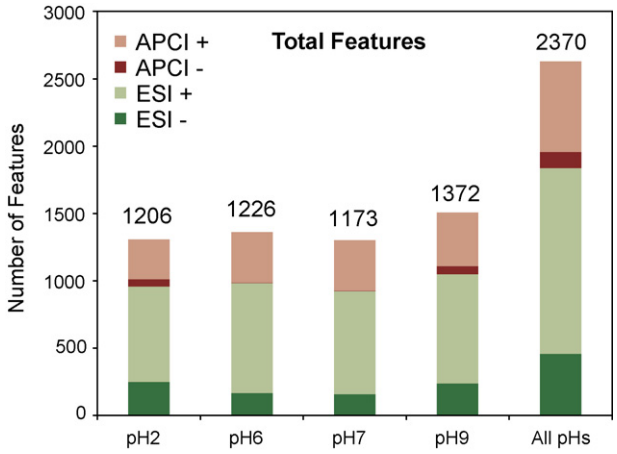

Fig. 2. Bar graph summarizing the total number of features for erythrocyte samples extracted at different solvent pHs and detected in APCI (+), APCI (−), ESI (+) and ESI (−) modes. The results are for all modes and polarities. The "All pHs" bar is the combined result for pHs 2, 6, 7 and 9, and has had feature redundancies (overlap) between the pHs filtered out.

expect it to be a suitable internal standard in negative-ion mode as well.

3.4. Varying extraction solvent pH increases total metabolite coverage by ESI and APCI

Fig. 2 summarizes the total number of features detected in erythrocytes with each extraction solvent at varying pH. A total of 2370 non-redundant features were detected, across all pHs, and in all ionization modes. Interestingly, each extracted sample recovered roughly the same number of observed metabolites (range: 1173–1372) at each pH. For each extract the total is approximately half of the total for all extracts combined. This can be accounted for due to metabolite redundancy (i.e. the same metabolite is recovered in different extracts). The result strongly suggests a significant portion of the metabolome is soluble in an aqueous solution of varying pH, and is not surprising given that biological systems use water as the solvent. Although the overall results are encouraging, the APCI (−) results were relatively poor and also displayed a pH-dependent effect. It is possible that under our chromatographic conditions, the acetic acid mobile phase modifier did not cause sufficient ionization in (−) mode. A single solvent system/gradient method by necessity implies compromising on some aspect of the analysis.

Next, we compared what effect adjusting the pH would have on metabolite recovery on individual pH conditions where the total number of features detected by all ionization modes has been combined. We included ammonium acetate buffer, pH 6 to see if there was significant overlap between a pH 6 buffered and non-buffered extraction solvent. In fact, the importance of sample pH adjustment using appropriate buffer mixtures for analyte extraction has recently been described in human blood plasma [34]. As expected there was a significant overlap between the pH 6 and 7 conditions where 60% of the metabolites were shared (Fig. 3A). Had we used only a single pH condition for extraction, such as a non-buffered pH 7 solvent that is typically used in sample extractions, the total number of detected features would be approximately halved. Fig. 3B shows a Venn diagram of three extraction conditions: pH 2, 7 and 9. Approximately half of the total number of features would be detected (313 + 263 + 515 + 82 = 1173) compared to a strategy including pH 2 and 9 extracts ((1173) + 483 + 126 + 468 = 2250). Therefore, using our sample extraction and analytical workflow, the inclusion of pH 2 and 9 extractions resulted in a 92% increase

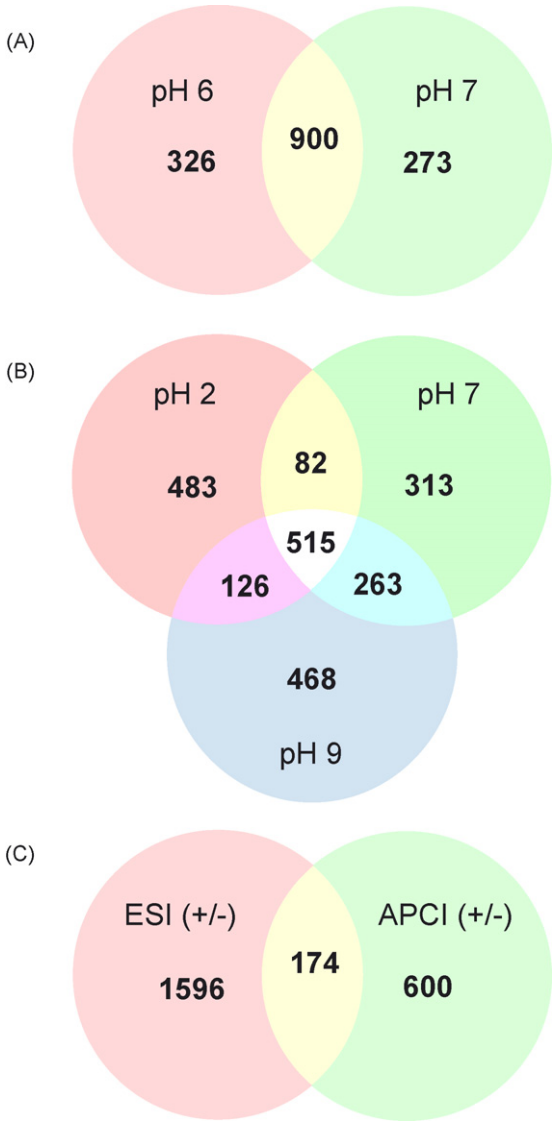

Fig. 3. Venn diagrams showing the number of overlapping features between different pH conditions and ionization modes. Comparison of the total number of detected features for (A) pH 6 and 7 extracted samples, and (B) pH 2, 7 and 9 extracted samples, by combining the results of ESI (+), ESI (−), APCI (+), and APCI (−) modes. (C) Comparison of the total number of detected features for ESI and APCI modes in both polarities (+/−), where the results for each condition: pH 2, 6, 7 and 9 were combined together in each ionization mode.

in metabolite coverage over pH 7 alone. This is consistent with these pH extractions providing the greatest aqueous solubility enhancement. In summary, a comprehensive and "unbiased" analysis missing half of the metabolites is neither comprehensive nor unbiased.

3.5. A comparison of detection coverage by APCI and ESI

The chemical structure variation of metabolites is substantial. It is therefore incumbent upon the analyst to incorporate different chromatographic separation techniques such as reverse phase chromatography, HILIC or aqueous normal phase (ANP) chromatography for polar compounds. In an untargeted analysis the researcher may be searching for molecules that are ionic (i.e. amino acids and organic acids), polar, neutral (i.e. sugars) and non-polar (i.e. steroids, triglycerides). These metabolites and others cannot be ionized by a single source technique or polarity. ESI is the generally

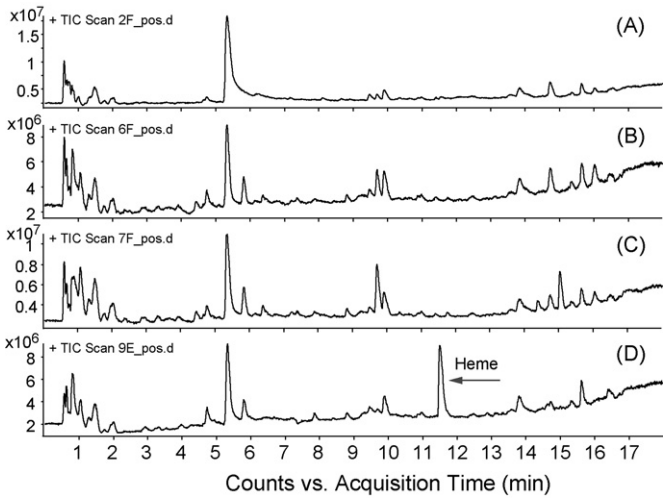

Fig. 4. Representative TICs for erythrocyte samples in ESI (+) mode extracted at various pHs: (A) pH 2, (B) pH 6, (C) pH 7 and (D) pH 9. An arrow highlights a peak later determined to be heme.

preferred ion source for metabolomic studies because it produces more ions via charge exchange in solution, where the charged ions are desorbed from the charged aerosol into the gas phase. APCI produces ions by charge exchange in the gas phase after an analyte is vaporized from an aerosol. While the mechanism of ion formation is different, the two techniques can also have some ion formation overlap for biologically based, small molecules. The overlap is not precisely known but based on the observation in this experiment it is approximately 7.3% (see Fig. 3C). Although we observed a 2.5-fold greater overall coverage by ESI compared to APCI, there was a 34% increase in the total number of detected metabolites when APCI was included, compared to ESI alone (Fig. 3C). Hence, the contribution of APCI in the analysis, particularly in (+) mode, suggests that they perform as complementary techniques.

3.6. Recovery of heme and its metabolite biliverdin is pH sensitive

The largest repository of heme in the human body is in erythrocytes, which are generated in the bone marrow and destroyed in the spleen, releasing hemoglobin. Heme is hydrophobic and has a porphyrin structure consisting of four rings that are connected into a larger ring. We detected one of the most abundant peaks in ESI (+) mode in the TIC of pH 9 extracted samples, but not in other extracts, that eluted around 11.5 min (Fig. 4). We also observed a similar trend by APCI (+) (data not shown). Because heme contains iron, its isotope pattern is very distinctive due to the natural distribution of iron isotopes. The observed average spectra from representative TICs, between RT 11.441 and 11.658 (Fig. 5A) was consistent with the calculated isotope pattern for heme (Fig. 5B). The mass errors for the observed isotope ions were (614, −0.8 ppm), (616, 1.3 ppm), (617, 0.7 ppm) and (618, 0.1 ppm). The proposed empirical formula of $C_{34}H_{32}N_4O_4Fe$ returned from the METLIN Personal database search produced a match, and based on the mass error, isotope pattern and combined with an understanding of the biology it was sufficient to confirm this peak as heme.

An EIC of the most abundant isotope for heme revealed that the pH 9 extracted samples were over 100-fold more abundant than in the pH 7 and 6 extracts, and 10-fold more abundant than pH 2 extracted samples (data not shown). It is likely that in addition to the physiological free heme that is present in erythrocytes, that the acidic and basic extraction conditions facilitated the dissociation from hemoglobin to varying degrees. This hypothesis is supported

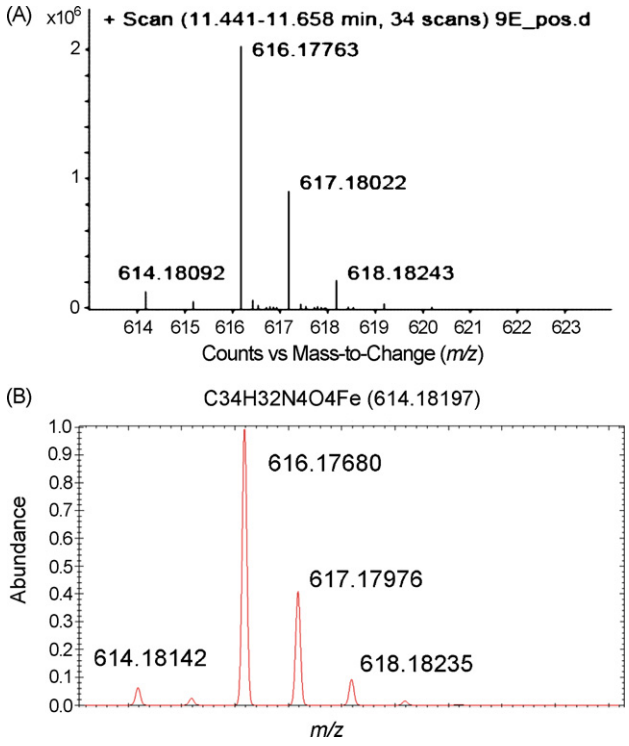

Fig. 5. (A) The observed average spectrum from 11.441 to 11.658 min of a data file for a representative pH 9 extracted sample in ESI (+) mode, showing a characteristic iron-containing isotope pattern, and (B) the calculated isotope pattern for molecular formula $C_{34}H_{32}N_4O_4Fe$ (heme).

by the results for biliverdin (*m/z* 583.25511), a structurally similar metabolite, that does not contain iron. EICs for Biliverdin (Fig. 6) revealed twin peaks between RT 12.5–13 min that are probably due to being diastereomers, and would be consistent with its structure. The pH 9 extracted samples (Fig. 6D) showed a 10-fold increase in abundance over the other extracts in ESI (+) mode, indicating that biliverdin is prone to better recovery at pH 9. The observation that biliverdin has the same pH 2–9 recovery ratio as heme is consistent with base extraction enhancement by the ionization

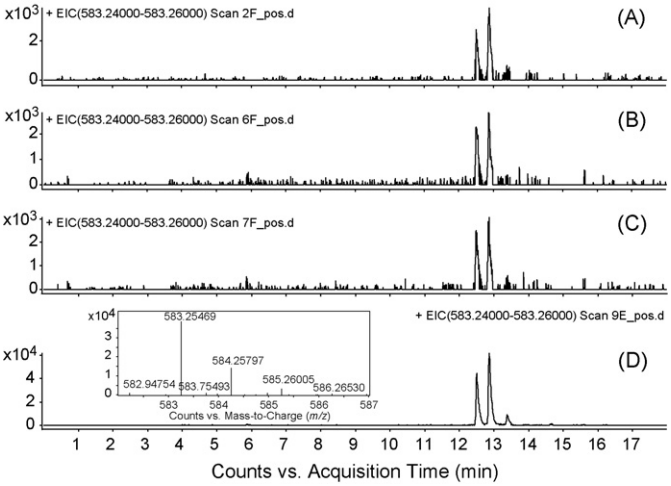

Fig. 6. Representative EICs generated for *m/z* range 583.24–583.26 in ESI (+) mode for samples extracted at (A) pH 2, (B) pH 6, (C) pH 7 and (D) pH 9. Averaged scans across both peaks contain the ion *m/z* 583.25469 $[M+H]^+$. A search of the METLIN Personal database identified this mass to be Biliverdin with a measurement error of 0.72 ppm.

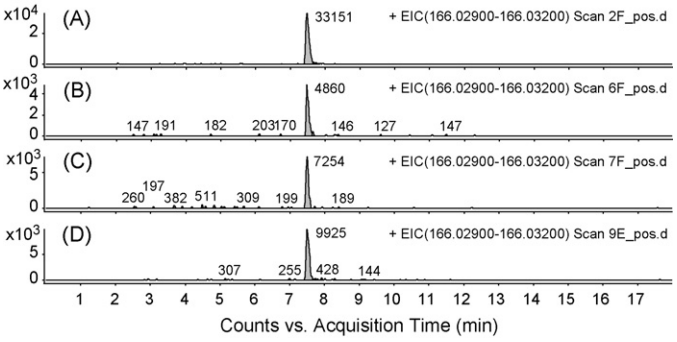

Fig. 7. Representative EICs generated for *m/z* range 166.029–166.032 in ESI (+) mode for samples extracted at (A) pH 2, (B) pH 6, (C) pH 7 and (D) pH 9.

of the carboxylic acid groups in both molecules. The observation that biliverdin does not show an increase in pH 2–6/7 recovery as heme, suggests that significant amounts of observed heme is being generated by denaturation of hemoglobin.

3.7. The significance of pH and using different ionization modes in the recovery of unknown compounds

With a water/methanol/chloroform LLE protocol the distribution of compounds is driven by the relative polarity of the analyte. Neutral but polar analytes will partition in both phases with the analyte enriched in the aqueous methanol phase. Analytes that are acid or basic will potentially experience significant changes in the distribution in the two phases based on pH. Fig. 7 shows a comparison of EICs, performed in ESI (+), on a compound with $[M+H]^+$ ion of 166.03117, and predicted formula of C_8H_7NOS. It has a strong pH effect on recovery into the aqueous phase. At pH 2 (Fig. 7A), the metabolite is recovered between 3 to 7 fold higher than at the other pHs. This strongly suggests that it contains a basic functional group in its structure. The recovery of this metabolite at other pHs also suggests the molecular structure is more complicated: at pH 6 the metabolite recovery reaches a minimum but at pH 7 and 9 it shows a noticeable increase in recovery. This suggests that this metabolite behaves as a zwitterion, with its aqueous solubility maximized at a point away from its isoelectric point. Since the isoelectric point for most zwitterions is between pH 5 and 8, the recovery data is consistent with this expectation.

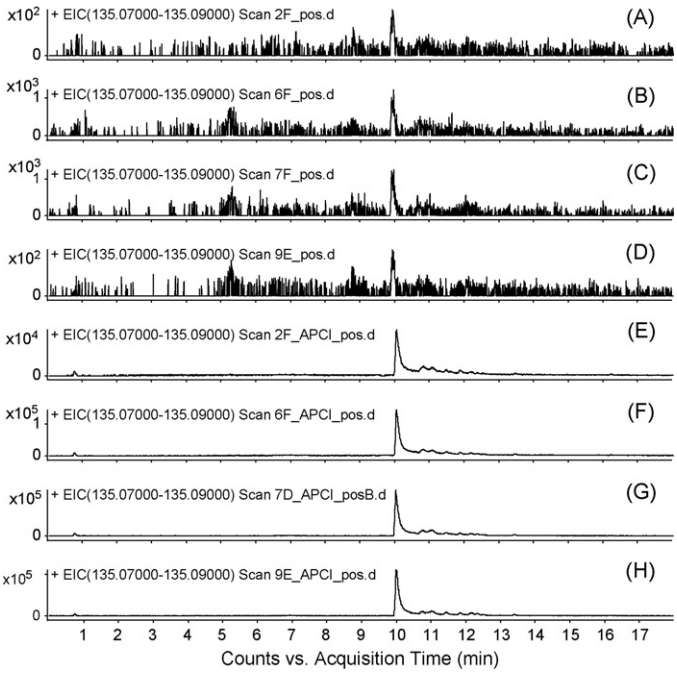

Fig. 8. Representative EICs generated for *m/z* range 135.070–135.090 in ESI (+):(A) pH 2, (B) pH 6, (C) pH 7 and (D) pH 9 and APCI (+) modes: pH 2 (E), pH 6 (F), pH 7 (G) and pH 9 (H).

An example of APCI ionizing a compound while ESI effectively does not is seen in Fig. 8. The chromatograms show that the ESI response in all extraction conditions is near zero whereas the APCI response is quite strong and uniform across pH extractions. This example of an APCI only responding molecule; low molecular weight and $[M+H]^+$ ion of *m/z* 135.07925, corresponding to a mass of 134.07197 suggests that it is likely to be volatile but not sufficiently polar to ionize by ESI (calculated formula: $C_9H_{10}O$). If the metabolite had been ionic it would have been expected to show recovery that was pH-dependent.

4. Conclusions

Our objective was to evaluate an LC/MS method for the dependency of extraction solvent pH on the number of erythrocyte metabolites detected by multiple ionization modes. We were able to accomplish this task by employing a reverse phase LC solvent system of water/methanol/0.2% acetic acid, followed by ESI and APCI detection in both (+) and (−) polarities. The RT drift was very low, using a single chromatographic system instead of one optimized for each ion source mode. This single LC system will facilitate the future incorporation of a RT to a RT/accurate mass library to permit the comparison of data for the same metabolite in different ionization modes. It will also greatly improve the confidence with which metabolites are tentatively identified. Our results indicate that a large number of additional compounds were detected with pH 2 and 9 extracted erythrocytes, particularly by ESI, and that a significant subset of them would not have been detected at neutral pH or by ESI alone. While for this paper we analyzed the different pH extraction conditions separately, it may be possible to combine the extracts to reduce the number of analytical runs needed for full metabolome coverage.

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