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Maximizing Metabolite Extraction for Comprehensive Metabolomics Studies of Erythrocytes

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Abstract

Metabolomics is the comparative analysis of metabolites found in sets of similar biological samples. Since metabolites play vital roles in biological systems, metabolomics can be useful for finding and identifying biomarkers, or for obtaining a better understanding of the effects of drugs or diseases on both known and unexpected biological pathways.

Successful metabolomic research requires effective metabolite extraction. For non-targeted metabolomics, extraction methods need to capture a broad range of cellular and biofluid metabolites, while excluding components such as proteins that are not intended for analysis. Extraction is made more challenging by the physico-chemical diversity of metabolites and by metabolite abundances that can vary by many orders of magnitude.

Biphasic, liquid-liquid extraction is often used to extract metabolites. The nature of the organic and aqueous solvents, their volumes, solvent ratios, and aqueous solvent pH, however, must be considered carefully. They can significantly affect the total number of metabolites extracted and experimental reproducibility.

This application note describes a method for liquid-liquid extraction of metabolites from erythrocytes. It demonstrates the importance of adjusting the aqueous/organic ratio to favor biphasic separation. It also demonstrates the effect of aqueous-phase pH on the number of metabolites extracted and shows that to obtain as many metabolites as possible, extractions need to be performed at multiple pHs.



Background

Extraction is the process by which compounds, such as metabolites, are selectively separated from other, often undesired, compounds. One of the most common extraction methods is liquid-liquid extraction, which takes advantage of differential solvent solubility and solvent immiscibility. Compounds are transferred from one liquid phase to another liquid phase by adding to the original solution an immiscible solvent in which the compounds are more soluble.

Polar, aqueous solutions are often paired with non-polar organic solvents such as chloroform—which is volatile, non-reactive, immiscible with and denser than water—to form a two-phase system for liquid-liquid extraction (Figure 1). This permits the separation of polar and non-polar metabolites for subsequent chromatographic separation and analysis. The “Folch”¹ method and its variant, the “Bligh & Dyer”² method, have traditionally been used in the extraction of lipids from tissues and in subsequent separation from more polar metabolites.

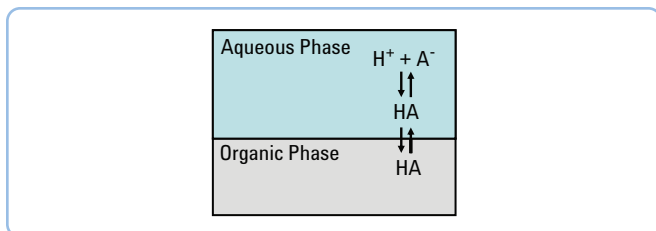


Figure 1. If a compound is added to a solution of two immiscible liquids, it will distribute between the two layers according to its relative solubility in the two liquids.

Alcohols such as methanol, ethanol, and isopropanol are miscible with water and may be used as aqueous phase co-solvents to enhance the solubility of less-polar metabolites during the extraction process.

Liquid-liquid extraction of metabolites is typically performed at neutral pH. However, by taking advantage of the differences in metabolite acid-base chemistry and varying the pH of the aqueous extraction solvent, extraction of metabolite classes from either complex biofluids or cells can be significantly improved.

For example, most compounds that contain acid functional groups (e.g., carboxylic acids) are insoluble or only slightly soluble in water. Addition of a dilute base such as 2% ammonium hydroxide (NH_4OH , $\text{pH} = 9$) results in a carboxylate anion with higher aqueous solubility (Figure 2). By adding an alkaline solution of dilute ammonium hydroxide/methanol and chloroform to cells, any organic acids that would otherwise be dissolved in chloroform at neutral pH, can instead be extracted into the basic polar phase.

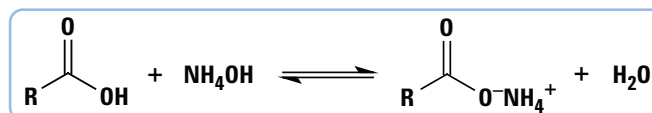


Figure 2. The addition of a dilute base such as 2% ammonium hydroxide (NH_4OH , $\text{pH} = 9$) to a protonated carboxylic acid results in a carboxylate anion with higher aqueous solubility. R- can be any group of atoms attached to the functional group COOH .

Similarly, in the presence of a weak acid, compounds containing basic groups form a soluble salt and become more water soluble (Figure 3). Adding a dilute acid such as dilute formic acid (HCOOH) to the aqueous phase can improve extraction of compounds with organic base groups. Formic acid is well suited to this application because it is miscible with water and with most polar organic solvents that might be used as aqueous phase co-solvents.

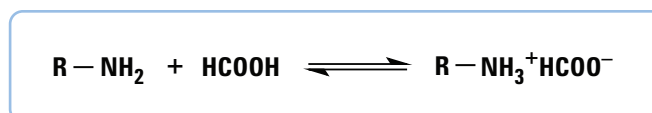


Figure 3. The addition of 1% formic acid (HCOOH , $\text{pH} = 2$) to a protonated amine results in a carboxylate anion with higher aqueous solubility. R- can be any group of atoms attached to the NH_2 functional group.

Formic acid and ammonium hydroxide are good choices for adjusting the pH of the aqueous phase because they are volatile and compatible with downstream LC/MS applications.

Experimental

The first experiment was designed to determine the volumes and ratio of aqueous and organic solvents that would favor biphasic separation and meet the other requirements for liquid-liquid extraction of metabolites from erythrocytes. The second experiment involved testing the aqueous/organic ratio determined in the first experiment on actual erythrocyte samples to verify its effectiveness and practicality. The third experiment involved performing actual extraction of metabolites from erythrocytes and determining via LC/MS analysis how variations in aqueous-phase pH would affect the number and class of metabolites extracted.

Optimizing phase separation

The first experiment was performed to find the ratio of aqueous solvent to organic solvent that would result in optimum phase separation. Equal volumes (0.75 mL) of 80:20 methanol/water were placed in six 1.7-mL capacity microcentrifuge tubes. This methanol/water ratio was used because at lower ratios there is a risk of the solution freezing during the extraction process. Increasing volumes of chloroform, from 0.1 mL to 0.6 mL in 0.1 mL increments, were added to each tube (Figure 4).

Sudan I, a lipid-soluble diazo dye³ that is preferentially soluble in non-polar solvents and imparts a bright yellow/orange color to chloroform, was added to permit visualization of the chloroform phase and provide an indication of the degree of miscibility between the polar and non-polar phases. None of the tubes exhibited biphasic separation at this point. An additional 0.20 mL of water was added to each tube to drive phase separation and each tube was centrifuged to make the phase separation more distinct.

No obvious phase separation occurred at the lowest chloroform volumes (0.1 and 0.2 mL), even after centrifugation. For tubes that did show separation, the chloroform phase was recovered and measured. Chloroform volumes over 0.4 mL all had similar recovery rates. Due to partial miscibility between chloroform and methanol, some of the dye was still visible in the aqueous phase. Similarly, the volume of the chloroform

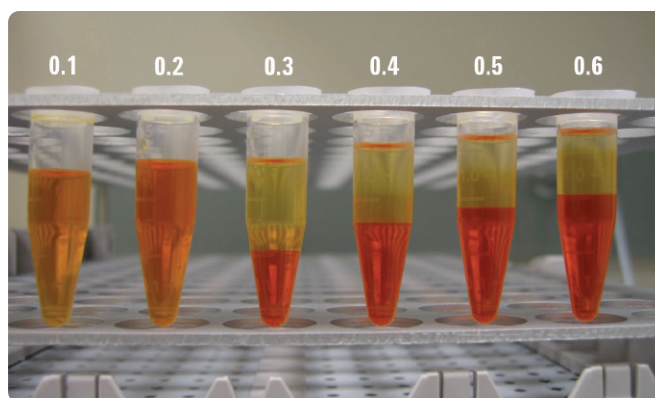


Figure 4. Increasing volumes of chloroform and 0.75 mL aliquots of 80:20 methanol/water were mixed to simulate the extraction step. An additional 0.20 mL of water was subsequently added to help drive phase separation. Sudan I, a lipid-soluble diazo dye was added to aid visualization of phase separation. Color banding within the aqueous (upper) half is due to rediffusion of chloroform into the aqueous phase.

phase was increased by about 10% due to methanol miscibility in that phase. Hence, some non-polar compounds would be expected to appear in the aqueous phase as well.

Test extraction

The next experiment applied the aqueous/organic ratio determined previously to the extraction of metabolites from erythrocytes. The experiment followed the workflow shown in Figure 5.

0.5 mL aliquots of human donor erythrocytes (Stanford University Blood Center) that had been pre-treated with sodium citrate anti-coagulant were placed in six 1.7 mL microcentrifuge tubes. They were centrifuged at 1000 g and 4 °C for 2 minutes, and then placed on ice while the supernatant was aspirated.

A wash cycle involving resuspension of the cells in phosphate buffered saline (PBS), centrifugation, and aspiration of the supernatant can be included at this point, although it was not in this experiment. The wash cycle removes non-erythrocyte metabolites and other compounds that may still be present outside the cells, but it also delays quenching and lysing, and may leave residual traces of phosphate salts.

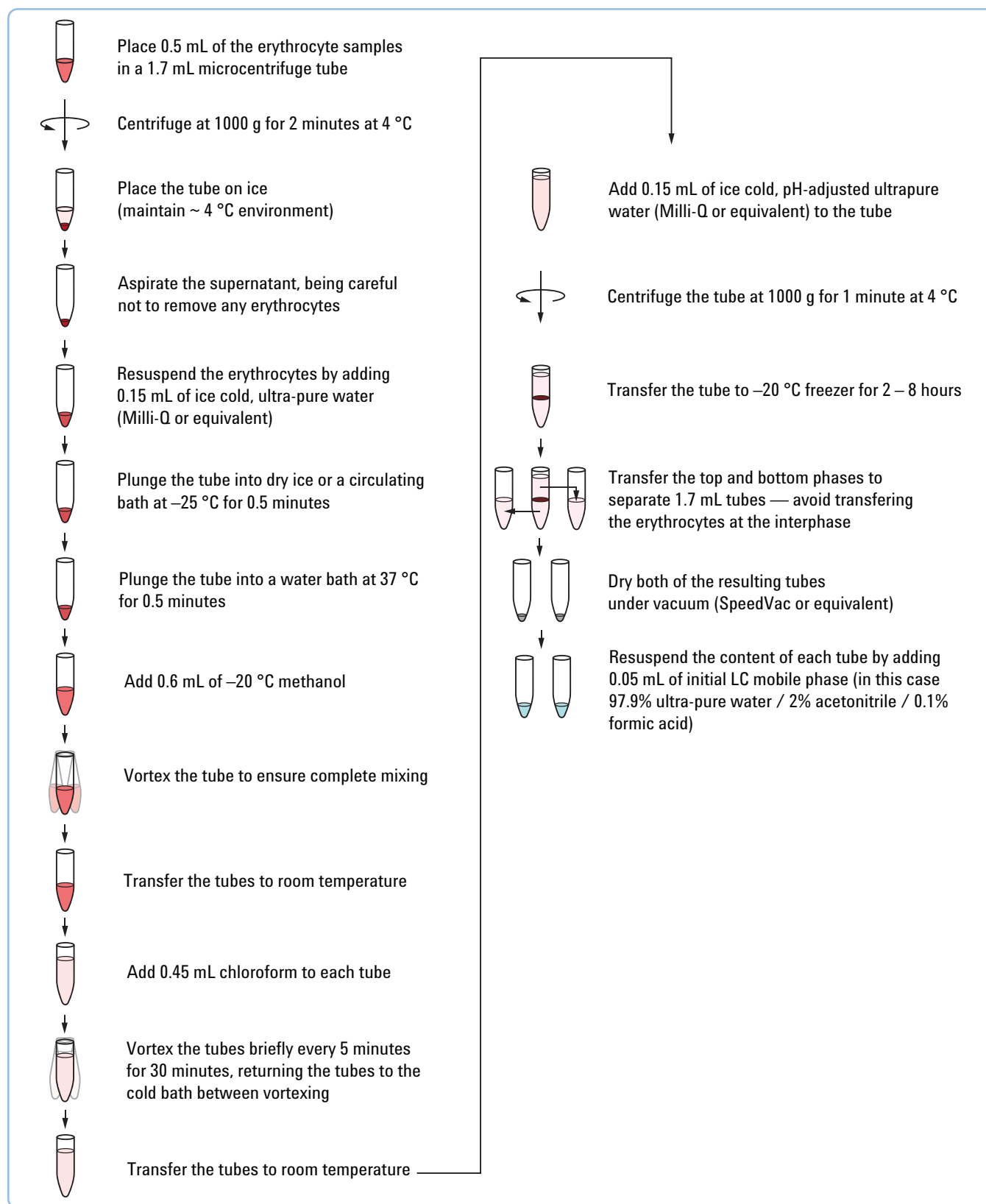


Figure 5. Workflow for liquid-liquid extraction of metabolites from erythrocytes using a single 1.7 mL microcentrifuge tube.

0.15 mL of ice cold, ultrapure (Milli-Q) water was added to each tube to resuspend the erythrocytes. The tubes were plunged into a circulating bath at $-25\text{ }^{\circ}\text{C}$ for 0.5 minutes and then into a water bath at $37\text{ }^{\circ}\text{C}$ for 0.5 minutes to quench metabolism and lyse the cells.

0.6 mL of $-20\text{ }^{\circ}\text{C}$ methanol was added to each tube and the tubes were vortexed to ensure complete mixing. The tubes were transferred to a circulating bath at $-25\text{ }^{\circ}\text{C}$. Differing amounts of chloroform, from 0.35 mL to 0.60 mL in 0.05 mL increments, were added to each tube (Table 1). The tubes were vortexed briefly every 5 minutes for 30 minutes, returning the tubes to the cold bath between vortexings. The tubes were transferred to room temperature and 0.15 mL of ice cold, ultrapure water was added to each tube to drive the phase separation. 0.15 mL was used instead of the 0.2 mL specified in the first experiment, because the erythrocytes take up volume in the tubes. The tubes were centrifuged at 1000 g for 1 minute at $4\text{ }^{\circ}\text{C}$ so a clear separation of the two phases could be observed above and below the compact disk of erythrocytes. The tubes were transferred to a $-20\text{ }^{\circ}\text{C}$ freezer and kept there overnight to allow residual chloroform to precipitate out of the aqueous methanol phase.

Figure 6 shows the results of the metabolite extractions. Although the aqueous phase volumes are actually the same in all tubes, they appear to be different due to solvent miscibility of methanol with chloroform.

A few granules of Sudan I were added to each of the six tubes (Figure 7). This would not be done with samples that were to undergo actual LC/MS analysis, but for purposes of this experiment, the dye made it easier to assess the final

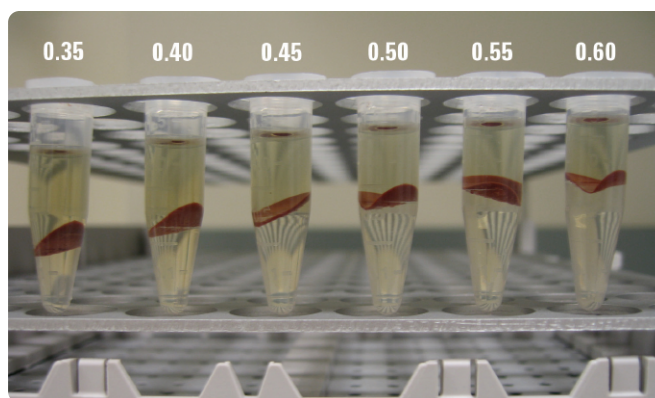


Figure 6. Extraction of erythrocytes using solvent volumes from Table 1. Although the same total aqueous volumes (top phase) were present in each tube, they appear to be different after mixing due to methanol and chloroform solvent miscibility.

chloroform phase volume and estimate how much chloroform remained in the aqueous phase due to chloroform/methanol miscibility.

The two liquid phases in each tube were transferred to separate 1.7 mL microcentrifuge tubes. Care was taken not to disturb the disk of erythrocytes or transfer any erythrocytes to the new tubes. Based on the original volume of chloroform recovered, the most complete phase separation occurred in tube #3 (see Table 1), corresponding to 0.45 mL of chloroform. Therefore, the solvent volumes of tube #3 were used in subsequent experiments. These volumes translated to a final methanol/water/chloroform ratio of 4:2:3 for extraction and phase separation. At this ratio, miscibility between the aqueous and organic phases is minimal, resulting in good phase separation for subsequent analyses.

Table 1. Amounts of aqueous and organic components added to each tube.

| Tube | Water added for cell lysis (mL) | Methanol added (mL) | Chloroform added (mL) | Water added for phase separation (mL) | Total volume (mL) |
|------|---------------------------------------|------------------------|--------------------------|---|----------------------|
| 1 | 0.15 | 0.60 | 0.35 | 0.15 | 1.25 |
| 2 | 0.15 | 0.60 | 0.40 | 0.15 | 1.30 |
| 3 | 0.15 | 0.60 | 0.45 | 0.15 | 1.35 |
| 4 | 0.15 | 0.60 | 0.50 | 0.15 | 1.40 |
| 5 | 0.15 | 0.60 | 0.55 | 0.15 | 1.45 |
| 6 | 0.15 | 0.60 | 0.60 | 0.15 | 1.50 |

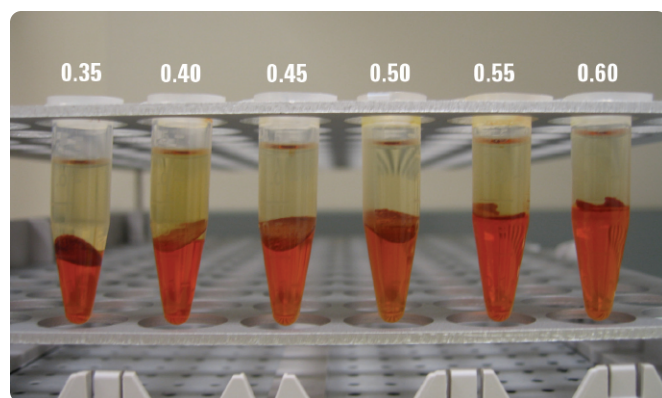


Figure 7. Sudan I was added to the tubes to help visualize the chloroform phase and estimate the extent of mixing with methanol/water.

The test samples in experiment 2 did not undergo further analysis by LC/MS, but for actual samples, at this point the two tubes would undergo drying under vacuum. The contents would then be resuspended in the initial LC mobile phase.

Extraction at multiple aqueous-phase pHs

The third experiment was performed to determine the extent to which aqueous-phase pH affects extraction of non-targeted metabolites, and to determine if performing extractions at multiple pH levels could improve metabolite coverage.

Three 50 mL Erlenmeyer flasks were filled with ultrapure (Milli-Q) water. One of the flasks was acidified with concentrated formic acid to achieve a final 1% formic acid concentration at pH 2. Similarly, in the second flask, a 30% concentrated solution of ammonium hydroxide was diluted with ultrapure water to a final concentration of 2% ammonium hydroxide, yielding pH 9. The third flask was left at an uncontrolled neutral pH 7.

Three erythrocyte samples were prepared using the workflow outlined in Figure 5. The solvent volumes and ratios previously determined to provide optimum separation were used. During initial resuspension (step 4), one sample was resuspended with 0.15 mL of the water and formic acid at pH 2. One sample was resuspended with 0.15 mL of ultrapure water at neutral pH. One sample was resuspended with 0.15 mL of water and ammonium hydroxide at pH 9.

At the end of the extraction and phase separation process, the aqueous phase of each sample was dried under vacuum and then reconstituted in the initial LC mobile phase for analysis.

Data acquisition and analysis

All three samples were analyzed using an LC/MS system consisting of an Agilent 1100 Series liquid chromatograph coupled to an Agilent 6210 Time-of-Flight LC/MS equipped with an electrospray ion source. The TOF system used external reference mass correction with ions at m/z 121.050873 and m/z 922.009798 to ensure the best possible mass accuracy.

The molecular feature extraction (MFE) algorithm in the Agilent MassHunter Workstation software was used to find the molecular features—unidentified, untargeted compounds—in each of the three data files. The MFE algorithm looks for mass signals (ions) that are co-variant in time, considers likely chemical relationships (isotopes, adducts, dimers, multiple charge states), and generates an extracted compound chromatogram and compound mass spectrum for each molecular feature. This approach finds compounds that are poorly resolved chromatographically and increases the total number of compounds found. The result was a list of features (compounds) in each sample, with related chromatograms and mass spectra for all features.

LC Conditions

| | |
|---------------------|---|
| Column: | Zorbax SB-Aq, 2.1 x 150 μ m, 3.5 μ m |
| Flow rate: | 0.4 mL/min |
| Column temperature: | 20 $^{\circ}$ C |
| Injection volume: | 2.0 μ L |
| Mobile phase: | A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile |
| Gradient: | 2% B at 0.0 min 100% B at 28.0 min Stop Run at 30.0 min |

MS Conditions

| | |
|----------------------|-----------------------|
| Ionization mode: | Positive electrospray |
| Drying gas flow: | 10 L/min |
| Drying gas temp.: | 250 $^{\circ}$ C |
| Nebulizer: | 40 psig |
| Vcap: | 4000 V |
| Max mass: | 1700 |
| Ref. mass flow rate: | 10 μ L/min |
| Scan range: | m/z 50–1000 |
| Acquisition rate: | 2 Hz |

To identify the solvent-extracted compounds in each sample, the three feature lists were queried against Agilent's METLIN Personal metabolite database. The METLIN database, compiled by the Center for Mass Spectrometry at the Scripps Research Institute, contains mass spectral data, chemical formulas, and structures for over 15,000 endogenous and exogenous metabolites, as well as di- and tri-peptides. Compounds that generated matches (mass agreement closer than 10 ppm) in the METLIN database, were imported into a Microsoft Excel spreadsheet.

Figure 8 shows a bar graph of the total number of compounds extracted at each pH. There was a significant difference in the number of extracted compounds, particularly between pH 7 and pH 9, which exhibited a two-fold increase in the number of compounds found. The raw numbers in Figure 8 reflect some redundancies, including instances where a feature matched multiple compounds with identical masses in the METLIN database. Each of the three compound (mass) lists was filtered separately in Microsoft Excel to remove such redundancies. The result was three compound lists in which a particular compound (mass) appeared only once per pH list. This enabled direct 1:1 comparison of compounds extracted at more than one pH.

Next, the three compound lists were merged to create a single non-redundant compound library. The library was imported into Agilent's GeneSpring MS data analysis software along with the three lists containing the compounds extracted at each pH. A Venn diagram (Figure 9) was constructed from the three lists and the library to reveal the number of compounds

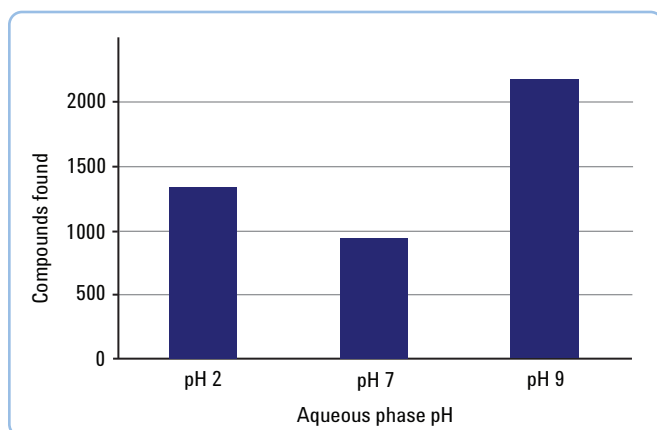


Figure 8. Total (raw) number of compounds detected by MassHunter at each pH.

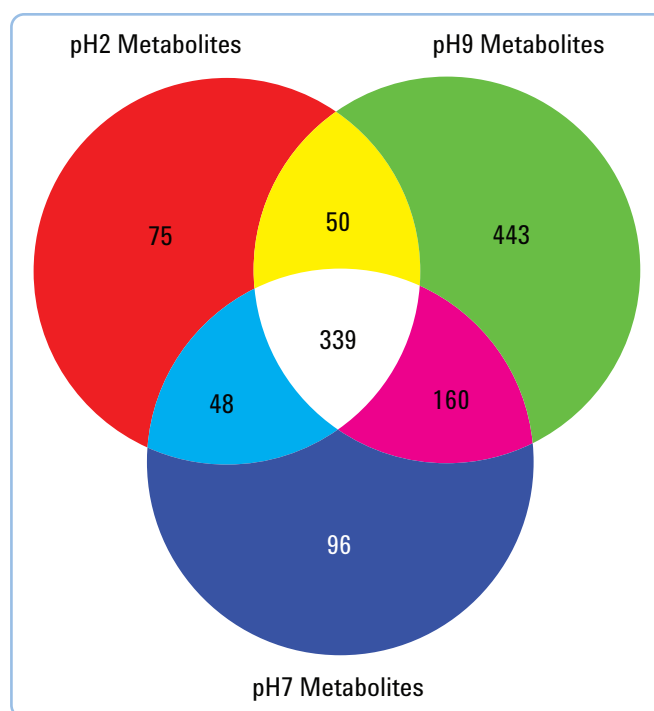


Figure 9. Venn diagram from GeneSpring MS shows the number of unique compounds (metabolites) that were extracted at one or more of the three pHs.

extracted at only a single pH, or those compounds that could be extracted at two or more of the three pHs.

From the total set of 1211 non-redundant compounds in the library, a significant number of compounds (339) were extracted at all three pHs, indicating that they are insensitive to changes in extraction pH. It is reasonable to expect that this set of compounds could be reproducibly extracted if extraction was performed at only a single pH, such as pH 7. However, over one third of the total extracted compounds, 443, were extracted at pH 9 only. This is the pH where one would expect to extract compounds with carboxylic acid groups. Many of the metabolites extracted at pH 9 were identified as fatty acids involved in arachidonic acid metabolism, leukotriene metabolism, and lipid synthesis. Although many compounds are stable at neutral pH and can be extracted at pH 7, these compounds would not have been found if extraction had occurred only at neutral pH, which is the common practice. A similar conclusion can be made for the 96 compounds that were extracted only at pH 7 and the 75 compounds extracted only at pH 2.

Conclusion

This work demonstrates a liquid-liquid extraction protocol in microcentrifuge tubes as a viable approach to the extraction of metabolites from erythrocytes. The correct ratio of aqueous and organic solvents is essential to achieving a good balance between quenching metabolism at a high organic solvent ratio and biphasic solvent partitioning after the addition of extra water. Biphasic partitioning allows polar and non-polar metabolites to be recovered separately. Finally, extraction of metabolites at multiple pHs (e.g. pH 2, 7, and 9) dramatically increases the number of unique metabolites found. For this particular sample, over 45% of the unique metabolites extracted would not have been recovered if the extraction had been performed only at a single, neutral pH—the common practice in the field today.

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