Towards a Universal LC/MS Method for Metabolomics

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Overview

Purpose: Demonstrate the challenges and progress towards creating a universal chromatography for metabolomics screening.

Methods: The use of an accurate mass instrument and new column technology were employed. In addition to these newer technologies, the use of several different modifiers and gradients were used.

Results: A significant progress has been accomplished in the quest of a universal metabolomics method. Here we present the retention of polar endogenous compounds like glutamate, as well as Gaussian peak shapes of problematic compounds, such as adenosine triphosphate (ATP), in one analytical method.

Introduction

Mass spectrometry lends itself to be an excellent tool for both metabolomics discovery and screening, due to the vast number of endogenous metabolites covering a wide range of analyte concentrations. The large number of compounds also presents a unique challenge for both the chromatography and mass spectrometry due to the wide range of physical chemical properties. More specifically, these challenges include limitations of modifiers that are compatible with mass spectrometry, a wide range of both lipophilic and hydrophilic compounds, and the lack of a universal ionization method. In typical LC/MS methods, a great number of compounds come out in the void volume (Figure 1), or have poor elution profiles like adenosine -5'- diphosphate (ADP), shown in Figure 2b. Here we present the method development of a universal chromatographic method for LC-MS analysis.

Methods

Sample Preparation

The LC/MS method was made using a list of nineteen compounds. These compounds represented many of the different classes and polarity for many of the different endogenous components in a metabolomics study.[1] Each sample was dissolved in mostly water. In the case of folic acid, ammonium hydroxide was added at 0.01% to help with solubility. The compounds were serial diluted into the appropriate solution depending on the method. The list of compounds are as follows: glutamic acid, glutamine, adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), cysteine, cystine, phenylaline, alanine, tyrosine, histidine, arginine, asparanine, pantothate, lysine, aspartic acid, glutathione, acetyl-coenzyme A and folic acid, as stated above.

Liquid Chromatography

The Thermo Scientific Accela 1250 pump and Accela Open Autosampler were used. The flow rate was consistently 400 µL/min for all method development. Varying concentrations of ammonium acetate, ETDA, formic acid and ammonium hydroxide were used, and the effects are discussed here.

HILIC methodology was employed on many different column types. The only two columns that were used that gave an elution profile of all nineteen compounds during method development included an amide column (100 x 2.1 mm, sub-2 micron) and the Thermo Scientific Syncronis HILIC column (250 x 2.1 mm, 5 micron).

Different solvent compositions were used, but acetone, acetonitrile, isopropanol, and water were the solvents used during this method development. All solvents were purchased from Thermo Fisher Scientific.

Mass Spectrometry

All data was collected on the Thermo Scientific Exactive benchtop Orbitrap™ mass spectrometer. Positive / negative mode switching was employed with resolution setting of 25,000 (FWHM at *m/z* 200). Generic source setting were used for a LC flow rate of 400 µL/min.

Results

FIGURE 1.

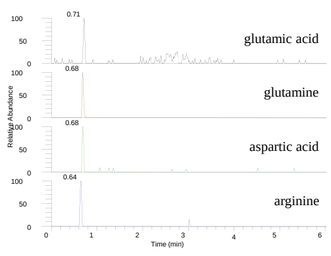


Figure 1 shows some of the very polar components that consistently elute near or in the void volume using reversed phase chromatography. The above data was obtained on a C-18 column running a 4 min. gradient starting at 0.5 minutes. The mobile phase was water and methanol with 0.1% formic acid with a flow rate of 350 µL/min.

The chromatograms in Figure 2 are data from the amide column using acetone and water with an ammonium acetate buffer gradient of 20 mM to 40 mM over four minutes. In Figure 2b, notice how ADP is present, and AMP has a narrow peak shape. The only difference between Figure 2a and 2b is the pH change from a 3.6 in Figure 2a and pH of 9 in 2b.

FIGURE 2a.

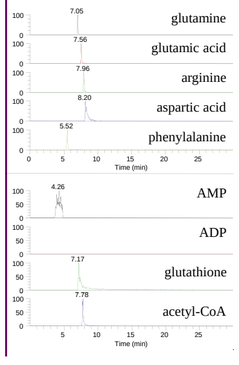


FIGURE 2b.

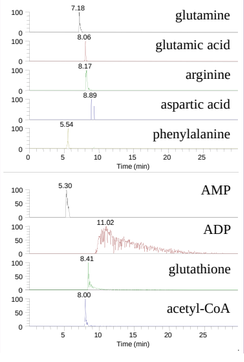
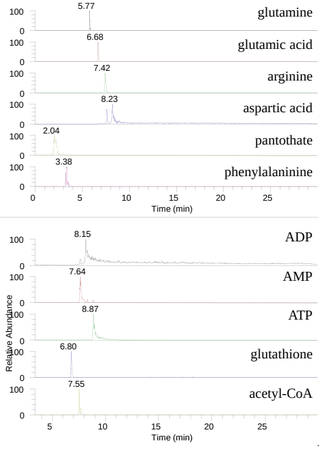


FIGURE 3.



The method in Figure 3 used water with 50 µM of EDTA in channel A, acetontirile in channel B and 400 mM ammounium acetate pH 9.8 in channel C. Channel C was ramped from 5% to 20% over 20 minutes as Channel A was ramped from 0 to 70% over 20 min. The mixture of components was dissolved in 20% water 20 mM ammonium acetate pH 10 and 80% acetonitrile.

The above chromatograms in Figure 3 were the most optimized methods achieved on the amide column. Here all components have Gaussian peak shape, or close to it, and are retained on the column beyond the void volume. A shallower gradient to give more chromatographic separation was tried, but peak shape was diminished (data not shown). Therefore, further improvements in this method would be enhancement in separation of components and improvement on the peak shapes of the ADP, ATP and aspartic acid.

Although this separation is compatible with mass spectrometry, there is an excess of buffer. Reducing the amount of the buffer would be beneficial in preventing ion suppression, as well as enhancing mass spectrometer cleanliness. An additional concern with the current method is that the concentration of ammonium acetate with acetonitrile is close to the solubility limited at the beginning of the gradient.

Figure 4 demonstrates data observed when using a Syncronis™ HILIC column, a new column technology recently released. The method uses three channels with water in channel A, isopropanol:acetonitrile (75:25) in channel B, and 100 mM ammonium acetate with an adjusted pH of 7.5 in channel C. The gradient below was employed.

No.	Time	A%	B%	C%	D%	µL/min
0	0.00	0.0	95.0	5.0	0.0	400.0
1	1.00	0.0	95.0	5.0	0.0	400.0
2	20.00	70.0	10.0	20.0	0.0	400.0
3	25.00	70.0	10.0	20.0	0.0	400.0
4	30.00	0.0	95.0	5.0	0.0	400.0
5	40.00	0.0	95.0	5.0	0.0	400.0

FIGURE 4a.

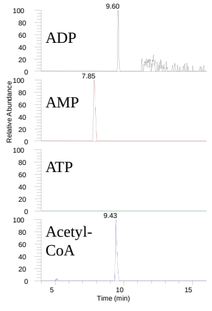
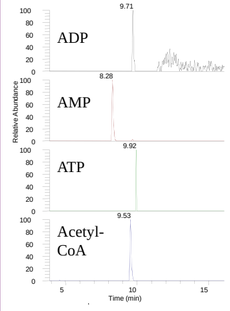


FIGURE 4b.



The difference between Figure 4a and Figure 4b is the pH of the samples. The sample in Figure 4a was brought up in 80% isopropanol and 20% water with 0.1% formic acid added. Whereas, Figure 4b had no modifier added to the sample. With the neutral pH of the sample, ATP was not only seen in the chromatograph, but yielded a peak that was narrow (6 sec. wide) and Gaussian. The 20% water is important for solubility of all the components, many of which are polar compounds (data not shown).

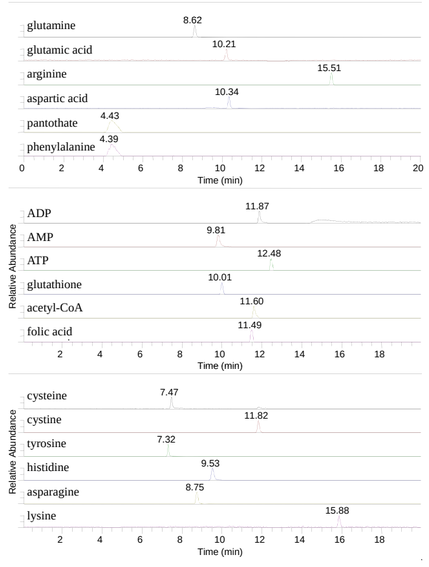
The method in Figure 4 worked well for the given components above, but for some of the amino acids, changing the pH to neutral appeared to have the opposite effects in chromatography. In the final method, the pH of the sample was basic by adding 0.05% ammonium hydroxide solution, as shown in Figure 5. In addition to the pH, some minor changes in gradient (shown below) are compared to the previous gradient shown, which was employed in Figure 3.

No.	Time	A%	B%	C%	D%	µL/min
0	0.00	4.0	95.0	1.0	0.0	400.0
1	1.00	4.0	95.0	1.0	0.0	400.0
2	11.00	28.0	65.0	7.0	0.0	400.0
3	20.00	50.0	10.0	40.0	0.0	400.0
4	25.00	50.0	10.0	40.0	0.0	400.0
5	27.00	89.0	10.0	1.0	0.0	400.0
6	30.00	4.0	95.0	1.0	0.0	400.0
7	40.00	4.0	95.0	1.0	0.0	400.0

The higher pH in the sample allowed all components to be chromatographed on one method with narrow peaks (average of 6 sec.) The shallower gradient method, shown in Figure 5, allowed for more separation of the components and used most of the gradient profile. These final changes to the method alleviated the concerns regarding the high concentration of ammonium acetate in the organic and used only mass spectrometer-friendly modifiers.

Figure 5 shows all components currently tested in this method. Further method development could attempt the removal of the buffer gradient, but all experiments up to this point suggest it is necessary for the elution of the phosphate compounds. Also, converting this to a two-channel system would simplify the method but might be problematic to deliver the appropriate buffer concentrations. Overall, the method is reproducible and yields separated, well-defined peaks for each component. Testing on biological samples still needs to be evaluated.

FIGURE 5.



Conclusion

- Method development of a "universal" method for metabolomics has to take into account a wide range of parameters which make the method development challenging.
- Here we present a LC/MS method that yields well separated endogenous components with a wide range of physical chemical properties.

References

1. Lu, Wenyun, et al. Metabolomic analysis via reversed-phase ion-pairing liquid chromatography coupled to a stand alone orbitrap mass spectrometer. *Analytical Chemistry*. 2010 Apr 15;82(8):3212-21.