Two pH Optimized LC-MS Methods for Metabolomics Analysis of Hydrophilic Compounds on a Silica Hydride Stationary Phase

ASMS 2010

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Introduction

The determination of hydrophilic metabolites is a crucial analytical problem for metabolomics scientists, where coverage needs to be both comprehensive and broad. Silica hydride columns have demonstrated good retention reproducibility, ruggedness and metabolite coverage. Classes of metabolites that can be separated in complex biological matrixes using the aqueous normal phase (ANP) technique on a silica hydride surface are sugars, amino acids (positive ionization mode) nucleotides and organic acids (negative ionization mode). The retention mechanism of ionizable compounds on the silica hydride column is highly pH dependent, whereas neutrals are not. Therefore, two general purpose ANP chromatographic methods were developed to achieve retention and separation of (1) neutral and acidic compounds by (-) ESI-MS, and (2) neutral and basic compounds by (+) ESI-MS.

Separation method for nucleotides was first developed using UV detection. When the method was transferred to LC-MS analysis, trouble with peak shape of triphosphate nucleotides was observed. It was discovered that trace sodium present in the solvents (leaching from the glass) was causing distorted peak shape.

Experimental

Standards: A mixture of amino acids, nucleotides and organic acids was prepared from individual components.

Samples: Urine samples were stored frozen at -20 ° C prior to analysis. Shortly before use, aliquots of the urine samples were thawed and then centrifuged at 13 000 g for 10 minutes at 4° C to pellet any cells. 0.5 mL of urine was subsequently filtered through a 10 kDa Biomax ultrafiltration membrane (Millipore, Milford, MA USA) to remove proteins. 10 uL of urine filtrate was diluted with 90 µL acetonitrile and formic acid was added at a final concentration of 0.1% prior to injection. Methods: In order to analyze a broad range of hydrophilic metabolites it was necessary to develop two different chromatographic methods. Since we discovered that ammonia alters the physio-chemical properties of the silica hydride surface, one Diamond Hydride (150mm×2.1mm) column was dedicated for each method. High acetonitrile to water gradients were used for both methods. (1) used a buffered ammonium acetate mobile phase, at neutral pH; with a pH gradient generated by the addition of increasing formic acid. (2) used a simple pH system with a constant amount of formic acid. The total analysis time for each method was approximately 20 minutes. HDPE bottles were used to minimize the presence of sodium ions that caused poor peak shape for some analytes.

Experimental

Materials: The silica hydride stationary phase used in this study was the Cogent Diamond Hydride (DH) material in 150 x 2.1 mm columns (MicroSolv Technology Corporation, Eatontown, NJ, USA). The phase contains a small amount of an organic moiety (~2% carbon as reported by the manufacturer) on a silica hydride surface.

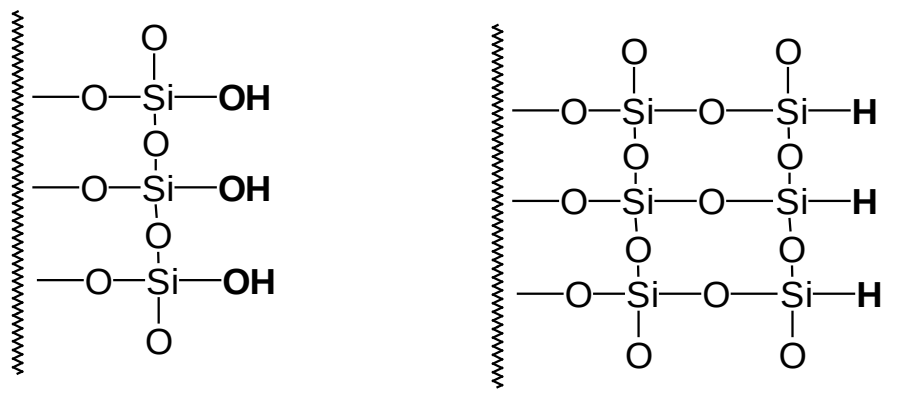
Instrumentation: An Agilent 1200 SL HPLC system with binary pump and degasser, well plate autosampler with thermostat, thermostatted column compartment, and an Agilent 6220 and 6224 Accurate-Mass TOF mass spectrometers with dual ESI source operated in the positive and negative ion mode were used. Dynamic mass axis calibration was achieved by continuous infusion of a reference mass solution using an isocratic pump connected to a dual sprayer electrospray ionization source.

Results and Discussion

Separation– Aqueous Normal Phase (ANP) is an excellent choice for separation of Hydrophilic metabolites on silica hydride column in Metabolomics analyses, because:

- *No derivatization required*
- *Compatible with LC/MS*
- *Rapid re-equilibration*
- *Reproducible chromatography*

ANP chromatography is done on Type C silica



Conversion of Type B Silica to Type C Silica

Type C material has been created that removes about 95% of silanols by replacing them with nonpolar Si-H groups. The fundamental difference between the two materials (Type B and Type C) is that silanol groups (Si-OH) are present on the surface of ordinary silica while Si-H moieties dominate the surface of hydride silica. This chemical change leads to profound differences in the surface properties of the two materials. Silica hydride has unique properties that can be exploited as a stationary phase for ANP - HPLC.

Results and Discussion

ANP Chromatography: Effect Of Trace Sodium Leaching From Glass Solvent Bottles

Several nucleotides were analyzed using Method 1 (Figure 1). After one day the peaks for ADP and ATP deteriorated. Fresh samples were prepared, The column was cleaned but the peaks still looked as in Figure 2. Next the glass bottles were changed to plastic bottles to eliminate traces of sodium leaching from the glass. The analysis was repeated with the same sample, column and fresh solvents. The results were identical to results presented in Figure 1. Note that traces of sodium do not affect AMP significantly.

Figure 1.

Glass Bottles: Fresh Solvent

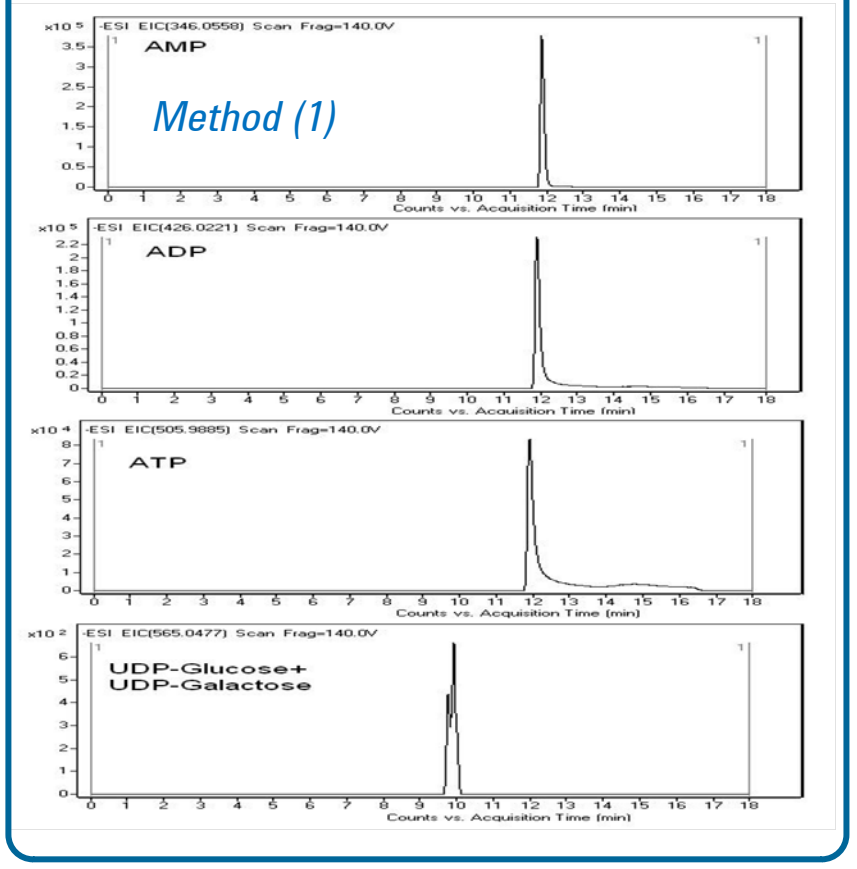


Figure 2.

Glass Bottles: Solvent Standing for One Day

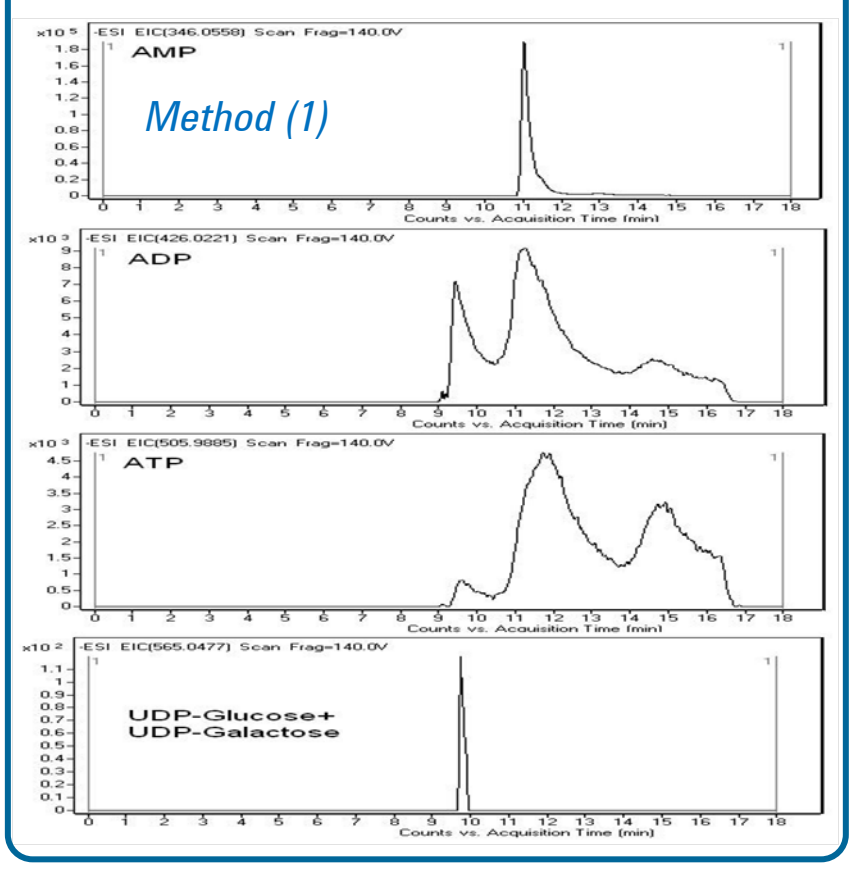


Figure 3.

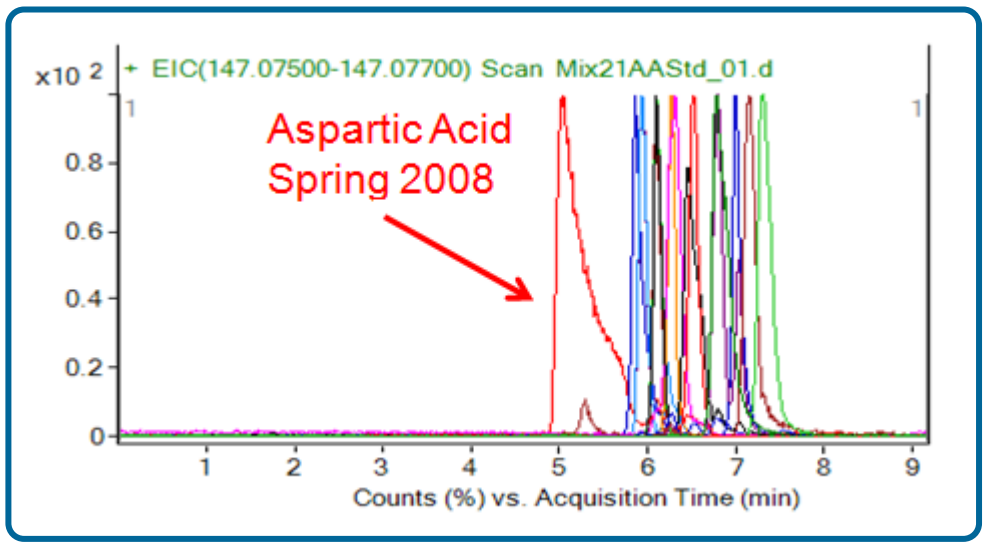
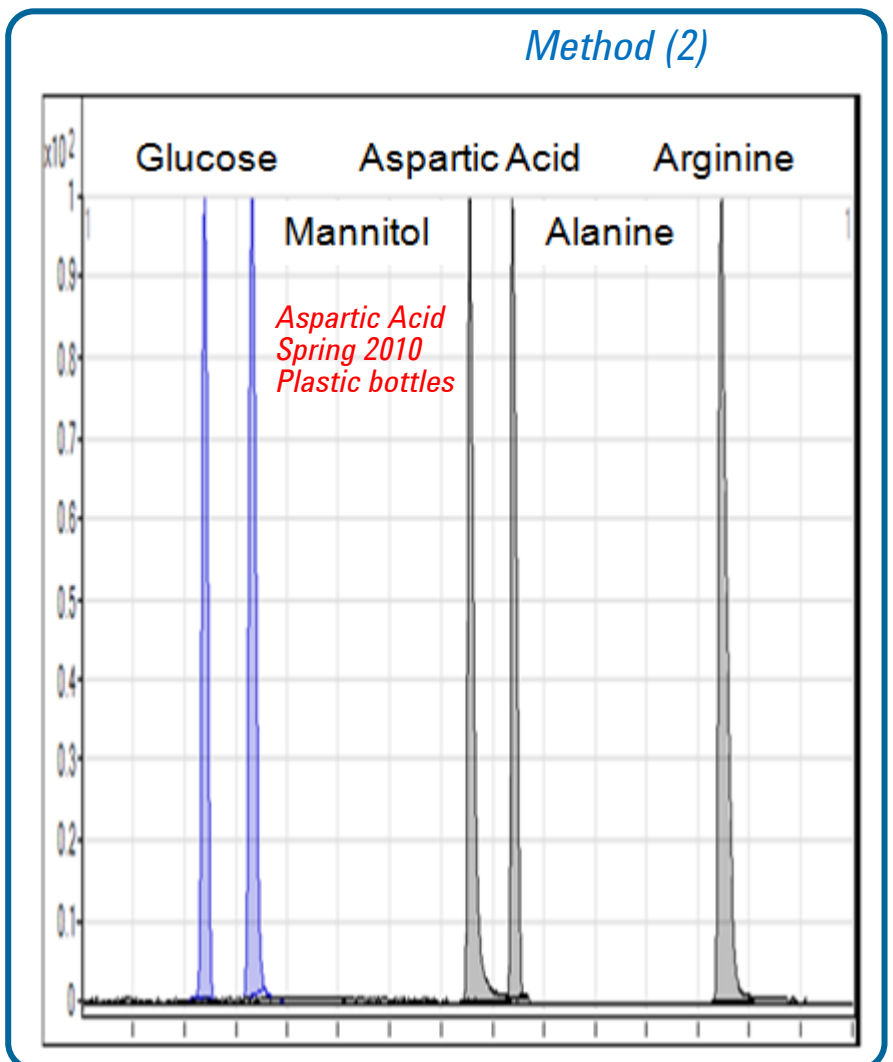


Figure 4.



Effect of trace sodium on neutral and basic compound separation Trace sodium effects were observed for aspartic acid and other di-carboxylic acids. The peak for aspartic acid was the most affected; it showed asymmetry and tailing (Figure 3). When the major sources of sodium were removed by switching to HDPE bottles, the peak for this compound was dramatically improved (Figure 4) and other affected compounds showed similar improvements.

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Results and Discussion

Chromatography for neutral and acidic compounds

Organic acids and nucleotide phosphates presented major development challenges for optimization. To achieve good retention of organic acids, the mobile phase must be in the 6-9 pH range (Figs. 5 and 6). Only volatile buffers , ex: ammonium acetate and ammonium formate that optimally buffer around pH 6 can be used. For tri-carboxylic acids such as citric acid, these conditions lead to poor peak shape. Development of a pH gradient was able to resolve this issue (Figure 5). Method 1 is universal for most neutral and acidic compounds, however small chromatographic adjustments have to be made to resolve isomeric compounds (Figure 6).

Figure 5.

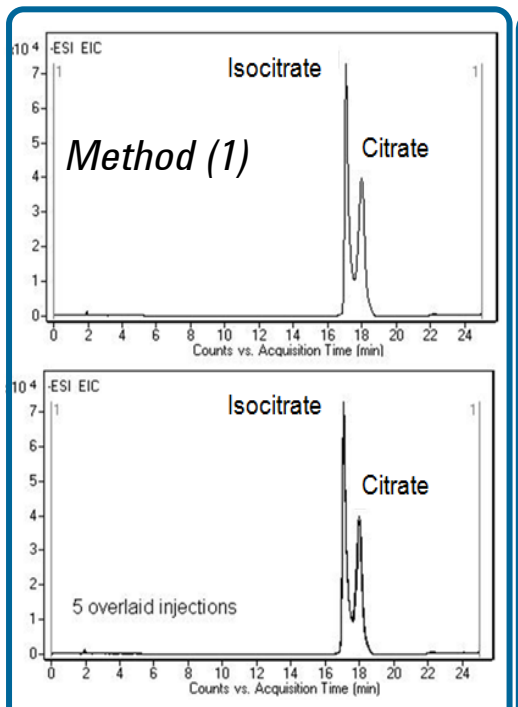
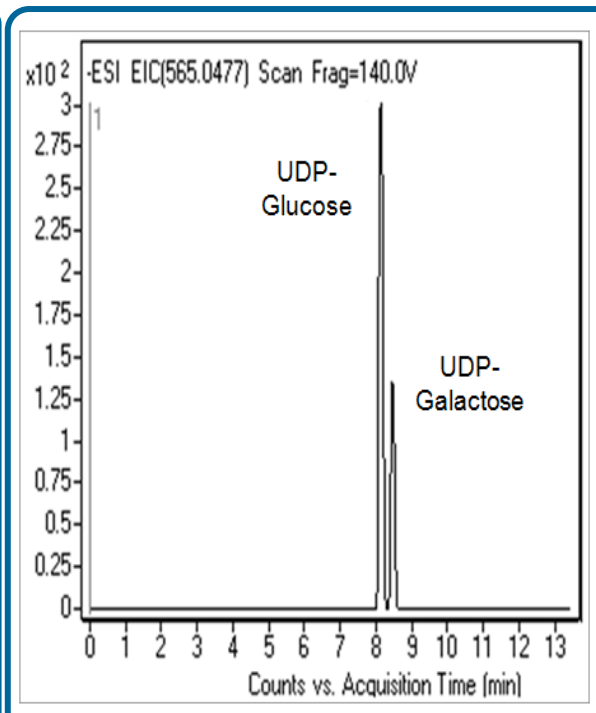


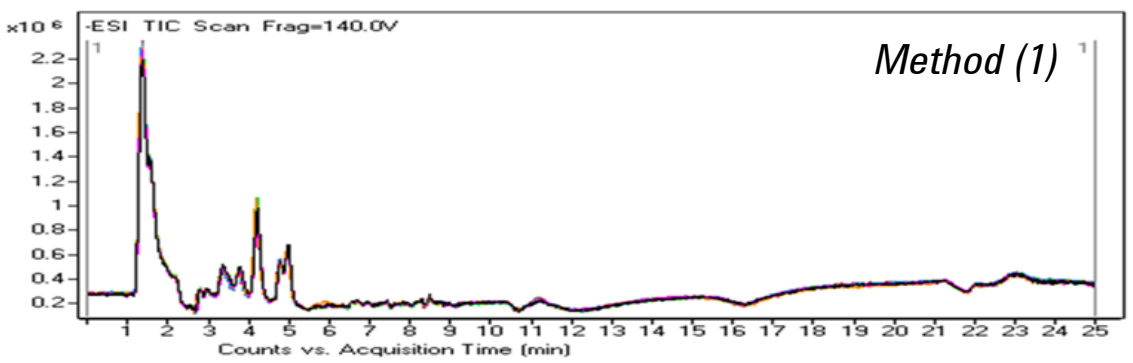
Figure 6.



Column:
MicroSolv Technology Corp
Cogent Diamond Hydride
2.1 x 150 mm
Solvents:
Bottle A: Strong Solvent
50% methanol / 50% water / 0.05% formic acid
Bottle B: Weak Solvent
90% acetonitrile / 10% water +10 mM NH_4 Acetate
pH adjusted To = 7 with ammoniaFlow Rate:
0.4 ml/min Stop time:
20 min Post time: 5 min

Figure 7.

ANP Method 1:
Reproducible Results With Urine – 10 Technical Replicate Overlays



Column: MicroSolv Technology Corp Cogent Diamond Hydride 2.1 x 150 mm

Bottle A: Strong Solvent 50% methanol / 50% water / 0.05% formic acid

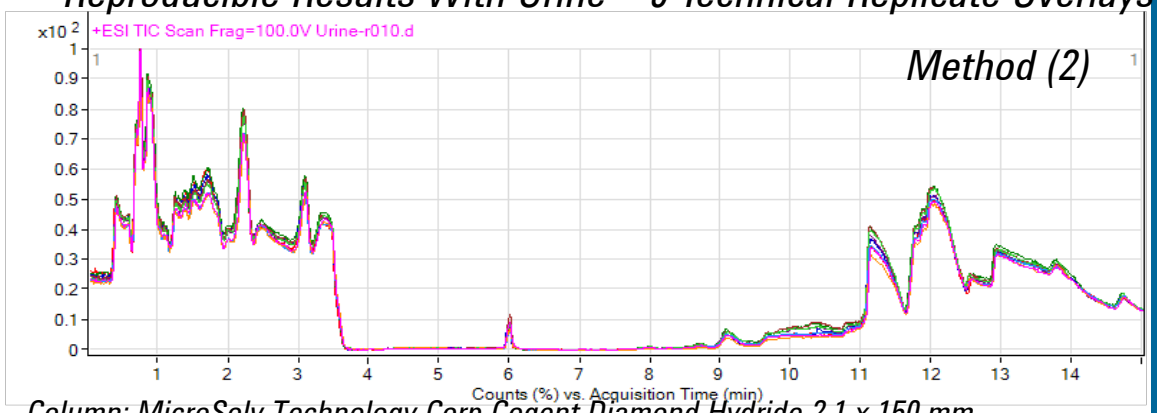
Bottle B: Weak Solvent 90% acetonitrile / 10% water 10 mM NH_4AcO

Gradient: (0, 100% B), (12, 50%B), (14, 50%B), (15, 0%B), (19, 0%B), (20, 100%B).

Flow Rate: 0.4 ml/min, Stop time: 20 min, Post time: 5 min

Figure 8

ANP Method 2:
Reproducible Results With Urine – 9 Technical Replicate Overlays



Column: MicroSolv Technology Corp Cogent Diamond Hydride 2.1 x 150 mm

Bottle A: Strong Solvent 50% methanol / 50% water / 0.10% formic acid

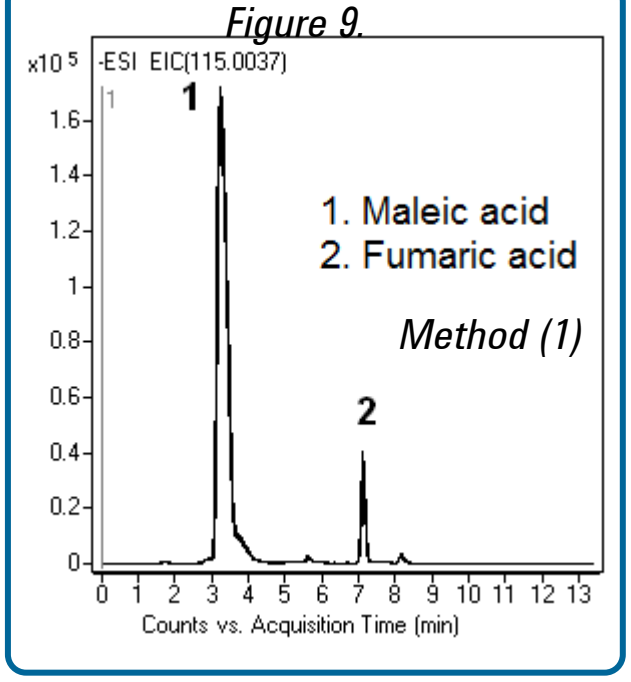
Bottle B: Weak Solvent 100% acetonitrile / with 0.10% formic acid

Gradient: (0, 95% B), (1, 95%B), (15, 20%B), Flow Rate: 0.6 ml/min, Stop time: 15 min, Post time: 5 min

Biological samples

The robustness of Methods 1 and 2 was tested in extracts of urine. Biological replicates were evaluated for consistency in the number of resolved compounds, retention time reproducibility, peak shape and the different classes of detected metabolites. Figures 7 and 8 show TIC's from 10 and 9 injections of urine samples. Figure 9 shows 10 overlaid injections (10 spiked urine samples) of the EICs at m/z 115.0037.

Figure 9.



Conclusions

1. *Two optimized LC/MS methods for comprehensive coverage of hydrophilic metabolites were developed to build an Accurate Mass and Retention Time database.*
2. *By switching from glass solvent bottles to HDPE trace sodium present in the solvents (leaching from the glass) was eliminated, which resulted in dramatic improvement of nucleotide (mainly ATP, GTP, ITP etc.) peak shape.*
3. *The methods developed are rugged and applicable to physiological samples such as urine.*