Mass Spectrometry Based metabolomics Defining Urinary Metabolome

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Abstract

The metabolites present in the urine are extensive and it is expected to have around 800 metabolites, until now around 300 metabolites have been identified. The use of high resolution LCMS to identify the repeatable features present in the urine samples is essential. The major composition of the urine is water and many compounds in it are highly polar. To obtain an optimum retention time and better identification of the metabolites an ideal column is required which can retain polar metabolites in the column for a considerable amount of time. To determine the best column for the analysis of the urine samples, two standard solutions were prepared for analysis by using high resolution Orbitrap mass spectrometry and these standard solutions were run on three different columns: ZICHILIC, ZICpHILIC and Cogent Diamond Hydride. The column which identified the most number of metabolites present in the standard solutions could be considered to be the ideal choice for the urine analysis. To determine the effect of storage conditions on the urine samples, the urine samples were frozen at -80^0C and thawed at room temperature, these cycles were repeated four times in one day and after each cycle the urine samples were analysed on LCMS. To determine the repeatable features of the urine, samples collected from various subjects, the urine samples were prepared for analysis on three different columns and each column depending upon their polarity retained and identified a variable number of repeatable features present in the urine samples. Software's including Mz Mine and Xcalibur were used to identify the metabolites which gave a good peak shape and by using Excel database the metabolites were putatively identified with the help of the high accuracy 'mz' values. PCA analysis was carried out by the using SIMCA P+ software and this procedure was used to determine the effect of freeze thaw on urine samples.

1. Introduction:

The main motto of our project is to better define a urinary metabolome and to find all the repeatable features present in the various urine samples. In a predictive screen of prostate cancer it was found that there were some differences between the patient and control samples. The metabolites present in the urine are extensive perhaps 800 metabolites, until now we are familiar with around 300 metabolites but would like to characterise the additional metabolites using MS/MS methods and standard hydrophilic interaction chromatography (HILIC) methods will be used in conjunction with high resolution mass spectrometry. There has been a lot of research going on all around the world to define the urinary metabolome. This research project will consist of running

the sample under three different experimental conditions.

1). Formic acid in combination with water and ACN with a ZICHILIC column

2). Ammonium carbonate in water and ACN with a ZICpHILIC column

3).Formic acid in combination with water and ACN with a Cogent Diamond Hydride column.

This project will involve a lot of data processing and it includes the confirmation of all repeatable features and uses them as signals for data analysis and to identify as many metabolites as possible. It also involves normalization methods and multivariate analysis.

Recently a high pH HILIC method has been added to the techniques used and also a retention time prediction algorithm has been developed. We will employ these techniques to better define the urinary metabolome.

INSTRUMENTATION:

1.7 Exactive Orbitrap LCMS:

The Exactive Orbitrap LCMS is a bench top full scan LCMS system designed for compound identification, high throughput and high performance screening. This is also used for quantitative and qualitative analysis. The Exactive gives high resolution accurate mass (HRAM) and provides fast, precise and reproducible results.

The availability of HRAM and full scan; all the data, all the time, allows retrospective data analysis for qualitative and quantitative applications. Moreover, it eliminates MS method development leading to improved and simplified analytical throughput in drug discovery laboratories.

Exactive is easy to use and it is cost effective to operate and coupled with work flow driven software.

The features of Exactive are specified below:

1) High resolving power up to (100,000) provides precise mass accuracy for complex sample analysis.
2) Mass accuracy of better than 2ppm in full scan using fully automated AGC and mass calibration procedures.
3) Scan speed operating at 10Hz scanning frequency Exactive is fully compatible with U-HPLC and ensures exact mass measurement for fast chromatography applications.
4) Fast polarity switching without sacrificing mass accuracy an any scan, a full scan of 1 positive and 1 negative scan performed within 1 second.
5) Intuitive software interface.

The Exactive Orbitrap is ideal for the following applications:

1) Environmental and food safety
2) Clinical.
3) Drug discovery metabolism and pharmacokinetics (DMPK).
4) Metabolomics.
5) Pharmaceutical.
6) The software used is thermo scientific Xcalibur processing and instrument control software.

This research project consists of running the sample under three different experimental conditions and for this

purpose three different columns are been used, they are:

1) ZICHILIC column.
2) ZICpHILIC column
3) Cogent Diamond Hydride column.

1.8 ZIC-HILIC COLUMN:

This column is ideal choice for the separation of polar compounds and hydrophilic compounds as it is completely antagonist to the RPLC columns. Analytes that usually have little or no retention and which may be affected by wettability problems in reverse phase liquid chromatography, generally have strong retention on ZIC-HILIC column. For example analytes such as amino acids, peptides, carbohydrates, plant extracts and various other polar compounds usually have little or no retention in RPLC.

The zwitter ionic ZIC-HILLIC stationary phase is attached to porous silica. The separation is achieved by a hydrophilic partitioning mechanism superimposed on weak electrostatic interactions. Typical eluents contain a high content of organic solvent (eg: Acetonitrile) mixed with buffer i.e. comparable to RPLC eluents, however, water being the strong solvent. Also, the low content of water in the eluents enables higher detection sensitivity in many LCMS applications.

1.9 ZICpHILIC column:

The ZICpHILIC Hydrophilic Interaction Liquid Chromatography column is the ideal choice for separation of polar and hydrophilic compounds as it is completely antagonist to the reversed phase chromatography (RPLC) columns. For example, compounds such as amino acids, peptides, carbohydrates, plant extracts and various other polar compounds that might have little or no retention in RPLC, generally have strong retention on ZICpHILIC column.

This column has zwitterionic stationary phase on porous polymer particles and the separation is achieved by hydrophilic interaction mechanism superimposed on weak electrostatic interactions.

Due to polymer support particles, the column can be operated in a broad pH range, which is used to enhance the selectivity and retention for many compounds. Also, the detection is sensitivity in LC-MS applications might benefit the use of eluents in alkaline pH range.

1.10 Cogent Diamond Hydride column:

The Cogent Diamond Hydride column is the best choice for metabolites, amino acids, carbohydrates and small organic acids. In Aqueous Normal Phase it can retain very polar compounds. This column is mostly used for metabolomics and also in environmental and food chemistry labs. It is completely free of surface silanols and this column can produce excellent peak shape and it has unique retention capabilities. The columns are highly reproducible and can be used in ANP, NPC and even reverse phase mode. The carbon load in the column is very low and hence the polarity is increased and this helps in the retention of more polar compounds.

2. Materials and Methods

2.1 Chemicals and Solvents

HPLC water was produced in the laboratory using MilliQ system (Millipore U.K.). Analar formic acid was

purchased from VWR International Limited Poole, U.K. Acetonitrile and methanol were purchased from Fisher Scientific Ltd. Leicestershire, U.K. Ammonium carbonate and analytical standards were obtained from Sigma-Aldrich, Dorset U.K.

2.2 HPLC COLUMNS

The specifications of the three columns used are shown in table 2:1

Table 2:1 HPLC columns used in the study:

Specifications	ZICHILIC column	ZICpHILIC column	Cogent Diamond Hydride column
Length of the column	15cm	15cm	15cm
Internal diameter	4.6mm	4.6mm	4.6mm
Particle size	5µm	5µm	4µm
Thickness of the silica layer	200Å	Polymeric beads	100Å

2.3 Standard Solutions:

Standard solutions were available from a previous study.

2.4 Sample Collection and Storage:

The research project consisted of the determination of the effects of storage conditions and mode of sample preparation of the metabolite profiles in urine samples. The number of samples which were prepared to determine the effect of storage conditions and mode of pre-treatment of the urine samples is summarised in table 2.2.

Table 2.2 Urine samples studied**:**

samples	Freshly prepared	Immediate freeze	Freeze and thaw	total
Subject A	1	1	4	6
Subject B	1	1	4	6
Subject C	1	1	4	6
Pooled sample	1	1	4	6
Total	4	4	16	24

2.5 Preparation of fresh urine samples :

200µl of freshly collected urine sample was transferred in to the 1ml Eppendorf tube with the help of a Gilson pipette. With the help of a Gilson pipette 800µl of acetonitrile was added to the 200µl of freshly collected urine sample. Then the Eppendorf tube was centrifuged at 8000 rpm for 8 minutes. After 8 minutes the protein and salts present in the sample were sedimented at the bottom and the clear supernatant layer was collected and transferred to HPLC vials and these are sent for LC-MS analysis on the ExactiveOrbitrap.

2.6 Preparation of immediate freeze urine samples:

The fresh urine samples were collected into 5 ml vials were immediately frozen at -80^{0}C. The samples were to frozen for one day and on the next day the sample was thawed at room temperature. Then 200µl of urine sample was transferred to the 1ml Eppendorf tube with the help of a Gilson pipette and then with the help of a Gilson pipette 800µl of acetonitrile was added to the 200µl of urine sample. The sample mixture was then centrifuged at 8000 rpm for 8 minutes. After 8 minutes the sedimentation of the protein and salts at the bottom of the Eppendorf tube could be found. The clear supernatant layer was separated and transferred to the HPLC vials which will be sent for LC-MS analysis on the Exactive Orbitrap.

2.7 Preparation of freeze and thaw urine sample:

10 ml of a fresh urine sample was collected in a vial. The sample was immediately frozen at -80^{0}C for 30 minutes and then thawed at room temperature. Then 200µl of urine sample is transferred to Eppendorf tube with the help of a Gilson pipette to this urine sample 800µl of acetonitrile is added with the help of a Gilson pipette. The sample was then processed as described above.

The urine sample was then refrozen at -80^{0}C for 30 minutes and then thawed again after thawing the sample was again prepared for LC-MS analysis as stated above. This procedure was repeated for 4 times and every time after the preparation of sample it was immediately sent for LC-MS analysis on the Exactive Orbitrap.

2.8 Determination of Metabolite coverage on three different columns:

Our research project also consists of the determination of the effect of different experimental conditions on the metabolite profile by running the sample on three different columns. The samples analysed are shown in the table 2.3

Table 2.3 Urine samples analysed in order to test the performance of different columns:

Samples	HILIC	pHILIC	Cogent Diamond Hydride	Total
Standard 1	1	1	1	3
Standard 2	1	1	1	3
Subject D	1	1	1	3
Subject A	1	1	1	3
Subject B	1	1	1	3
Total	5	5	5	15

2.9 Urine sample preparation to determine the effect of experimental conditions:

Freshly prepared urine samples are used to determine the effect of various experimental conditions. 200µl of fresh urine sample was transferred into 1ml of Eppendorf tube with the help of a Gilson pipette. Then 800µl of acetonitrile is transferred to Eppendorf tube with the help of Gilson pipette. The mixture was then treated as described in section 2.5.

2.10 HPLC conditions:

Various mobile phases are used for LCMS analysis on various columns. The mobile phases used were as follows

A 0.1% v/v Formic acid in combination in Water and Acetonitrile with a ZICHILIC column.

B 0.1% v/v Formic acid in Acetonitrile.

C 20mM of Ammonium Carbonate in water.

D Acetonitrile.

The HPLC flow rate used for LCMS analysis was 0.3ml/min.

The mobile phase gradient used with the ZICHILIC column and the Cogent Diamond Hydride column are shown in table 2.4

Table 2.4

Time	Solvent A %	Solvent B %
0	20	80
30 min	80	20
31 min	92 %	8%
36 min	92 %	8%
37 min	20%	80 %
46 min	20 %	80 %

The HPLC conditions used with the ZICpHILIC column are shown in table 2.5

Table 2.5

Time	Solvent C%	Solvent D%
0	20 %	80 %
30 min	80 %	20 %
31 min	92 %	8 %
36 min	92 %	8 %
37 min	20 %	80 %
46 min	20 %	80 %

2.11 Mass Spectrometry Conditions:

An ExactiveOrbitrap mass spectrometer (Thermo Electron Corporation) was operated in polarity switching mode and coupled to the HPLC by Electrospray ionisation (ESI). ESI spray voltage is 4KV in positive ionisation mode and 3,000 V in negative ionisation mode. Capillary temperature is maintained at 275^0C. Tube lens in 100V. Sheath gas is set at 55psi and auxiliary gas at 10psi. HESI temperature is set at 35^0C if available. The instrument was calibrated each day according to manufacturer's instructions and the lock masses used were:

For HILIC column:

Pos: 80.04948 83.06037 88.07569 195.08765 391.28428; Neg: 89.02442 91.00368

For pHILIC column:

Pos: 86.09642 88.07569 195.08765 391.28428; Neg: 89.02442 91.04006 96.9601 121.0295 122.02475 165.01933 166.01458.

For Cogent Diamond Hydride column:

Pos: 86.06037, 88.07569, 195.08765, 391.28428, ;Neg : 89.02442, 91.00368.

3.0 Results and Discussion

Urine samples were diluted with acetonitrile filtered and run on four different columns in order to assess metabolite coverage. Appendix 1 shows the metabolite lists obtained in positive and negative ion modes for each column. The metabolites can be putatively identified according to their elemental compositions since the Exactive

instrument routinely delivers a mass accuracy < 2ppm often < 1 ppm. To make a definitive assignment matching against standards is required; however, isomers do not occur for all compounds or are much less commonly found in biological systems. The data for each column is discussed below.

3.1 Cogent Diamond Hydride Column

Analysis of urine on the Cogent column gave 257 putatively identified metabolites in positive ion mode . Figure 3.1 shows extracted ion traces for some representative high and low abundance metabolites. The quaternary amine choline is most strongly retained although the relatively non-polar compound hypoxanthine also exhibits good retention. The level of the neurotransmitter dopamine is low however it is possible to see a peak for it on this column. Figure 3.2 shows extracted ion traces for metabolites of neurotransmitters. Dopamine is mainly present in urine as its sulphate conjugate and a clear peak for it can be seen. 269 metabolites putatively identified in urine using the Cogent column with negative mode ESI. The column also works quite well for acidic metabolites and figure 3.3 shows traces for some Krebs cycle acids, Succinate, Fumarate, Pyruvate and Ketoglutarate in negative ion mode. Acidic compounds appear to be less strongly retained than basic compounds. Neutral compounds are also retained and figure 3.4 shows extracted ion traces for some sugar molecules in negative ion mode which are retained quite strongly by this column.

Figure 3.1 Extracted ion traces of for Glycine, Choline, Hypoxanthine and Dopamine on the Cogent Diamond Hydride column in positive ion mode

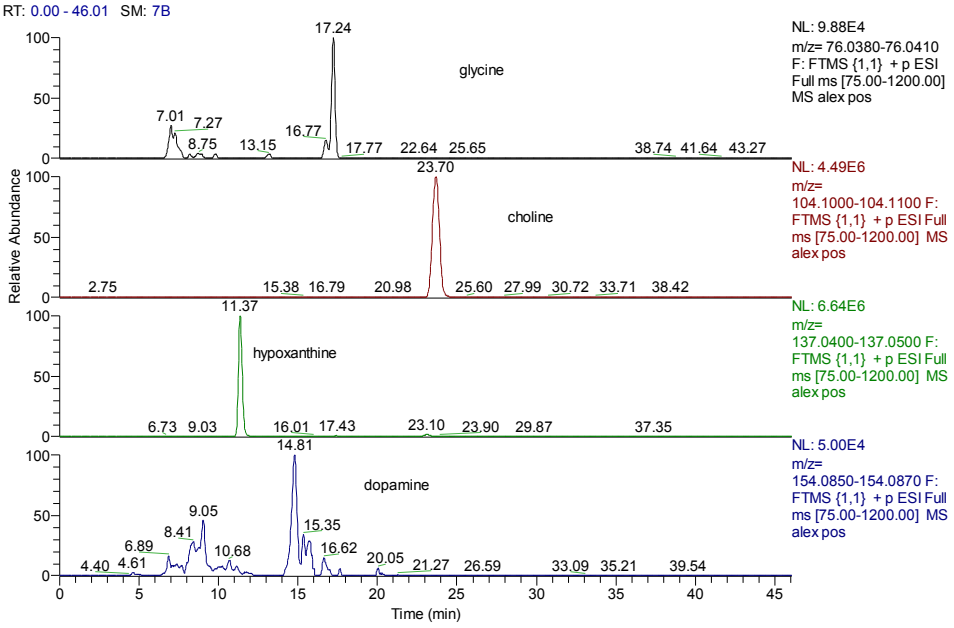


Figure 3.2 Extracted ion traces of neurotransmitter substances Dopamine Sulphate, Dopamine Glucuronide, Tyramine and Normetanephrine on the Cogent Diamond Hydride column in positive ion mode.

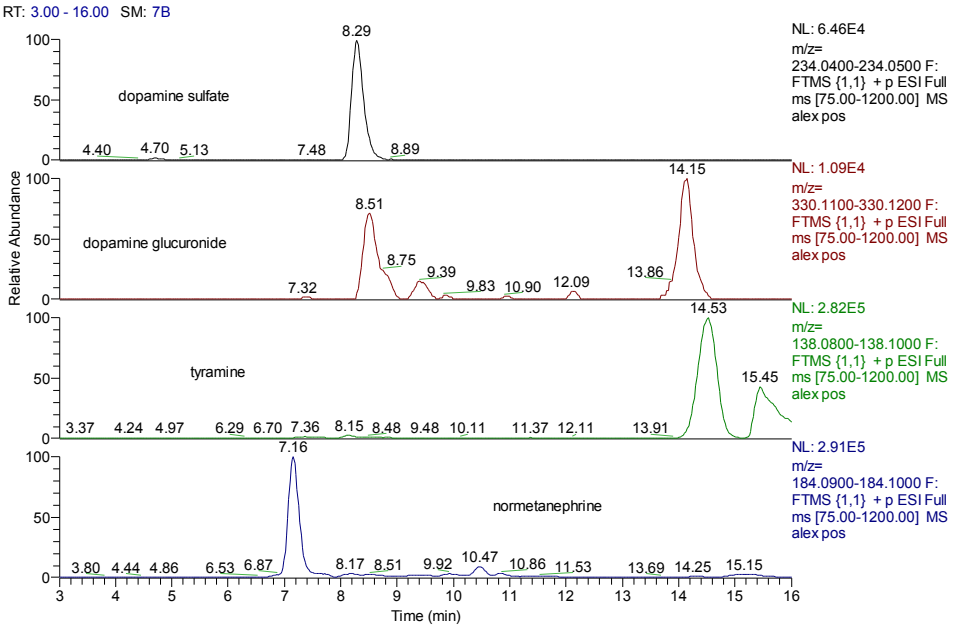


Figure 3.3 Extracted ion traces of acids Succinate, Pyruvate, Fumarate and Ketoglutarate on the Cogent Diamond Hydride column in negative ion mode.

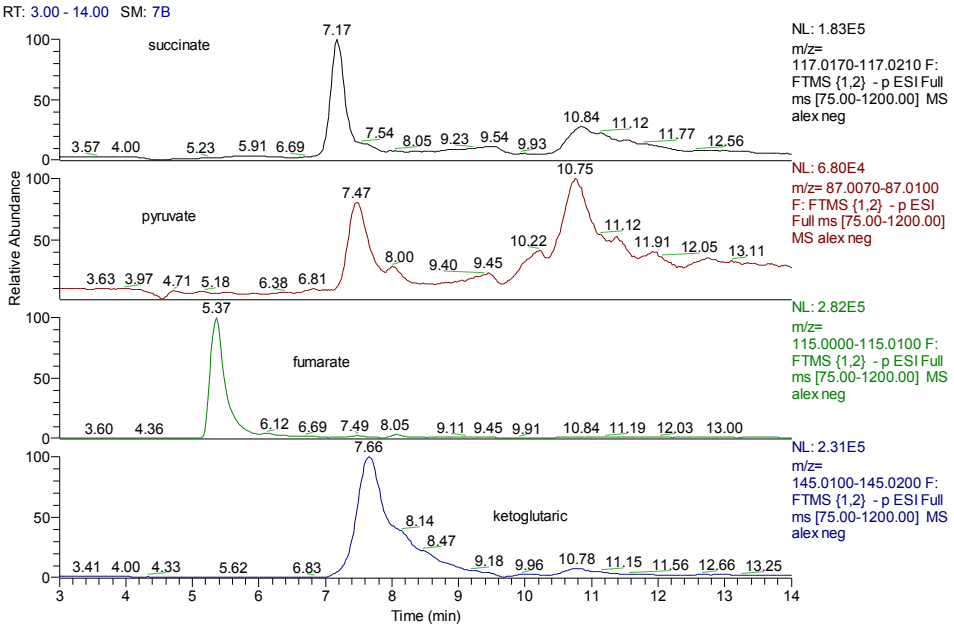
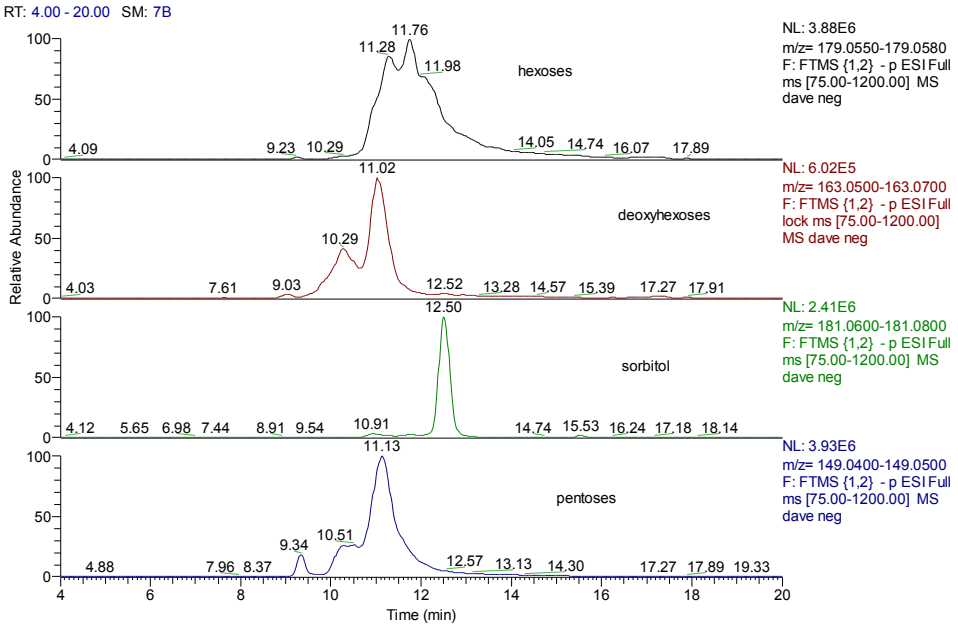


Figure 3.4 Extracted ion traces for sugar molecules, Hexoses, Deoxyhexoses, Sorbitol and Pentoses on the Cogent Diamond Hydride column in negative ion mode.



3.2 ZICpHILIC column:

There were 416 metabolites putatively identified by using the ZICpHILIC column with negative ESI mode detection. Figure 3.5 shows extracted ion traces for Succinic, Pyruvic, Fumaric and Ketoglutaric acids in urine separated on the ZICpHILIC column. The peak shapes for the acids are better than those obtained on the Cogent Diamond Hydride column. Figure 3.6 shows extracted ion traces for sugars on the ZICpHILIC column. The separation of the Hexoses on the column is better than that obtained on the Cogent column. In positive ion mode on the ZICpHILIC column 377 metabolites were putatively identified. The peak shapes were largely good. Some metabolites were not seen as well. For instance Dopamine was not seen under these conditions. Figure 3.7 shows extracted ion traces for Glycine, Choline, Hypoxanthine and Leucine. The column delivers good peak shapes for these analytes and they are detected even through the analysis is carried out at high pH. The conditions used seem to deliver more separation in terms of peak shape thus Leucine and Isoleucine are well separated on this column while they are incompletely separated on the ZICHILIC column as discussed below.

3.3 ZICHILIC column:

There were 353 compounds putatively identified in urine by using the ZICHILIC column in positive ion mode and 361 putatively identified in negative ion mode.

Figure 3.5 Extracted ion traces of acids Succinate, Pyruvate, Fumarate and Ketoglutarate on the ZICpHILIC column in negative ion mode.

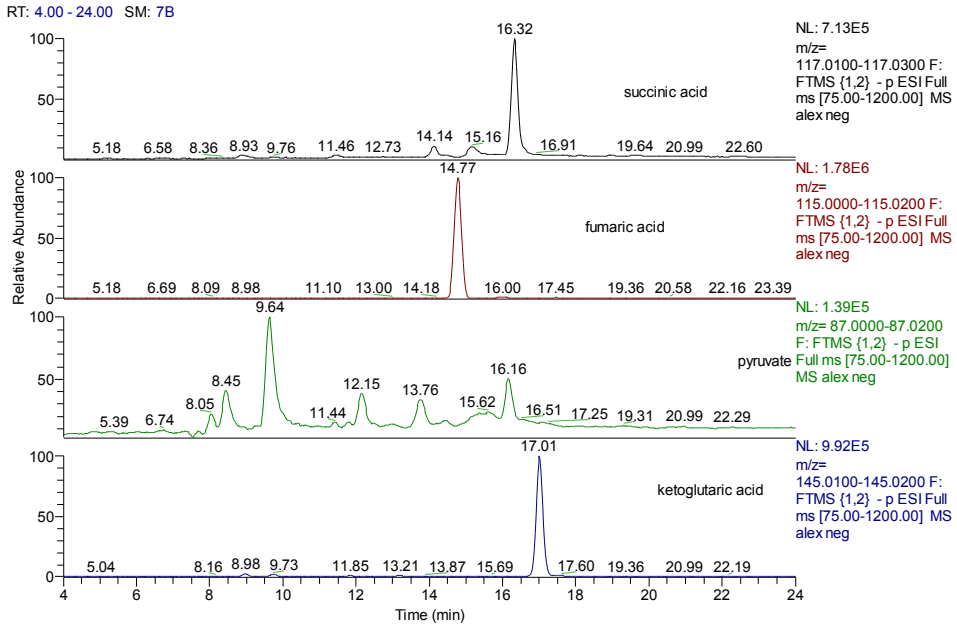


Figure 3.6 Extracted ion traces for sugar molecules, Hexoses, Deoxyhexoses, Sorbitol and Pentoses on the ZICpHILIC column in negative ion mode.

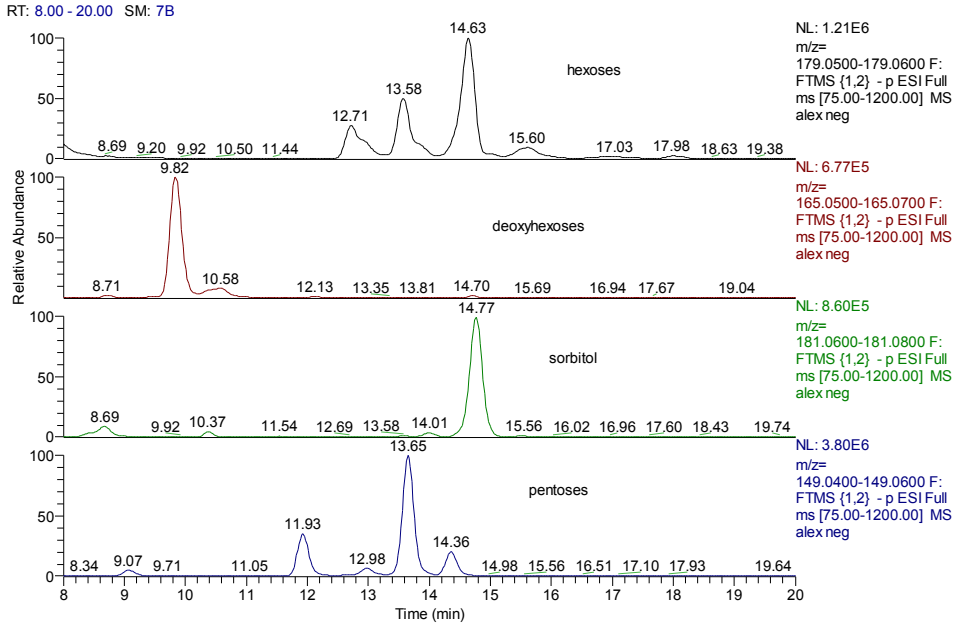


Figure 3.7 Extracted ion traces of Glycine, Choline, Hypoxanthine and Isoleucine/Leucine on the ZICpHILIC column in positive ion mode.

Figure 3.7

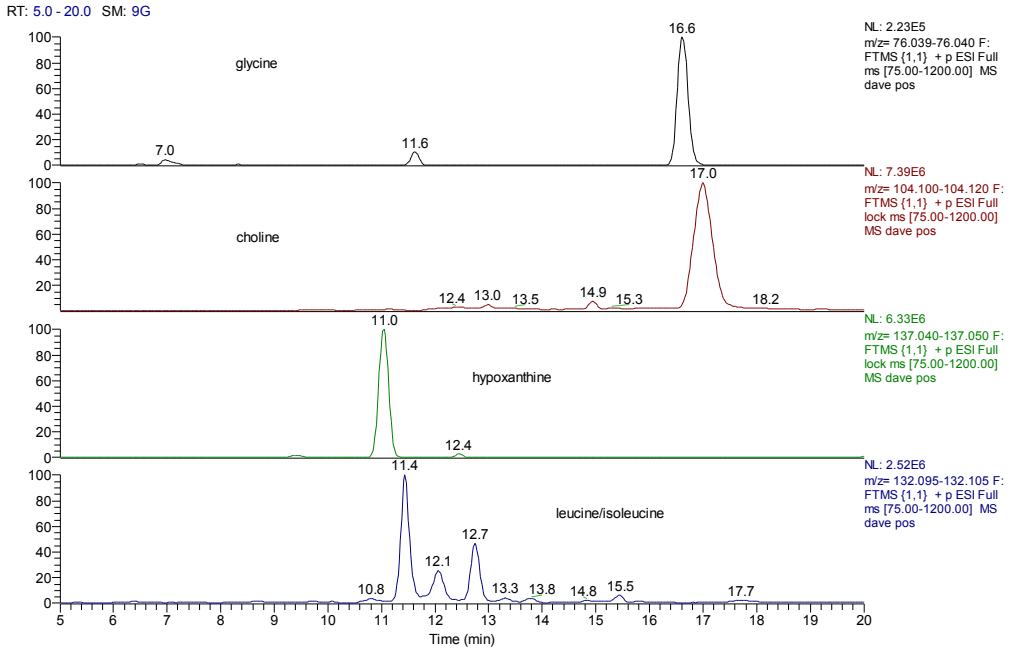


Figure 3.8 shows extracted ion traces for Glycine, Choline, Hypoxanthine and Leucine/Isoleucine on the ZICHILIC column. Leucine and Isoleucine were not resolved as has been observed on the ZICpHILIC column and generally retention times were longer than on the ZICpHILIC column probably due to the bases being more charged at low pH. Figure 3.9 shows extracted ion traces for Succinate, Pyruvate, Fumarate and Ketoglutarate on the ZICHILIC column. The peak shapes for some of the acids are not as good as on the ZICpHILIC column and the peaks are less well retained.

Figure 3.8 Extracted ion traces of Glycine, Choline, Hypoxanthine and Isoleucine/Leucine on the ZICHILIC column in positive ion mode.

Figure 3.8

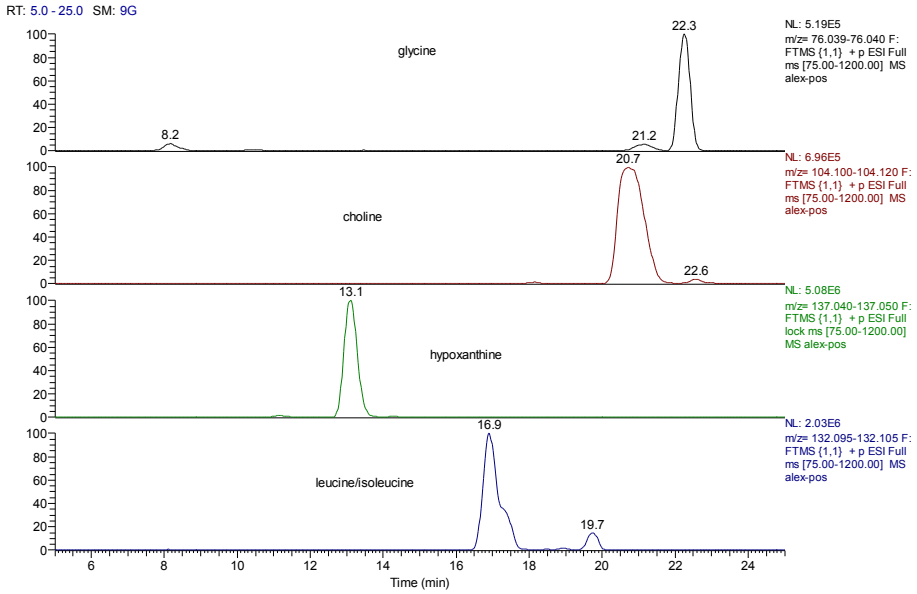
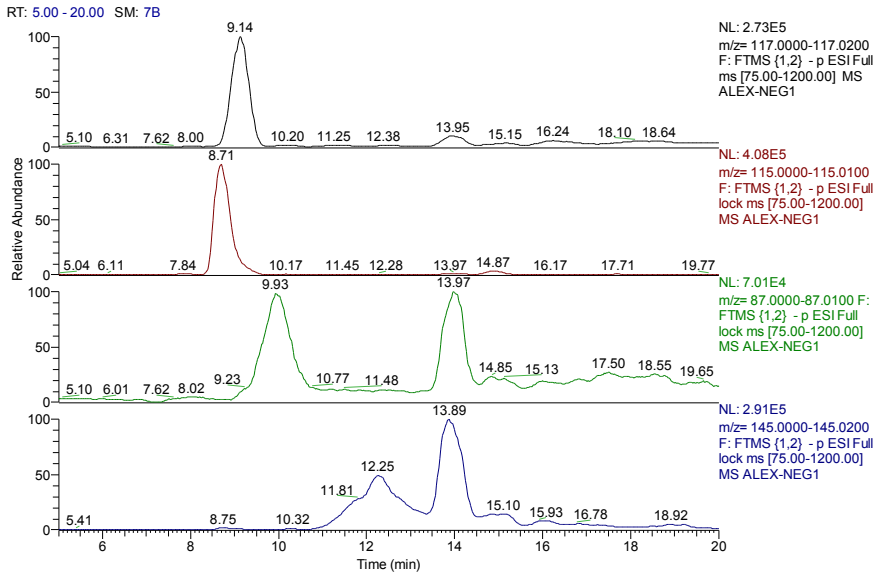


Figure 3.9 Extracted ion traces of Succinate, Fumarate, Pyruvate and Ketoglutarate on the ZICHILIC column in negative ion mode.

Figure 3.9



4.0 Conclusion:

The evaluation of three different columns (ZICHILIC, ZICpHILIC and Cogent Diamond Hydride column.) was carried out by using two different standard solutions and by the analysis of urine samples. The raw data obtained after the analysis of the standards are processed by the use of Xcalibur and mz-mine software's. The columns all produced similar number of putatively identified metabolites but there was evidence that columns were better for particular metabolites. The ZICpHILIC column looked to best overall since it produced good peak shapes and retention for acids and produced a wider separation of sugars such as the Hexoses. However, ZICpHILIC was less good from strongly basic compounds such as the amino acids Lysine and Histidine which worked well on ZICHILIC.

The effect of storage conditions on the urine samples was determined by using three urine samples which are collected from three different individuals and these urine samples are subjected to freeze and thaw at -80^{0}C and at room temperature. After each cycle the samples were run on ZICHILIC and ZICpHILIC columns in both positive and negative ionisation mode. The data obtained after the LCMS analysis was processed with the help of Xcalibur and mz-mine software's, to determine the real peaks of the metabolites. The freeze thaw samples were compared with those of the fresh samples with the help of Simca P+ software and by PCA analysis the differences between the fresh and freeze thaw samples are determined. There was evidence of an effect of freeze thaw on some of the metabolites in the samples.

References :

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