Evaluation of Coupling Reversed Phase (RP), Aqueous Normal Phase (ANP) and Hydrophilic Interaction (HILIC) Liquid Chromatography with Orbitrap Mass Spectrometry for Metabolomic Studies of Human Urine

*Tong Zhang[1], Darren J. Creek[2,3], Michael P. Barrett[2] Gavin Blackburn[1] and David G. Watson[1]

1. Strathclyde Institute of Pharmacy and Biomedical Sciences, 161 Cathedral Street, Glasgow G4 0NR, UK
2. Wellcome Trust Centre for Molecular Parasitology, Institute of Infection, Immunity and Inflammation,University of Glasgow, 120 University Place, Glasgow G12 8TA, UK
3. Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Victoria, 3010, Australia

*Corresponding author:

Tong Zhang,

SIPBS

161, Cathedral Street

Glasgow G4 0RE

Telephone : +441415482651

Fax: +441415526443

E mail: tong.zhang.101@strath.ac.uk

Abstract

In this study, we assessed three liquid chromatographic platforms: reversed phase (RP), aqueous normal phase (ANP) and hydrophilic interaction (HILIC) for the analysis of polar metabolite standard mixtures and for their coverage of urinary metabolites. The two zwitterionic HILIC columns showed high-quality chromatographic performance for metabolite standards, improved separation for isomers and the greatest coverage of polar metabolites in urine. In contrast, on the reversed phase column most metabolites eluted very rapidly with little or no separation. Using an Exactive Orbitrap mass spectrometer with a HILIC liquid chromatographic platform approximately 800 metabolites with repeatable peak areas (RSD ≤ 25%) could be putatively identified in human urine, by elemental composition assignment within a 3 ppm mass error. The ability of the methodology for the verification of non-molecular ions, which arise from adduct formation, and the possibility of distinguishing isomers could also be demonstrated. Careful examination of the raw data and the use of masses for predicted metabolites produced an extension of the metabolite list for human urine.

Introduction

Metabolomic studies of human urine have been receiving increased interest[1] and can be used to find biomarkers that diagnose disease or provide early warning at the pre-clinical stage. NMR based metabolomics offers highly repeatable and non-discriminatory outcomes, and in conjunction with pattern recognition methods provides an effective approach for diagnostics but cannot always identify individual biomarkers due to limited resolution between spectra of individual molecules and poor sensitivity.[2, 3] MS provides higher sensitivity, wider dynamic range and the possibility of distinguishing metabolites by their accurate m/z signals and fragmentation patterns. Chromatography can be easily coupled with MS to differentiate metabolites by their retention times (Rt) providing an additional dimension for identification. Thus LC-MS or GC-MS are more suitable for qualitative and quantitative measurement of individual metabolites [4-6] and have been widely used in metabolite profiling studies of human urine for biomarker discovery.[7-12]

Pasikanti *et al*[13] reported a method for GC-MS profiling of human urine where 150 putative metabolites were detected and 144 of them were assigned a name by using retention indices and mass spectral matching scores with the NIST library. However, GC-MS is limited to analysing non-polar and volatile compounds. In addition spectra resulting from electron impact ionization (EI) are fragment-rich which complicates deconvolution of overlapping chromatographic peaks.[14] LC-MS is attracting increasing interest [5] as it requires minimal sample preparation and offers diverse LC selectivities.

Reversed-phase (RP) chromatography has been commonly used to analyse human urine[15, 16] and it has been successfully used in human urine metabolomic studies for disease diagnostics and biomarker discovery.[7, 8, 12] Separation and retention of metabolites under these conditions are predominantly determined by hydrophobic interaction. However, human urine contains a large number of highly polar metabolites such as amino acids, organic acids, sulphate and glucuronide conjugates and sugars. The polar metabolites generally elute together close to the dead time (t_0) from columns under reversed-phase LC conditions and thus retention time makes no contribution to identification. Additionally ion-suppression is more likely occur for co-eluting polar metabolites present at trace levels. Hydrophilic interaction chromatography (HILIC) is able to separate polar compounds and the use of an organic solvent-rich mobile phase improves the sensitivity of ESI-MS.[17, 18] Under HILIC conditions the retention/separation is mainly based on the hydrophilic partitioning of metabolites between an organic solvent-rich mobile phase and an aqueous layer formed on the stationary phase. HILIC phases can be classified into: neutral, charged and zwitterionic. Selectivity can be improved for ionisable compounds on charged or zwitterionic HILIC stationary phases when electrostatic and/or ion-exchange interactions are introduced into their chromatographic behaviour.[19-20] Use of HILIC columns alone[9] or together with RP columns[10] has been reported in human urine metabolomics studies. Cubbon *et al*[21] measured human urine under both RP-LC-MS and HILIC-MS conditions and their multivariate analysis results showed that the correct classification of gender, diurnal variation and age could be obtained by using either method. Aqueous normal phase (ANP) chromatography is

another effective LC method for separating polar compounds. A silica hydride-based stationary phase bonded to a small amount (~2%) of carbon provides potential for the co-existence of hydrophilic and hydrophobic interactions, and the mobile phase can be either organic solvent-rich or water-rich in composition depending on the polarity of analytes of interest.[22] Callahan *et al*[23] reported that approximately 1,000 features in human urine could be detected by an ANP-LC-MS system and a similar number of features were obtained by a RP-LC-MS system. However, none of the studies above evaluated chromatographic performance of LC conditions in respect of repeatability and linearity for urinary metabolites. In addition, no comprehensive assignment of detected features to putative metabolites has ever been carried out.

Time-of-Flight (ToF) mass spectrometers are the most widespread HRMS instruments used in metabolomics.[5] From our literature research the average mass error produced by ToF around 5 ppm but can be better than this. The Orbitrap MS is able to provide excellent resolution (>100,000) and mass accuracy (routinely < 2 ppm).[24-26] Recently a benchtop Orbitrap MS system (the Exactive) has been introduced into the market. Which has high scan speed and fast polarity switching and high is ideal for fast and comprehensive metabolite profiling of biofluids or tissue extracts when coupled with LC separation. The accuracy of the Orbitrap means that there is often only one sensible elemental composition for a feature, although this becomes less likely as the mass of an analyte increases to above *ca* 300 amu,. Amajor issues which arises in profiling when high resolution mass spectrometry is used are with regard to isomer separation and identification. In the current study we have used standard mixtures containing 176 metabolite standards to test the chromatographic performance of one reversed phase, one ANP and two zwitterionic HILIC columns. The columns were then tested for their ability to measure the features with repeatable peak areas in human urine. Finally by combining retention times with accurate elemental composition assignment the feasibility of qualitative and quantitative analysis of individual metabolites in human urine was evaluated. In addition an extensive evaluation of the raw

data led to putative identification of many metabolites which are not currently in the metabolite data bases.

Experimental

Chemicals and materials

HPLC grade acetonitrile (ACN) was purchased from Fisher Scientific, UK. HPLC grade water was produced by a Direct-Q 3 Ultrapure Water System from Millipore, UK. AnalaR grade formic acid (98%) was obtained from BDH-Merck, UK. Ammonium carbonate and ammonium acetate were purchased from Sigma-Aldrich, UK.

Standard sample preparation

Authentic standard stocks and standard mix solutions were prepared as described previously [27] and diluted 5 times with a solvent of H_2O/ACN (20/80) before LC-MS analysis.

Urine sample collection and preparation

Urine was collected from 6 healthy volunteers, who had no diet or lifestyle restrictions and processed as described in supporting information 1 (SI.1).

LC-MS analysis

Measurement of standard samples and pooled urine samples was carried out on a Dionex Ultimate 3000 HPLC system (Camberley, UK) combined with an Exactive (Orbitrap) mass spectrometer from Thermo Fisher Scientific (Bremen, Germany). The mass spectrometry settings are described in S .The 5 LC conditions tested in this study are listed in SI.1.

Data processing

Raw data were sliced to individual positive and negative data sets using the RecalOffline tool from the Xcalibur software package before importing them into MZMine 2.2 software.[28] After the process of chromatogram building, chromatogram deconvolution, de-isotoping, alignment, gap filling and identification (adduct and complex search) each data set was converted to hundreds of extracted unique chromatographic features (peaks) based on the combination of m/z and retention time. The settings for each step are listed in

SI.1. After removing a few non-peak-shape features by visualization both data sets were exported from MZMine 2.2 to an Excel 2007 workbook for further statistical analysis.

Data analysis

The calculation and statistics were performed by Excel 2007 and Visual Basic for Application (VBA). The identification of putative metabolites for features was achieved by using a VBA macro searching their accurate masses with a threshold of ±3ppm from an in-house database containing 41,623 potential metabolites from KEGG, MetaCyc, HMDB and Lipidmaps public databases.[27]

Results and discussion

Chromatographic evaluation of standards

Two mixtures of standards, as described in SI.2, containing a range of amino acids, organic acids, sugars and other metabolites were run in triplicate under 5 LC conditions. The mixture represents many of the compounds present in urine but is lacking in conjugates such as sulphates and glucuronides which are not commercially available. The repeatability of the retention time and peak area for each metabolite between replicates was calculated, and an additional visual inspection of peak shapes was performed on the peak list generated by MZMine 2.2 in both positive and negative modes. Finally all metabolites were categorised into 3 groups based on their peak shapes and the repeatability of their retention times and peak areas. At the ‘good’ level, metabolites showed narrow and symmetric peak shapes and more importantly the shift of their retention times and peak areas between replicates was in the range of ±5% and ±10% respectively. Results for individual metabolites were still ‘acceptable’ if retention times and peak areas varied in the range of ±10% and ±25% respectively. The cause of these variations was often found to be due to variation in peak shapes including broadening, tailing, fronting and splitting. The remaining metabolites were classified as ‘unacceptable’ which could usually be attributed to broad or multiple-peaks. The C18 column was excluded from this comparison because more than 80% of metabolites eluted as sharp spikes in the solvent front (Rt < 3 minutes). Figure 1 shows the

distribution of metabolite standards at the three classification levels for each remaining LC condition.

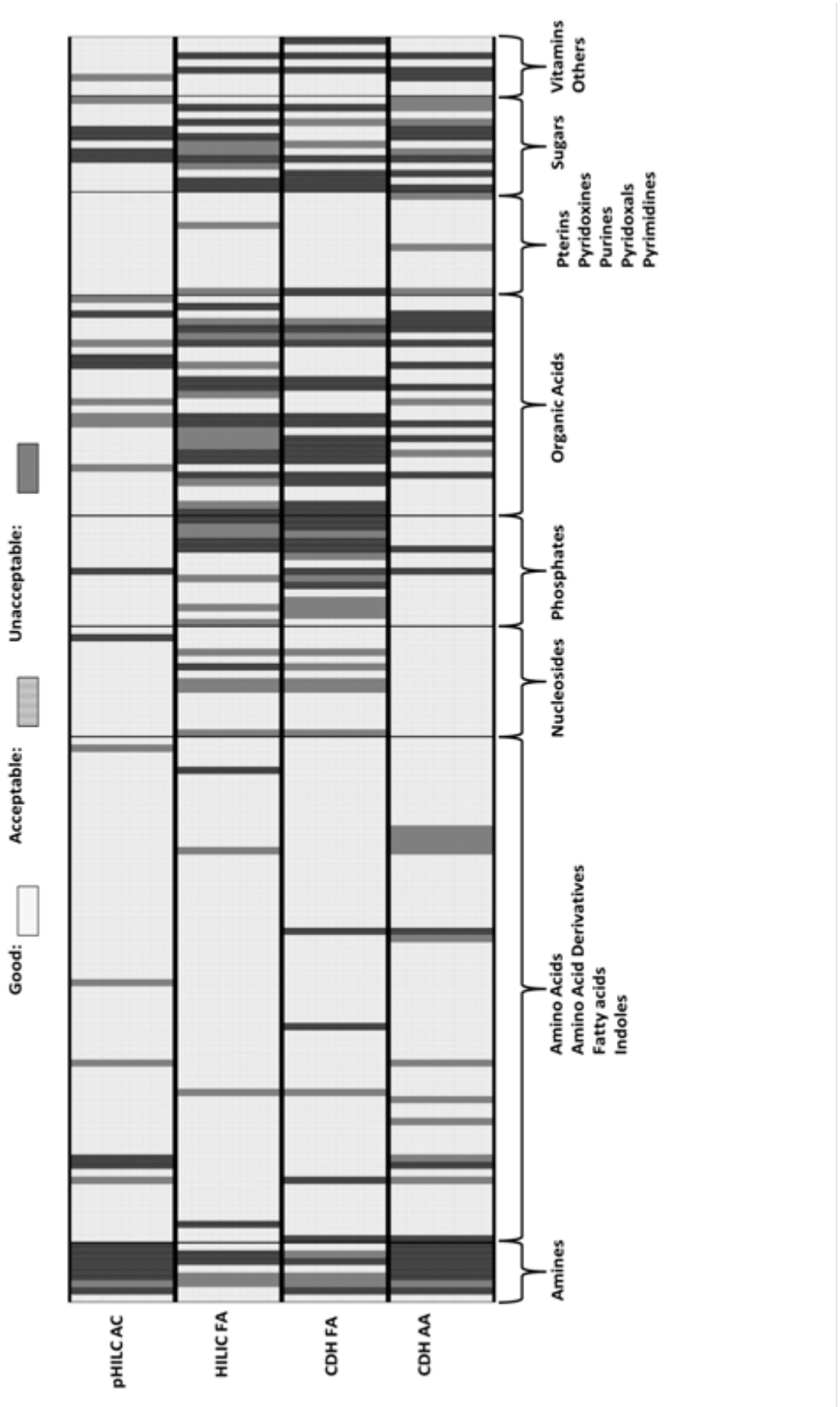

Figure 1 Distribution of metabolite standards at good, acceptable and unacceptable levels under different LC conditions with their biochemical classifications.

At first glance it is clear that most of the amino acids, amino acid derivatives and nitrogen heterocyclic metabolites performed well under all four LC conditions, while generally the performance of amines and sugars was poorer. It also should be noted that phosphates and organic acids show much better performance under the conditions used for ZIC-pHILIC+AC. Figure 2 shows a clear example of difference in peak shapes for citrate and 6-phospho-D-gluconate produced by the different conditions. Although tailing can be observed under ZIC-pHILIC+AC conditions, high repeatability of retention times and peak areas could be achieved for these two metabolites. Therefore they were assessed as being at the “good” level under this LC condition. As indicated in Figure 1 it seems that compared to the other three conditions ZIC-pHILIC+AC gives the greatest number of metabolites at ‘good’ and ‘acceptable’ levels in each classification, except for amines which are probably largely unionised under the mobile phase conditions used. The poor performance of amines and basic amino acids under the conditions of ZIC-pHILIC+AC and CDH+AA is shown in Figure 2. L-histidine shows a tailing and broad peak shape and putrescine cannot be detected. In contrast, good symmetric and sharp peak shapes can be achieved for these analytes under the conditions ZIC-HILIC+FA and CDH+FA. The combination of ZIC-pHILIC+AC and ZIC-HILIC+FA is able to offer good or acceptable chromatographic results for almost all tested metabolite standards (173 out of 176).

The retention of polar compounds on ZIC-HILIC and ZIC-pHILIC is caused by a combination of hydrophilic partitioning and electrostatic interaction of polar/ionised solutes between the mobile phase and the water-rich/zwitterionic stationary phase. Poor peak shapes are likely to be produced by a competition between hydrophilic partitioning and electrostatic interaction for partially ionized metabolites. That is why acidic metabolites show poor peak shapes under the conditions of ZIC-HILIC+FA (mobile phase A pH=2.8) and the same argument can be used for basic metabolites under the conditions of ZIC-pHILIC+AC (mobile phase A pH=9.2). Sugars perform inconsistently and

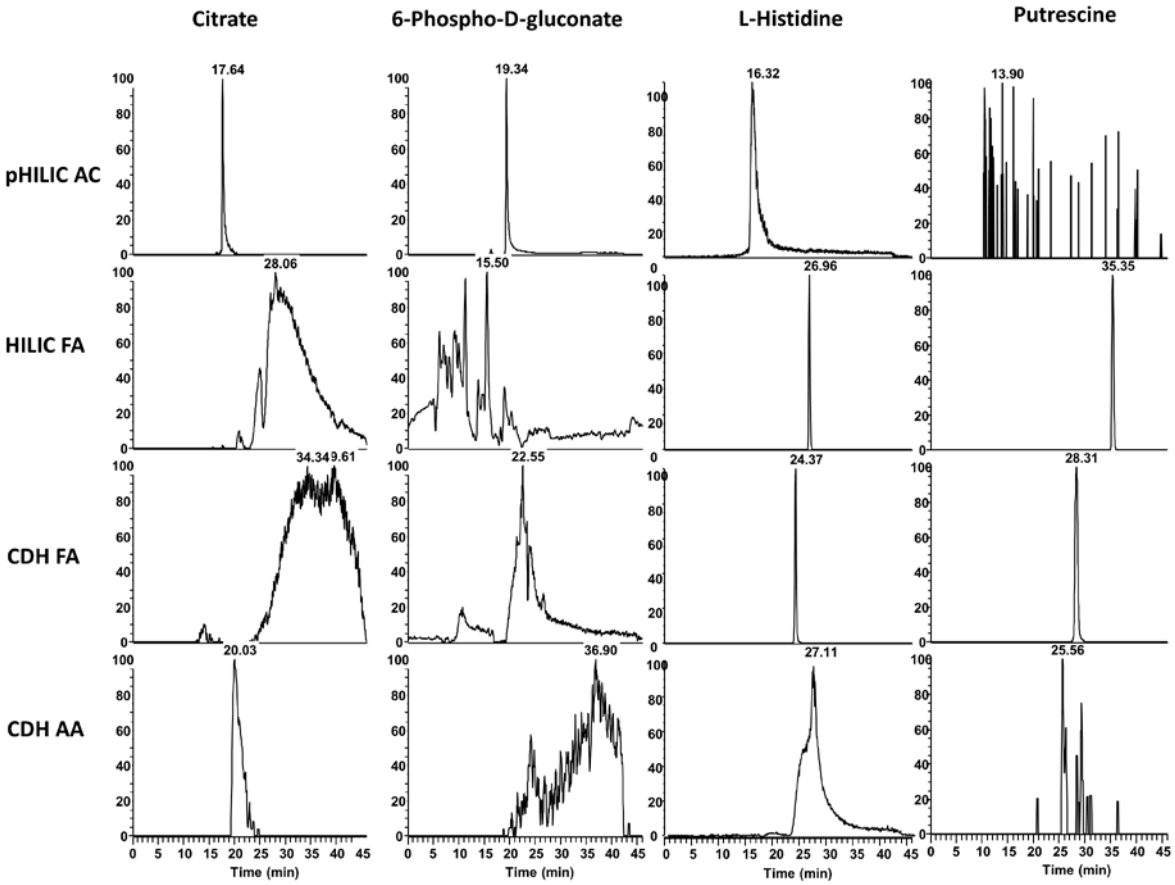

Figure 2 EICs of citrate, 6-phospho-D-gluconate, L-histidine and putrescine under different LC conditions.

overall the best performance is with ZIC-pHILIC+AC. All reducing sugars can exist as four isomers in equilibrium, the α- and β-forms of the pyranose and furanose types. This can potentially produce four peaks for each sugar or potentially broad peaks. This can be seen for the disaccharide standards (table 2) where the non-reducing sugar sucrose produces a single peak whereas its reducing sugar isomer maltose produces multiple peaks. At high pH, as used with ZIC-pHILIC+AC, the rate of interconversion between the different sugar forms is more rapid and thus narrower peaks are more likely.

Isomer separation evaluation of standards

In the study 19 pairs of isomers were used to test the separation ability of each set of LC conditions and their retention times and brief comments on peak shapes are shown in SI.3. It is clear that none of the pairs of isomers could be separated on the C18 column because of insufficient retention on the column except for 4-coumarate and phenylpyruvate which were poorly separated. Therefore the C18 column was excluded from the comparison once again. Visual inspection of exctracted ion chromatograms (EICs) for each pair of isomers in MZMine 2.2 gave a quick assessment of the separation ability of each set of conditions. Apart from sugars all pairs of isomers were separated well under the conditions of ZIC-pHILIC+AC. Methylmalonate and succinate show close retention times but the resolution is still more than 1.5. The condition of ZIC-HILIC+FA shows poor separation ability for acidic isomers. Similar results could be observed on the CHD column using the same mobile phase. However, on the same column some overlapped peaks of the isomers e.g methylmalonate and succinate could be separated if the pH of the mobile phase was increased to 7. Based on the current results the order of separation ability of LC conditions for the tested isomers, except for sugars, is ZIC-pHILIC+AC > CDH+AA > HILIC+FA > CDH+FA.

It has been reported that separation on the CDH column is mainly caused by hydrophilic interaction, and that hydrophobic interaction may also play a role because of the introduction of small amount of bonded carbon on the surface of stationary phase.[22] However, 4-coumarate and phenylpyruvate, which are

relatively hydrophobic metabolites, were not separated at low pH, but when the pH was increased to 7, where both of them are more ionized/polar, a separation was achieved. This suggests that the hydrophilic interaction is the major factor in the separation for this pair of isomers on the CDH column. Some experimental factors are capable of improving the separation of isomers. Figure 3 illustrates some good examples. In order to compare peak width and shape all the time widows in Figure 3 are fixed as 10 minutes wide. For acidic isomers Figure 3A shows that methylmalonate and succinate are completely overlapped at low pH but were separated at high pH on both ANP and HILIC columns. The reason for this on the HILIC columns could be increased electrostatic interaction at high pH for these two acidic metabolites. Better separation of these two isomers could be achieved on the ANP column at high pH which might be due to the stronger hydrophilic interaction of the isomers in their more ionized states.

It can be seen in Figure 3B that the separation of amino acid isomers L-leucine and L-isoleucine was improved when the pH was raised. This might be due to increased hydrophobic interaction since the CDH column does have some hydrophobicity and the ZIC-pHILIC column is based on an organic polymer base so it is likely that it also has some hydrophobic properties. Figure 3C shows that good peak shapes are essential for good separation. Two nucleotide isomers show similar broad and tailing peak shapes at low pH on both HILIC and ANP columns. Good peak shapes were obtained at high pH but only with the conditions of ZIC-pHILIC+AC and delivered a good separation of the isomers.

Urinary metabolite profiling

A pooled urine sample from six healthy volunteers was measured in triplicate under each LC condition and the data obtained was processed using MZMine 2.2 with the same settings as above. In order to achieve a reasonable evaluation for the purpose of quantitative analysis for metabolomics all LC conditions were compared at three levels of filtering (with classification into detectable charge modes). Table 1 shows a summary of the results. Features were calculated separately in ESI positive (P) and negative (N)

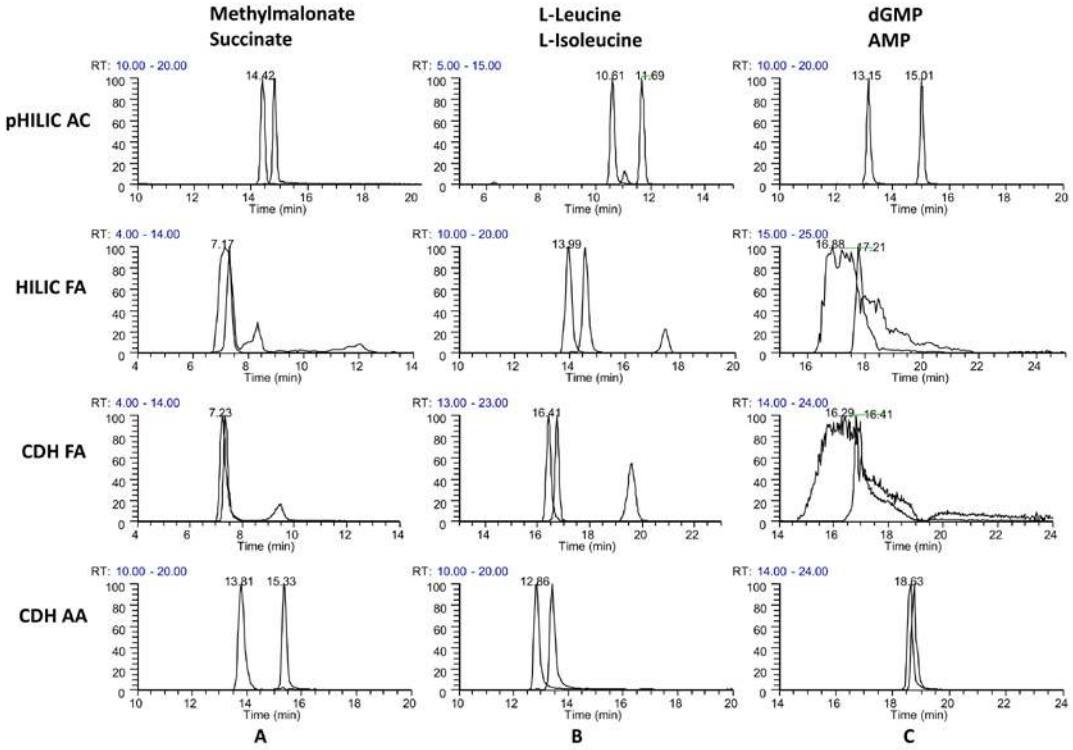

Figure 3 Extracted ion chromatographs of three pairs of isomers under different LC conditions.

Table 1 Numbers of features detected at different levels of data filtering under five different chromatographic conditions. P/N =detected in both positive and negative ion mode.

	Detected				**Repeatable**				**Identified**			
	Pos	Neg	P/N	Total	Pos	Neg	P/N	Total	Pos	Neg	P/N	Total
C18	769	1034	83	1886	313	501	77	891	192	306	66	564
pHILIC	872	1427	132	2431	487	871	101	1459	248	451	90	789
HILIC	943	1193	154	2290	680	732	137	1549	417	284	123	824
CDH FA	1178	970	142	2290	520	525	120	1175	256	207	111	574
CDH AA	467	1416	50	1933	269	822	42	1175	113	421	36	570

modes under each LC condition and some were considered as single features (P/N) if they were detected by both modes with mass difference of 2.0146 ± 0.001 m/z within a Rt window of ±0.1 min. It can be observed that the total numbers of features detected by C18+FA and CDH+AA are lower than the other three LC conditions and with high pH mobile phases (AC and AA) a greater number of features can be detected in ESI negative compared with positive mode. In order to select reliable chromatographic results the detected features for replicates showing RSD values > ± 25% for their peak areas were filtered out at the 'Repeatable' Level. Based on the number of repeatable features a clear classification of LC conditions can be seen now. More than half of the detected features were removed by this repeatability filter under C18+FA conditions, which leads to a clear gap between C18+FA and the other LC conditions. The dramatic cut-off for detected features under C18+FA conditions at the Repeatable Level could be due to an ion-suppression/enhancement effect on polar metabolites eluting together at early retention times and MS signal variation due to the lower ESI efficiencies obtained with high mobile phase aqueous content. The difference in the level of cut-off between HILIC and ANP LC conditions could be caused by inaccurate integration for chromatographic peaks with poor shapes. The combination of the two HILIC conditions produces about 650 more features than was obtained under ANP LC conditions. The number of identified features is greatest for HILIC at the Identified level (where there is a database match to one or more metabolite SI.4) and interestingly the C18+FA conditions show a similar number of identified features to ANP-LC conditions at this level. The decrease in the identification of the number of features between these two levels could be due to the limitation of the metabolite matches available in the database and the removal of the signals of non-molecular ions (in-source fragments, complex ions, isotope peaks and non-proton adducts). Only a small decrease is observed in the number of P/N features from Repeatable to Identified level which suggests that the features are more likely to be generated by real metabolites if they can be reproducibly detected with both ESI modes.

Following the classification at the Repeatable level the five LC conditions were regrouped as RP, HILIC and ANP chromatography. The unique m/z features (±0.0005) were filtered out from each chromatographic method. For the three chromatography modes 1260 and 1823 unique m/z features were recognized in ESI positive and negative mode respectively (SI.5). A similar shareholding pattern is observed for both ESI modes. More than half of the pie is shared by HILIC-only (35%) and ANP-only (21%). 12% of the unique features are found with all three chromatography modes, which are believed to be the major components in human urine. HILIC is able to cover 70% of unique features in total, followed by ANP (53%) and RP-LC (25%). The table below summarises the percentages of features identified as metabolites in each share by accurate m/z and the ratios of metabolites with molecular weight <250 to metabolites with MW >250. From that data it would appear that a higher percentage of features can be identified as real metabolites if they are found with more than one chromatography mode. In the share of all three chromatography methods more than 80% of the identified features show a MW less than 250 amu which means small molecules are the major components of human urine. HILIC covers more small molecules and larger molecules, generally more lipophilic molecules, are found with RP-LC. These results match well with the characteristics of the tested chromatography and again prove that comprehensive coverage of metabolite profiling in human urine cannot be achieved by a single type of chromatography.[21, 23]

The threshold for database matching was ±3ppm and more than 80% of hit features showed mass error within ±1ppm for all LC conditions and only few features with large m/z were simultaneously matched to two metabolites with different elemental compositions present in the database (IS.4). During the ESI process it is possible for a single compound to form various non-proton adducts with Na^+, K^+ and NH_4^+, to generate complex ions with co-eluting molecules and to produce in-source fragments by degradation. These phenomena were more likely to be observed for highly abundant metabolites in human urine. In this study non-proton adducts and complex ions were identified using MZMine 2.2 by their accurate mass difference from the parent compounds if their retention times matched the molecular ions of the parent.

In-source fragments were evaluated by the increase in response of an MS signal at the same retention time as the parent with higher energy collisional induced dissociation (HCD) cell on. By these examinations some non-molecular ion features were accurately identified and assigned to the related molecular ion. Table 2 illustrates some examples of non-molecular ions obtained under ZIC-pHILIC+AC conditions where the features were not generated by actual metabolites in spite of the fact that they could be assigned to some common metabolites by accurate mass. After excluding the possibility of non-molecular ion signals isomer identification should be performed in order to assign the features to individual metabolites. Recently Creek et al[27] developed a retention time prediction model for HILIC chromatography based on the physicochemical nature of the analyte-column interactions. By using this method many structurally distinct isomers can be rapidly recognized without confirmation with standard metabolites. Table 3 shows some examples of the distinction of isomeric metabolites by calculated retention times under ZIC-HILIC+FA conditions. Amine and amide isomers were well modelled by the method leading to a good prediction of their retention times, which are different enough to distinguish them. Carboxylic acid and ester isomers could also be distinguished by this method under the condition of ZIC-pHILIC+AC by simply adjusting the pH-dependent variables in the prediction model. The non-selective nature of HCD meant that it was not possible to assign clear fragmentation patterns to a particular feature. However, in many cases it was possible to predict and observe the masses of fragments of overlapping features such as the acylium ions derived from different acyl glycines. It would be better to use a hybrid mass spectrometer for more selective and precise fragment pattern analysis, however, even in this case poor MS^2 spectra might be obtained from low level metabolites . In a previous study using a LTQ-Orbitrap mass spectrometer a constantly presenting feature (185.1284 m/z ESI-positive) in human urine was identified as N-(3-acetamidopropyl) pyrrolidin-2-one which was not available in our in-house database.[29] Many of the stable features in urine were not in available data bases and the metabolite identification was extended by including obvious compounds which might be expected in urine such as a wide range of acylcarnitines and acylglycines and sulfate and glucuruonide conjugates. Key

examples of these conjugates were confirmed from HCD fragmentation. In addition close inspection of the raw data enabled assignment of many false metabolite identifications resulting from minor fragments or adducts not removed by the filtering process. The ultimate confirmation of individual metabolites should be completed by comparison of retention times and fragment patterns with authentic standard compounds or further analysis of collected HPLC fractions by NMR.[30]

Table 2 Some examples of incorrect assignments of metabolites due to adducts or fragments derived from other features.

m/z	Polarity	Elemental composition	Mass error in ppm	Possible metabolite	Source ion and relationship
145.0621	N	C5H10N2O3	1.36	L-Glutamine	Fragment[a] of 263.014 m/z
103.0390	P	C4H6O3	0.03	2-Oxobutanoate	Fragment[a] of 162.112 m/z
284.0776	N	C12H15NO7	0.19	N-Glucosylnicotinate	Complex[b] of 194.046 and 89.024 m/z
389.0994	N	C18H18N2O8	0.93	Dopaxanthin	Complex[b] of 194.046 and 194.046 m/z
117.1022	P	C5H12N2O	-0.33	5-Aminopentanamide	ACN+H adduct[c] of 76.076 m/z
112.0869	P	C5H9N3	-0.18	1H-Imidazole-4-ethanamine	[M+NH3] adduct[c] of 95.060 m/z

[a] Fragment confirmed by increasing signal intensity with HCD fragmentation
[b] Complex determined by accurate mass and retention time in MZMine 2.2
[c] Non-proton adduct determined by accurate mass and retention time in MZMine 2.2

Table 3 Some examples of distinguishing isomers on the basis of their calculated retention times on HILIC+FA chromatography.

m/z	Polarity	Rt(min)	Elemental composition	ppm	Met Name	Std Rt(Min)	Cal Rt(Min)
133.0607	P	19.15	C4H8N2O3	-0.60	L-Asparagine	19.20	20.89
133.0607	P	8.97	C4H8N2O3	-0.37	3-Ureidopropionate	-	9.48
156.0768	P	26.86	C6H9N3O2	0.05	L-Histidine	26.97	23.43
156.0767	P	14.23	C6H9N3O2	-0.10	3-(Pyrazol-1-yl)-L-alanine	-	15.97
171.0414	N	19.07	C6H8N2O4	1.75	(R)-AMAA*	-	20.80
171.0415	N	7.90	C6H8N2O4	2.12	Hydantoin-5-propionate	-	9.26

* (R)-AMAA = (R)-2-Amino-2-(3-hydroxy-5-methyl-4-isoxazolyl) acetic acid

Linear responses for metabolites in urine

The correlation of metabolite levels with LC-MS peak areas was examined by calculating the Pearson correlation coefficient (R^2) for features obtained from diluted pooled urine samples (0, 2, 5, 10 and 25 times dilution, following the initial dilution step used to remove salts, with H_2O/ACN=20/80). Under the conditions of ZIC-pHILIC+AC 92.6% (900 out of 972) and 87.2% (513 out of 588) features showed R^2 values greater than 0.9 at the Repeatable level for ESI negative and positive modes respectively (SI.6). A similar result was also obtained for ZIC-HILIC+FA. The poor linearity for a few of the features is due to their low abundance in urine causing them to fall below their limit of detection. It is believed that metabolomic quantification would be satisfied by such good linearity across this dynamic range. In addition, it is an essential condition to perform normalization for urine samples either based on the signal for creatinine or using the strategy of mass spectrometer total useful signal (MSTUS).[31]

Conclusions

By comparing RP, ANP and zwitterionic HILIC chromatography it was demonstrated that a combination of two HILIC methods gave the most extensive coverage of highly polar metabolites in urine. The mixed separation mechanism of zwitterionic HILIC offers an enhanced separation of isomers and the improvement of peak shapes of organic acids and sugars at high pH on the ZIC-pHILIC column is very useful for extending the coverage of polar metabolites in human urine. The Exactive Orbitrap mass spectrometer provides high confidence for the elemental composition assignment of individual metabolites and its dynamic range is also satisfactory for quantitative analysis in metabolomic studies. Combining orthogonal liquid chromatographic platforms it is a powerful tool for achieving a more comprehensive metabolite profiling of human urine. Simple metabolite prediction has permitted the annotation of a substantial number of new metabolites not in current data bases.

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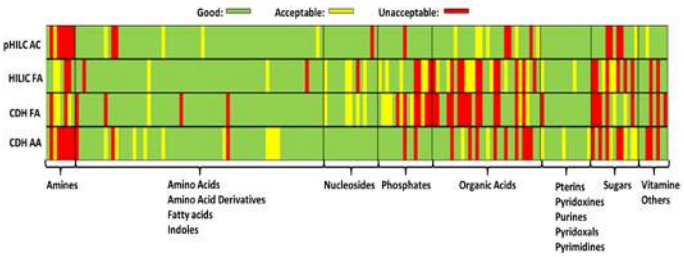
Good:
Acceptable:
Unacceptable:
pHILC AC
HILIC FA
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Amines
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Pyrimidines
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