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An approach towards method development for untargeted urinary metabolite profiling in metabonomic research using UPLC/QToF MS☆

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ABSTRACT

The application of LC–MS for untargeted urinary metabolite profiling in metabonomic research has gained much interest in recent years. However, the effects of varying sample pre-treatments and LC conditions on generic metabolite profiling have not been studied. We aimed to evaluate the effects of varying experimental conditions on data acquisition in untargeted urinary metabolite profiling using UPLC/QToF MS. In-house QC sample clustering was used to monitor the performance of the analytical platform. In terms of sample pre-treatment, results showed that untreated filtered urine yielded the highest number of features but dilution with methanol provided a more homogenous urinary metabolic profile with less variation in number of features and feature intensities. An increased cycle time with a lower flow rate (400 μl/min vs 600 μl/min) also resulted in a higher number of features with less variability. The step elution gradient yielded the highest number of features and the best chromatographic resolution among three different elution gradients tested. The maximum retention time and mass shift were only 0.03 min and 0.0015 Da respectively over 600 injections. The analytical platform also showed excellent robustness as evident by tight QC sample clustering. To conclude, we have investigated LC conditions by studying variability and repeatability of LC–MS data for untargeted urinary metabolite profiling.

1. Introduction

Metabonomic research using LC–MS as an analytical platform has attracted a great deal of interest in recent years in toxicological and pharmaceutical research, as well as in disease biomarker discovery [1–3]. Although advances have been achieved in generating comprehensive metabolite profiles, in data analysis, and biomarker identification [4,5], the actual methodology for undertaking untargeted profiling using LC–MS is still under development. One of the major challenges in untargeted metabolite profiling is to optimise experimental procedures for studying thousands of unknown metabolites in biological samples to 'appreciate' both the analytical and biological variability that exists. Recently, several laboratories have investigated different approaches to evaluate and monitor the performance of analytical platforms used in generic metabolite profiling [6–8] as well as testing the stability of biofluids under various sample handling and storage conditions [9]. The use of internal standards has also been commented failing to improve precision in data acquisition [6]. Therefore, standardisation in experimental procedures is of paramount importance to yield quality and reproducible data when conducting LC–MS-based metabonomic research.

In recent years, the development of higher performance LC systems in separation technology has provided a promising high throughput platform to separate thousands of metabolites in biological samples over a shorter cycle time with increased resolution. Such a development has expedited method development and validation in LC–MS-based metabonomic research [3,10]. An effective generic method with high repeatability is essential to generate quality analytical data for metabonomics. It is not known to what extent the variation in analytical conditions would affect the quality of urinary metabolite profiles. In this study we aimed to evaluate various sample pre-treatment and LC conditions in order to optimise a method for untargeted urinary metabolite profiling that would yield high quality and reproducible data using UPLC/QToF MS. We tested the effects of varied experimental procedures on total ion chromatograms, total number of features and the repeatability of selected endogenous metabolites. Quality control (QC) to

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monitor stability of the analytical system as described elsewhere [11] was also applied with some modifications.

2. Experimental

2.1. Chemicals

Acetonitrile and methanol were HPLC grade, formic acid was laboratory reagent grade and sodium hydroxide (0.1 M) was analytical grade (Fisher Scientific, Loughborough, UK). Water was purified using an Elga PureLab Ultra system (Elga, High Wycombe, UK). Leucine Enkephalin and all test-mix metabolites which including acetaminophen, sulfaguanidine, sulfadimethoxine, Val-Tyr-Val, terfenadine, reserpine and erythromycin (final concentration at 2 ng/μl each) were purchased from Sigma–Aldrich, Poole, UK.

2.2. Sample preparation

Twenty-four hour urine samples ($n = 3$) were collected without the use of preservatives from healthy male volunteers (average age and body mass index: 23 ± 3 years and 22.2 ± 1.7 kg/m^2 respectively). Samples were divided into aliquots and stored at −25 °C immediately after collection. An in-house QC was prepared by pooling the same volume of each sample and 8 ng/μl (final concentration) of sulfadimethoxine was added and mixed thoroughly.

Urine samples were either analysed untreated after filtering through a 0.22-μm filter, or pre-treated by diluting the urine either in water or 100% methanol in a ratio of 1:1 (v/v). The samples were then vortex-mixed for 30 s and centrifuged at 13,000 rpm for 10 min to remove any particulates. The supernatant was then transferred to autosampler vials (Waters, Manchester, UK) for UPLC/MS analysis. Blanks (ultrapure water) were also included and treated in exactly the same way as the samples.

To establish inter-day repeatability, urine samples ($n = 3$) were treated in the same way as described above and were analysed on 3 separate days, 6 injections for each day. The robustness of the method was also tested by injecting random healthy male urine samples ($n = 80$, 6 injections each) in a randomised order over a 120-h period, with the QC, metabolite test-mix and blank injected following every 20 sample injections in order to monitor retention time (RT) and mass drift within a run.

2.3. Instrumentation: liquid chromatography

Chromatography was performed on a Waters Acquity UPLC™ system (Waters, Manchester, UK) using an Acquity HSS T_3 column (1.8 μm, 2.1 mm × 100 mm; Waters, Manchester, UK). The column oven temperature was maintained at 40 °C and the autosampler temperature maintained at 4 °C. The mobile phases were (A) 0.1% formic acid in water, and (B) acetonitrile with 0.1% formic acid. The linear gradient program began with 100% (A) for 0.5 min, then proceeded to 100% (B) over 8.5 min, then returned to initial conditions (100% A) and maintained for 1 min. Total cycle time was 10 min with a flow rate of 600 μl/min and an injection volume of 5 μl using partial loop mode. Depending on the experiment, the cycle time was set at either 5 min, 10 min, 15 min or 20 min while the flow rate was set at either 400 μl/min or 600 μl/min. Except for the 5-min cycle time, all LC gradients began with 100% (A) for 0.5 min and maintained for 1 min at the end of each cycle for equilibration. For the 5-min cycle time, conditions started with 100% (A) for 0.4 min, then proceeded with a linear gradient to 100% (B) over 3.8 min, then returned to initial conditions and maintained for 0.8 min.

The effect of different elution gradients was assessed under either linear (described above), curve or step gradient which was controlled by the Waters MassLynx software (Ver. 4.1). The curve gradient changed from 100% (A) to 100% (B) over 8.5 min in a concave fashion (curve 7). The gradient then returned to initial conditions and maintained for 1 min for equilibration. The step gradient increased from 0% (B) to 70% (B) over 8.5 min in a linear fashion. Then the gradient increased linearly to 100% (B) within 0.3 min. The gradient returned back to initial conditions and maintained for 0.7 min for equilibration.

2.4. Instrumentation: mass spectrometry

Mass spectrometry was performed by using a Waters Micromass QToF Premier™ (Waters, Manchester, UK) operating in both positive and negative ion electrospray modes. The source temperature was set at 120 °C while the desolvation temperature was at 450 °C, with nebulisation gas set to 800 l/h. The collision energy was set at 4 eV. The capillary and cone voltages were set at 3.1 kV and 30 V respectively in positive ionisation mode, and 3.0 kV and 35 V respectively in negative ionisation mode. A LockSpray™ interface was used to ensure mass accuracy. For this, leucine-enkephalin (m/z 556.2771 or 554.2615 in positive and negative ionisation modes respectively) was infused at a concentration of 200 pg/μl at a flow rate of 30 μl/min. Data were collected in centroid mode over the range 100–1000 m/z with an acquisition rate of 0.2 s, interscan delay of 0.02 s, with dynamic range enhancement activated.

2.5. Data analysis

The acquired data were analysed and visualised using Waters MassLynx (Ver 4.1) and Waters MarkerLynx (Ver 4.1). The noise elimination level was set at 8, with 15 masses per retention time collected. Background ions from associated blank injections were excluded. The mass and retention time windows were set at 0.05 Da and 0.1 min respectively. Multivariate analysis of the processed data were performed and visualised using SIMCA-P + (Ver 11.5, Umetrics, Sweden). All the variables were set to Pareto scaling. The dataset was then "autofit" and visualised by principle component analysis (PCA) to discriminate any differences between groups.

3. Results and discussion

Urine represents the most used biological fluid for metabonomics research but for LC–MS-based applications it is important to optimise both LC and MS conditions for this type of analysis. Analytical conditions including the column [12], temperature [13], rapid gradients [14] have been tested, as well as the influence of sample separation techniques [15] and sample storage time [9] on urinary metabolite profiles. In the present study, we have compared various LC conditions and sample pre-treatments in order to further optimise the methodology for untargeted metabolite profiling. The use of QC in monitoring the performance of the analytical platform has also been applied to evaluate the robustness of the system as previously described [11].

3.1. Effect of cycle time and flow rate

With UPLC a higher resolution can be achieved in a shorter cycle time compared to traditional HPLC [3,10]. However, the mass spectrometer may not acquire enough data points which may influence the quality of the MS data. As the effect of varying cycle times and flow rates under the same LC–MS conditions on data acquisition has not been reported, we investigated the effect of four different cycle times under two different flow rates using UPLC/QToF MS.

From visual inspection, peak shapes were increasingly resolved with increasing cycle times (Fig. 1). However, peak width (defined as RT window at 50% peak height under base peak intensity

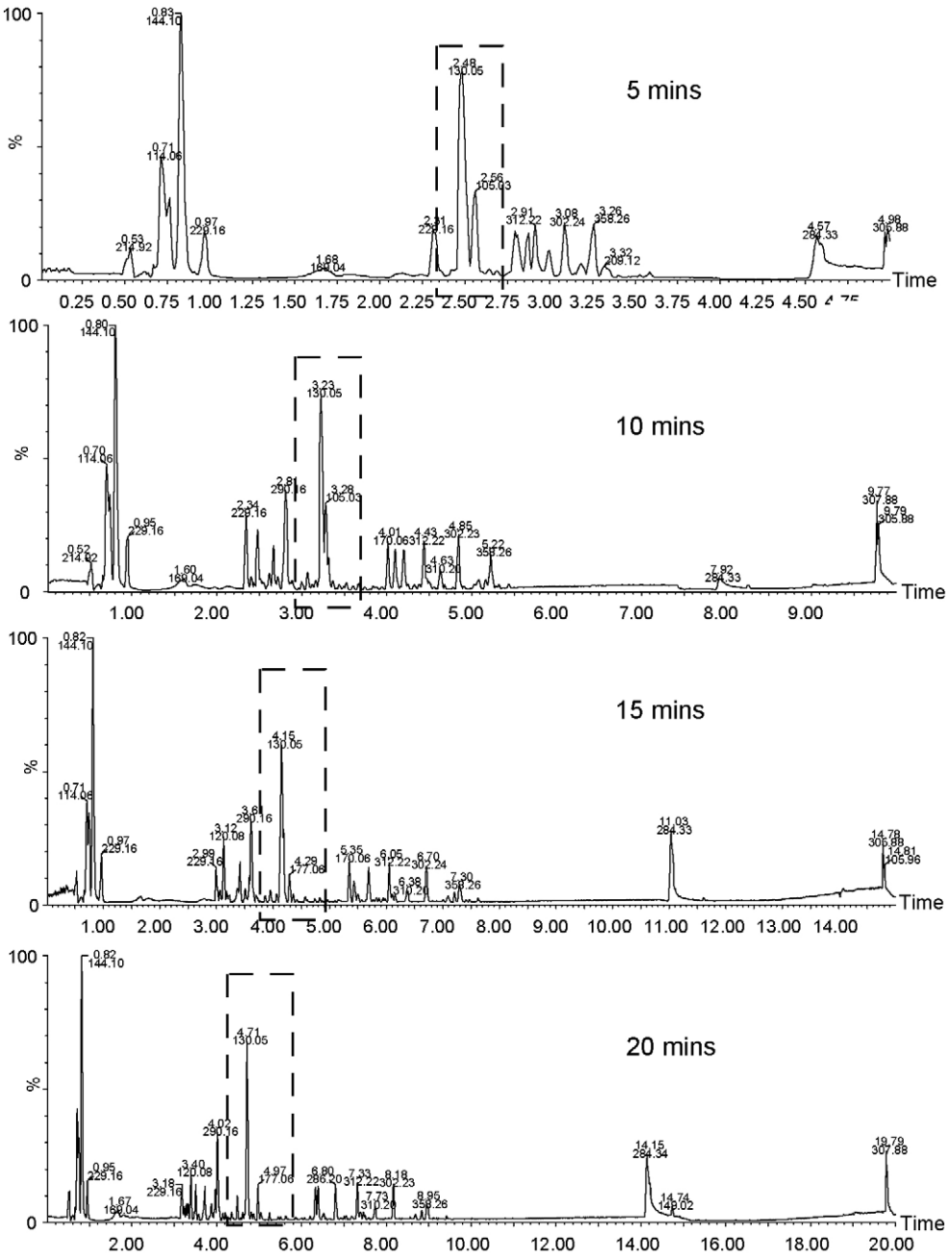

Fig. 1. Typical base peak intensity (BPI) chromatograms showing urine profiles analysed with four different cycle times under the same elution gradient at a flow rate of 400 μl/min. The area highlighted by the dotted line shows an example of improvement in peak resolution with cycle time.

chromatogram) of selected peaks increased only slightly from an average of 0.0443 min (5 min RT) to 0.0488 min (20 min RT). In order to have a better understanding of the effects of different cycle times, the average total number of features detected in the samples of all individuals was compared. In positive ionisation mode increasing the cycle time increased the number of features detected but also increased the intra-day variability in number of features (as determined by coefficient of variation, %CV) under the same noise threshold setting (Fig. 2). A flow rate of 400 μl/min resulted in lower variability compared to 600 μl/min at various cycle times but the number of features detected was comparable between the two flow rates. Similar patterns were also observed in negative ionisation mode (data not shown).

These results show that although the urine metabolites can be separated and analysed under the same gradient conditions, a longer cycle time results in better peak resolution and thus enhanced sensitivity and detectability (as evidenced by the increased number of features detected), while a lower flow rate yielded a more reproducible number of features (as indicated by less intra-day variability). The reasons behind these are not clear but a possible explanation could be that the capacity of the ionisation process is not sufficiently optimised for higher flow rates and/or a lower flow rate reduces any matrix effects thus enhancing the sensitivity for feature detection. In terms of analytical cycle times, although a longer cycle time results in better chromatographic separation and increased number of features detected, a

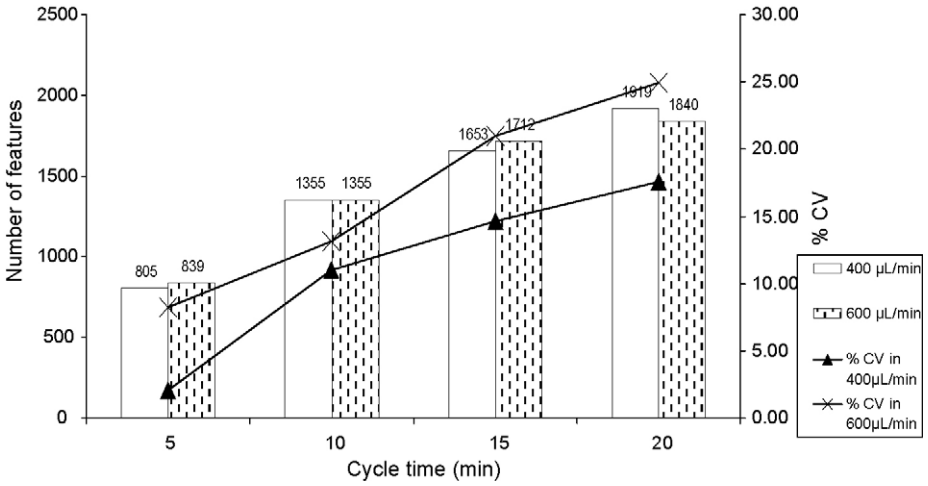

Fig. 2. Average total number of features obtained from different flow rates at four different LC cycle times in positive ionisation mode. Data are presented as mean for the total number of features (bar chart) and associated %CV (line chart).

Table 1
A summary of the average variation in intensity of selected endogenous species analysed under two different flow rates in positive ionisation mode (average of three urine samples)

Species	RT (min)	*m/z*	Intensity variation (%CV)	
			400 μl/min	600 μl/min
1	0.69	114.067	16	14
2	0.77	143.095	6	7
3	0.82	153.068	7	26
4	2.07	119.082	7	12
5	2.31	125.095	15	26
6	4.35	169.074	14	25
7	4.61	286.202	30	23
8	5.58	302.233	15	25
9	6.36	256.240	14	17
10	6.43	328.248	14	19
Average			14	19

10-min cycle time represents a practical solution for high throughput urinary profiling that yields a reasonable number of features and results in a relatively low %CV in both ionisation modes. However, we appreciate that this is dependent on individual analytical requirements.

We then compared the effect of varying flow rates on the repeatability of feature intensity. The average feature intensity of 10 selected endogenous species, which covered a range of RT and *m/z*, was calculated from all the samples studied (Table 1). With a flow rate of 400 μl/min the variation in intensity (%CV) of the selected endogenous species ranged from 5.7% to 30.4% demonstrating species-specific analytical behaviour in LC–MS detection, and the average %CV of these selected species was 13.9% in positive ionisation mode. At the higher flow rate of 600 μl/min the %CV calculated from the same set of selected species ranged from 6.8% to 26.3%, averaging 19.5% again in positive ionisation mode. A similar pattern was also observed in negative ionisation mode showing an average %CV of 9.4% at 400 μl/min and 13.7% at 600 μl/min (individual data not shown). These results further suggest that a reduction in flow rate to 400 μl/min produces a more stable and reproducible dataset.

3.2. Effect of varying the elution gradient

To optimise peak resolution and maximise the use of the chromatographic space, three different elution gradients were evaluated for urinary metabolite profiling. The column used is designed to retain and separate polar compounds so as to attain a better chromatographic resolution of polar samples such as urine. Nevertheless, with a linear elution gradient there was a "peak free" period at high % (B) region (around and above 60% B). Therefore, in order to achieve better separation, both curve and step elution gradients were evaluated. Both elution gradients expanded the time at low % (B) to allow for a longer interaction time between the analytes and the column, and therefore maximising chromatographic peak separation. The chromatograms generated from three different elution gradients were visually compared and the total number of features detected was used as an indicator of data quality.

Total ion chromatograms generated from the different gradients are shown in Fig. 3. With a linear elution gradient the majority of peaks appeared before 5.5 min over a 10-min cycle time. The curve elution gradient, as expected, shifted the peaks to higher RTs, while the step elution gradient resulted in an even greater peak separation with an increased RT (Fig. 3). The peak width of selected peaks also decreased from an average of 0.0507 min (linear) to 0.0455 min (step). This suggests a better chromatographic resolution for urine samples with a step elution gradient. Further data analysis showed that linear elution gradient yielded the lowest number of features detected in both ionisation modes. The step elution gradient, which had a linear elution up to 70% (B) over 8.5 min, yielded 9% and

Table 2
A summary of the number of features detected under different elution gradients in both ionisation modes (average of three urine samples)

	Positive ionisation mode			Negative ionisation mode		
	Linear	Curve	Step	Linear	Curve	Step
Mean	1394 ± 27	1519 ± 15	1523 ± 18	1454 ± 28	1598 ± 24	1687 ± 20
%CV	1.9	1.0	1.2	1.9	1.5	1.2

Data are presented as mean ± S.D. Linear: the elution gradient changed from 100% A to 100% B in a linear fashion over 8.5 min. Curve: the elution gradient changed from 100% A to 100% B under a concave (curve 7 setting) fashion over 8.5 min. Step: elution gradient changed from 100% A to 70% B over 8.5 min in a linear fashion.

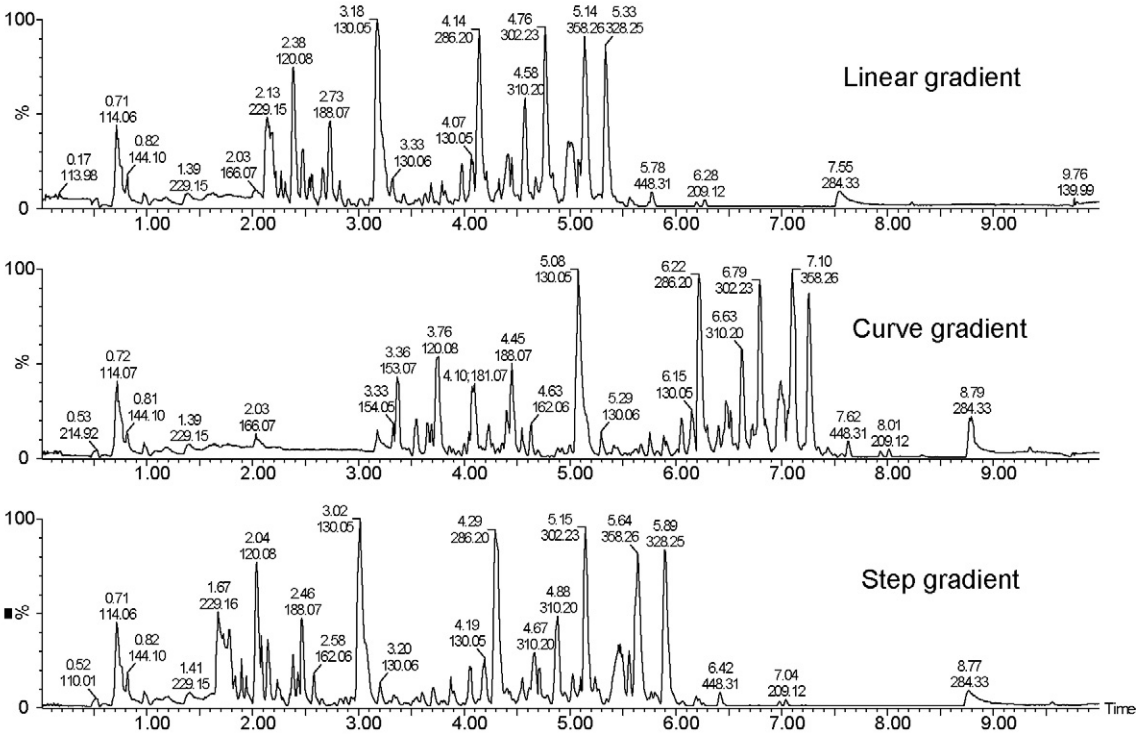

Fig. 3. Typical base peak intensity (BPI) chromatograms showing urine profiles analysed under three different elution gradients over 10 min at a flow rate of 400 μl/min.

14% more features, in both positive and negative ionisation modes respectively, than the linear elution gradient (Table 2). The number of features detected using the step elution gradient was also higher compared to the curve elution gradient in both ionisation modes. The increase in feature detection observed in the step elution gradient is presumably due to the improved separation in the shallow gradient region and hence increased capacity for ion detection. Indeed, step elution gradients have been used previously in other urinary metabolite profiling studies, possibly due to its better resolution of the complex metabolite profiles in urine [16]. Based on the aforementioned reasons, the step elution gradient was adopted to further evaluate the effects of sample pre-treatment on untargeted urinary metabolite profiling in the present study.

3.3. *Effects of different sample pre-treatments and repeatability*

Previous sections have demonstrated that a step elution gradient for 10 min at 400 μl/min provided the most "optimal" LC conditions studied for urinary metabolite profiling. The next step was to assess different sample pre-treatments, comparing them based on the quality and repeatability of the metabolite profiles produced.

Urine is an aqueous-based biofluid which usually contains a trace amount of macromolecules. Therefore, filtration through a 0.22-μm filter under centrifugation is usually sufficient for sample clean-up with LC–MS analysis. In some studies, urine samples are diluted prior to LC–MS analysis, possibly as a means to dilute interferences in the matrix [16,17]. However, one study demonstrated that dilution of human urine with water (1:4, v/v) reduced ion signals and some low abundant metabolites were undetectable after dilution [8]. Although dilution with water is the easiest and most convenient way of reducing matrix effects, a possibility of over-dilution may occur. Hence, it is important to identify alternative methods to clean-up the sample for untargeted urinary metabolite profiling. In the present study, the supernatants of urine samples after different sample pre-treatments (untreated or diluted with water or methanol) were subjected to LC–MS analysis. The total number of features detected and the variation in intensity of 10 selected endogenous species were compared between treatments. Repeatability of sample pre-treatments on different occasions over 3 days was assessed by PCA to visualise the similarity between sample groups.

The untreated filtered urine sample yielded the highest number of features while the numbers of features detected in the samples diluted with either water or methanol were comparable (Table 3). The variability (%CV) in features detected was lower in the methanol diluted samples (1% and 0.6% in positive and negative ionisation modes respectively) than that of the water diluted samples (5% and

Table 3
A summary of the number of features detected from different pre-treated samples in both ionisation modes (average of three urine samples)

	Positive ionisation mode			Negative ionisation mode		
	Untreated filtration	1:1 dilution water	1:1 dilution methanol	Untreated filtration	1:1 dilution water	1:1 dilution methanol
Mean	1532 ± 25	1355 ± 62	1344 ± 13	1622 ± 16	1454 ± 33	1402 ± 7
%CV	1.7	4.6	1.0	1.0	2.3	0.5

Data are presented as mean ± S.D. Untreated urine was prepared by filtering the urine with 0.22-μm filter and centrifuged at 13,000 rpm for 10 min. Dilution of urine was performed by vortex-mixing the urine with either water or 100% methanol (1:1, v/v) for 30 s and centrifuged at 13,000 rpm for 10 min.

Table 4
A summary of the number of features detected at separate occasions over 3 days in both ionisation modes using different sample pre-treatments (average of three urine samples)

	Positive ionisation mode		Negative ionisation mode	
	1:1 dilution water	1:1 dilution methanol	1:1 dilution water	1:1 dilution methanol
Intra-day				
Mean	1386 ± 34	1387 ± 29	2462 ± 59	2559 ± 25
% CV	2.5	2.1	2.4	1.0
Inter-day				
Mean	1364 ± 29	1376 ± 26	2227 ± 39	2355 ± 36
% CV	2.1	1. 9	1.7	1.5

Data are presented as mean ± S.D.

2% in positive and negative ionisation modes respectively). These results suggest that more reproducible spectra are obtained following methanol dilution of the samples. The variability in intensity of the 10 selected endogenous species was also used to monitor data quality. The average %CV in intensity obtained from the untreated urine samples was 4% which was comparable to that obtained from water and methanol diluted samples (also 4%) in positive ionisation mode. A similar pattern was also observed in negative ionisation mode (data not shown). These results indicated that urine samples diluted with methanol result in a more homogenous metabolite profile but untreated urine may be more suitable for comprehensive metabolite profiling.

Untreated urine samples have been used extensively for LC–MS-based metabonomic studies as untreated urine has been suggested to be an ideal sample that represents the most comprehensive profile of metabolites in urine [18]. However, the effects of high concentration of salts, such as phosphate and sulfates, as well as trace amounts of proteins are not negligible in LC–MS analysis [19]. It has been shown that the matrix effect of urine samples causes a drift in chromatographic RT, in particular to those endogenous metabolites that are less hydrophobic [8]. However, Waybright et al. [8] did not demonstrate any amelioration in RT drift after reduction in matrix effects by sample dilution. Therefore, the effect of sample dilution on the repeatability of RT has not been fully elucidated. In our study however, the drift in RT was within 0.03 min, and was similar for untreated, water diluted and methanol diluted urine samples over the course of 100 injections. It could be argued that the differences in RT drift between the study of Waybright et al. [8] and ours may be due to the difference in instrumentation (HPLC vs UPLC). The use of sub-2 μm column particle sizes and the smaller injection volume required for UPLC has improved the sensitivity and also reduced matrix effects and therefore the drift in RT is comparatively small [20]. Furthermore, there have been no studies demonstrating the effect of the sample matrix on electrospray ionisation for untargeted urinary metabolite profiling. Waybright et al. [8] commented that dilution of urine (1:4, v/v) was not required for retaining low abundant metabolites, however, signal enhancement or suppression with co-eluted analytes could affect quantitative analysis [21,22]. Based on the aforementioned reasons and findings, the water and methanol diluted urine samples were chosen and used further to investigate a matrix effect on data variability over 3 different days. The methanol diluted sample yielded the highest number of features detected with less variation between 3 different days in both ionisation modes (Table 4). Further data analysis showed that the average variability (%CV) in intensity of the 10 selected endogenous species in water diluted and methanol diluted samples was very similar in both positive and negative ionisation modes, Table 5.

Table 5
Average variation (%CV) in intensity of 10 selected endogenous species analysed over 3 days in three urine samples diluted with two different solvents

Species	RT (min)	*m/z*	Positive ionisation mode	
			1:1 dilution water (%)	1:1 dilution methanol (%)
1	0.69	114.067	4	3
2	0.77	143.095	13	8
3	0.82	153.068	4	7
4	2.07	119.082	7	8
5	2.31	125.095	5	5
6	4.35	169.074	3	3
7	4.61	286.202	3	4
8	5.58	302.233	6	6
9	6.36	256.240	6	4
10	6.43	328.248	5	6
Species	RT (min)	*m/z*	Negative ionisation mode	
			1:1 dilution water (%)	1:1 dilution methanol (%)
1	0.50	273.958	3	7
2	0.53	159.978	3	4
3	0.53	161.986	8	4
4	3.04	213.001	7	7
5	3.15	264.104	8	7
6	3.61	188.006	4	4
7	4.13	573.255	5	7
8	4.18	386.170	6	6
9	4.62	601.286	4	5
10	6.36	512.291	7	5

The average %CV of individual species was calculated from variability data obtained over 3 days.

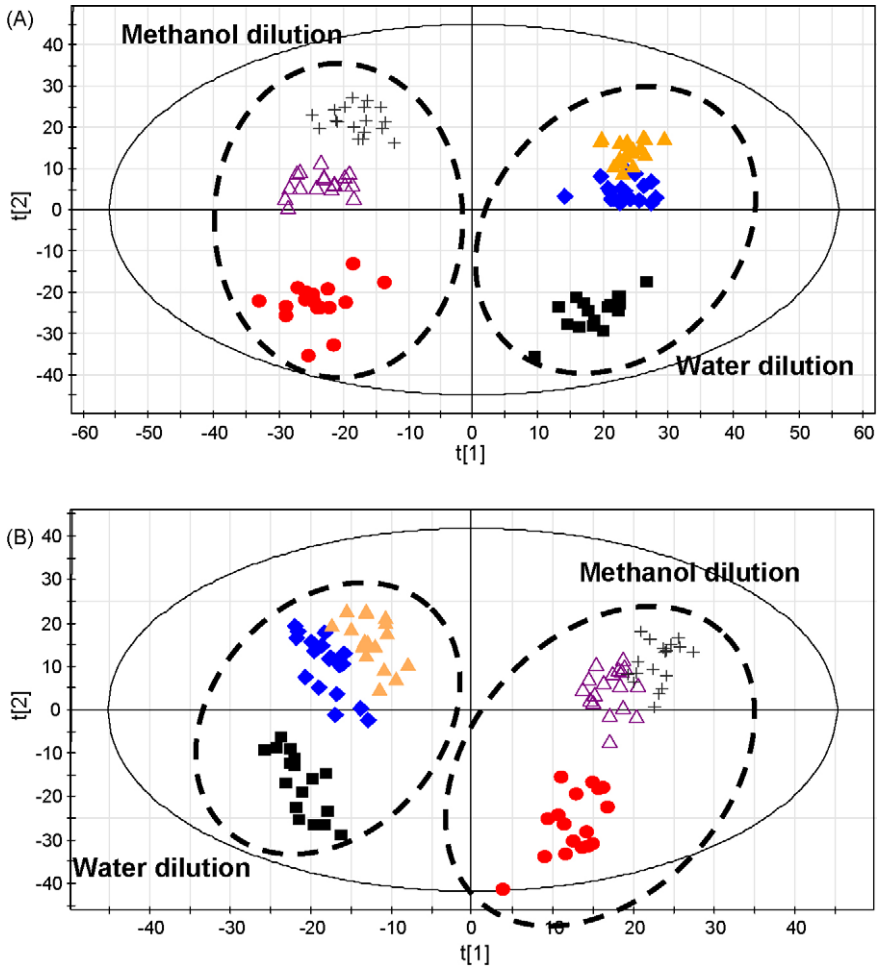

Fig. 4. Influence of sample pre-treatments on inter-day variation on multivariate analysis. PCA scores plots of metabolite profiles of urine diluted with either water or 100% methanol on separate occasions in 3 days in positive (A) and negative (B) ionisation modes. Square (Day 1, water dilution); diamond (Day 2, water dilution); triangle (Day 3, water dilution); circle (Day 1, methanol dilution); open triangle (Day 2, methanol dilution); cross (Day 3, methanol dilution).

The overall quality of the methanol diluted urine samples appeared to be better than those diluted with water, however, the repeatability of the data were further investigated with PCA. Scores plots were used to visualise the similarity of the dataset between the two sample pre-treatments. The PCA scores plot show clear discrimination between the metabolite profiles obtained following either water or methanol dilution in positive ionisation mode, with 15% of variance between treatments (Fig. 4A). A similar finding was also observed in negative ionisation mode with 12% variance between treatments (Fig. 4B). This data suggest that different sample pre-treatments result in different metabolite profiles and also clearly demonstrate the importance in standardising sample pre-treatment procedures for untargeted urinary metabolite profiling.

Furthermore, the inter-day repeatability of these two pre-treatments, as visualised by PCA scores plots, was considerably similar. Samples under the same pre-treatment were clearly separated into clusters along principle component 1 (PC1). With both treatments there was a shift in clustering along PC2 with date of analysis, i.e. inter-day variation. Furthermore, there was a similar variation (average %CV of 5%) in the intensity of the 10 endogenous species in both treatments on each day of analysis. Based on the highest number of features detected and lowest variability, together with the advantage of removing trace amounts of protein, phosphates and sulfates, we conclude from the present study that dilution of urine with methanol (1:1) is the preferred sample pre-treatment for high throughput untargeted urinary metabolite profiling.

Finally, the robustness of the experimental conditions described above was investigated by analysing the quality control injections (pooled urine spiked with sulfadimethoxine at 8 ng/μl final concentration) after every 20 injections over a total of 600 injections of non-QC urine samples. The sulfadimethoxine was used to assess the drift of RT and mass shift, while the QC profiles were visualised in PCA as recommended for monitoring the performance of the analytical system [7,11]. Over the course of 600 injections over a 5-day period the drift in RT was only 0.03 min and mass shift was less than 0.0015 Da. This suggests a stable performance of the system throughout this experimental time frame. Fig. 5 shows a PCA plot of the first three components where 600 injections, including QC and urine samples diluted with methanol (1:1, v/v), were analysed over a 10 min step elution gradient at 400 μl/min flow rate in positive ionisation mode. The tightly clustered QC samples demonstrate that the analytical conditions chosen in the present study provided a robust and reproducible method for untargeted urinary metabolite profiling using UPLC/QToF MS. Furthermore, the inclusion of QC also provides a means to monitor the stability of instrumental performance including mass accuracy, drift of RT, ionisation process and MS sensitivity when handling a large number of samples.

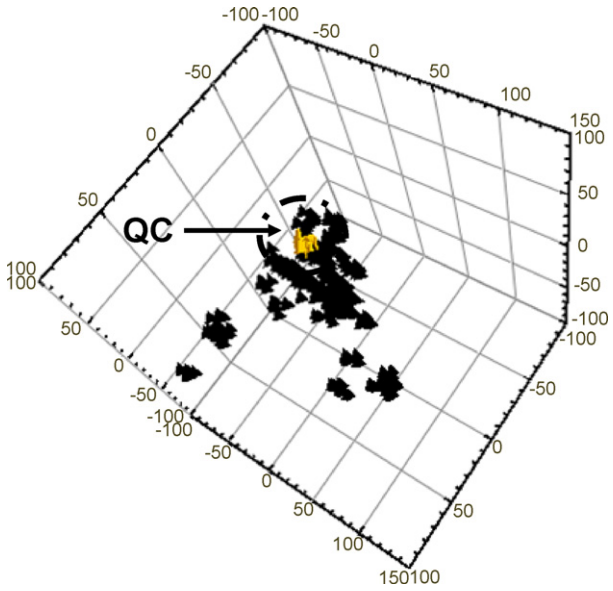

Fig. 5. 3D PCA score plot of urinary metabolite profiles (QC and non-QC urine samples) in positive ionisation mode. The stability of the analytical platform is monitored by the tightness of QC sample clustering as shown.

4. Conclusion

We have evaluated the effects of different sample pre-treatments and experimental conditions for the untargeted metabolite profiling analysis of human urine samples using UPLC/QToF MS. We have demonstrated the importance of optimising the LC conditions as different flow rates and elution gradients have significant effects on the quality of the metabolite profiles. We have also optimised a method which yields acceptable data quality and robustness over a 5-day period as demonstrated by feature variability and tightness of QC clustering. Although UPLC/QToF MS provides a high throughput analytical platform, this study provides further evidence of the importance of monitoring the stability of the analytical platform by using QC samples when handling a large volume of samples. It merits further studies to understand the effect of sample dilution on minimising interferences and/or modifying urinary metabolic profiles.

References

[1] J.K. Nicholson, J. Connelly, J.C. Lindon, E. Holmes, Nat. Rev. Drug Discov. 1 (2002) 153.
[2] R.S. Plumb, C.L. Stumpf, M.V. Gorenstein, J.M. Castro-Perez, G.J. Dear, M. Anthony, B.C. Sweatman, S.C. Connor, J.N. Haselden, Rapid Commun. Mass Spectrom. 16 (2002) 1991.
[3] X. Zhao, W. Wang, J. Wang, J. Yang, G. Xu, J. Sep. Sci. 29 (2006) 2444.
[4] K. Dettmer, P.A. Aronov, B.D. Hammock, Mass Spectrom. Rev. 26 (2007) 51.
[5] I.D. Wilson, R. Plumb, J. Granger, H. Major, R. Williams, E.M. Lenz, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 817 (2005) 67.
[6] H.G. Gika, G.A. Theodoridis, J.E. Wingate, I.D. Wilson, J. Proteome Res. 6 (2007) 3291.
[7] T.P. Sangster, J.E. Wingate, L. Burton, F. Teichert, I.D. Wilson, Rapid Commun. Mass Spectrom. 21 (2007) 2965.
[8] T.J. Waybright, Q.N. Van, G.M. Muschik, T.P. Conrads, T.D. Veenstra, H.J. Issaq, J. Liq. Chromatogr. Relat. Technol. 29 (2006) 2475.
[9] H.G. Gika, G.A. Theodoridis, I.D. Wilson, J. Chromatogr. A 1189 (2008) 314.
[10] R. Plumb, J. Castro-Perez, J. Granger, I. Beattie, K. Joncour, A. Wright, Rapid Commun. Mass Spectrom. 18 (2004) 2331.
[11] T. Sangster, H. Major, R. Plumb, A.J. Wilson, I.D. Wilson, Analyst 131 (2006) 1075.
[12] H.G. Gika, G.A. Theodoridis, I.D. Wilson, J. Sep. Sci. 31 (2008) 1598.
[13] R.S. Plumb, P.D. Rainville, W.B. Potts III, J.M. Castro-Perez, K.A. Johnson, I.D. Wilson, Rapid Commun. Mass Spectrom. 21 (2007) 4079.
[14] H. Pham-Tuan, L. Kaskavelis, C.A. Daykin, H.G. Janssen, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 789 (2003) 283.
[15] R.N. Xu, L. Fan, M.J. Rieser, T.A. El Shourbagy, J. Pharm. Biomed. Anal. 44 (2007) 342.
[16] G. Lu, J. Wang, X. Zhao, H. Kong, G. Xu, Se. Pu. 24 (2006) 109.
[17] P. Jonsson, S.J. Bruce, T. Moritz, J. Trygg, M. Sjostrom, R. Plumb, J. Granger, E. Maibaum, J.K. Nicholson, E. Holmes, H. Antti, Analyst 130 (2005) 701.
[18] R.E. Williams, E.M. Lenz, M. Rantalainen, I.D. Wilson, Mol. Biosyst. 2 (2006) 193.
[19] N.B. Cech, C.G. Enke, Mass Spectrom. Rev. 20 (2001) 362.
[20] E. Chambers, D.M. Wagrowski-Diehl, Z. Lu, J.R. Mazzeo, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 852 (2007) 22.
[21] T.M. Annesley, Clin. Chem. 49 (2003) 1041.
[22] P.J. Taylor, Clin. Biochem. 38 (2005) 328.