biocrates
The future of research and health

Quantitative metabolomics using urine samples with biocrates' MxP® Quant 500 kit across mass spectrometer platforms

Markus Langsdorf, Gregor Ömer, Stephen Dearth, biocrates life sciences ag, Innsbruck, Austria

1 Introduction

Over the past decade, biocrates kit technology has become the gold standard in quantitative metabolomics, opening new possibilities in the study of human health and disease. More recently, the WebIDQ companion software has streamlined the entire process by guiding users through the workflow from start to finish.

While blood plasma or serum have proven to be the preferred choices for many research or diagnostic purposes, urine offers numerous advantages and serves various application areas due to its non-invasive collection method and rich metabolite profile. Urine collection is patient-friendly, being both easy to obtain and available in large volumes, allowing samples to be used for multiple tests and studies or repeated analysis. Disease diagnosis, biomarker discovery, nutritional metabolomics, pharmacometabolomics, toxicology, and sports science have emerged as key application areas benefitting from urinary metabolomics.

biocrates has a longstanding history with urine metabolomics, contributing to the creation of the Urine Metabolome Database[1], which lists more than 5,000 microbial, endogenous and exogenous metabolites found in human urine. These metabolites have been shown in the literature to be associated with more than 300 medical conditions.

For urine analysis with the MxP® Quant 500 and AbsoluteIDQ® p180 kits, a urine extension set has been developed. The extension set consists of calibration standards and urine-based quality controls tailored for urine samples. The extension set allows for accurate metabolite quantification and data normalization across studies, laboratories, and instruments. The urine extension set was validated on human urine and has been successfully tested on rat urine as well. The performance results using the MxP Quant 500 kit with three different mass spectrometer platforms are summarized in this application note.

2 Materials and methods

The MxP Quant 500 kit consists of system suitability test samples and a patented 96-well filter plate with internal standards already integrated. The urine extension set has been used for sample analysis, consisting of:

- **Calibration standards**
  7 lyophilized concentration levels with ranges typically observed in human urine for accurate quantification.
- **Urine-based quality controls (QCs)**
  3 lyophilized QC levels for performance check and accurate data normalization.
- **Zero sample**
  Urine-like matrix with similar salt composition and background signal for calculation of limits of detection (LODs).
- **Instrument-specific methods**
  Optimized MRMs around expected urine metabolite abundances.
- **Sample preparation protocol**
  Modified workflow to improve derivatization of urine metabolites.

The calibration standards and QCs were reconstituted according to a new revised urine protocol. Experimental samples, consisting of 31 human and 4 rat urine samples from healthy subjects (absence of disease diagnosis), were registered in WebIDQ software and arranged together with the calibration and QC samples on a 96-well plate layout. The QCs were measured in replicates of three. The worklist was directly exported to the mass spectrometer software and the layout printed for kit preparation. The kit was prepared according to the protocol for urine samples and kit user manual with 10 µL of sample pipetted per well followed by derivatization, extraction, and finally dilution into two separate measurement plates: one for LC-MS/MS and one for FIA-MS/MS.

Plates were measured on three different triple quadrupole mass spectrometers:

- Agilent 6495C triple quadrupole
- SCIEX Triple quad 5500+
- Waters Xevo TQ-XS triple quadrupole

Data files were directly processed in WebIDQ with automated quantification, validation, and normalization. The urine-based QCs at different concentration levels were used to automatically assess performance, checking both accuracy and reproducibility. QC level 2 represents endogenous metabolite concentrations in urine samples of healthy human subjects and was used for data normalization as an essential part of the workflow to correct for any deviations caused by preparation, measurement, or batch effects. Creatinine is used specifically for normalization of urine concentrations[2] and has also been used here. Quantified data was exported and evaluated in R.

3 Results and discussion

Detectability

The detectability was defined as the number of metabolites above LOD with coefficient of variation (CV) below 30%. The detectability across the three instrument types was comparable. Up to 61% of the 147 small molecules and acylcarnitines (14% of the total kit panel of 630 metabolites including all lipids) were found to be above the LOD in more than 50% of both human and rat urine samples, whereas the overall concentrations in rat urine were found to be higher. As expected in urine samples, lipid detectability showed a very low coverage.

Out of the 107 small molecules, 72 (67%) could be detected across all biochemical classes of the MxP Quant 500 kit on all three mass spectrometers in more than 50% of the human samples. Only fatty acids were not detected among the small molecules.

Out of the 523 lipids and acylcarnitines measured in the FIA part, 17 of the 40 acylcarnitines were detected in more than 50% of the human samples. Lipids were either not above LOD or sporadically detected in nano molar ranges. With the exception of SM 34:1, there was no lipid to be found above LOD on all three mass spectrometers in more than 25% of the samples.

An overview of the detectability of individual metabolites is shown in section 6.

Reproducibility

The urine QCs, measured in triplicates, showed comparable intra-plate CV distribution per instrument, as well as inter-instrument. All median CVs were below 5% for metabolites measured above LOD (**Figure 1** and **2**).

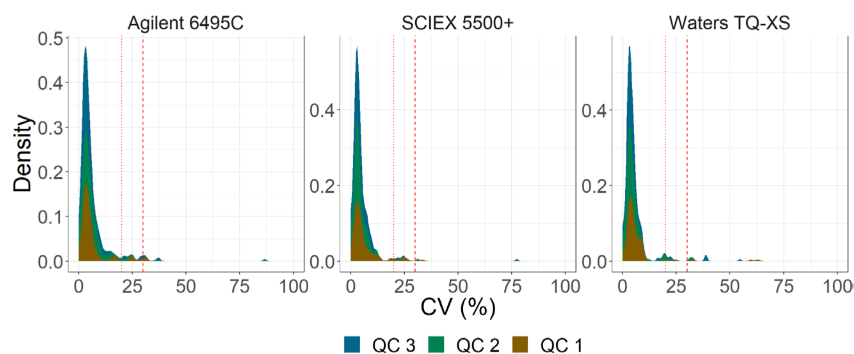

Figure 1: Inter-plate CV for each instrument (n = 3) after QC2 and creatinine normalization for each QC level.

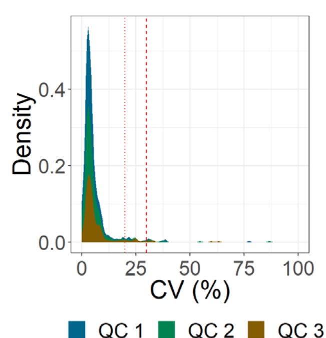

Figure 2: Inter-plate (n= 3) and instrument (N= 3) CV after QC2 and creatinine normalization for each QC level.

Inter-instrument comparability

Analyte concentrations were comparable across all tested LC-MS platforms after target value normalization to the urine-based QC level 2. **Figure 3** shows the benefit and need for data normalization using a sample of the same matrix.

Inter- instrument correlation

Figure 4 shows the correlation of concentration values of the SCIEX 5500+ to Agilent 6495C and Waters Xevo TQ-XS, respectively. The results show an excellent inter-instrument correlation.

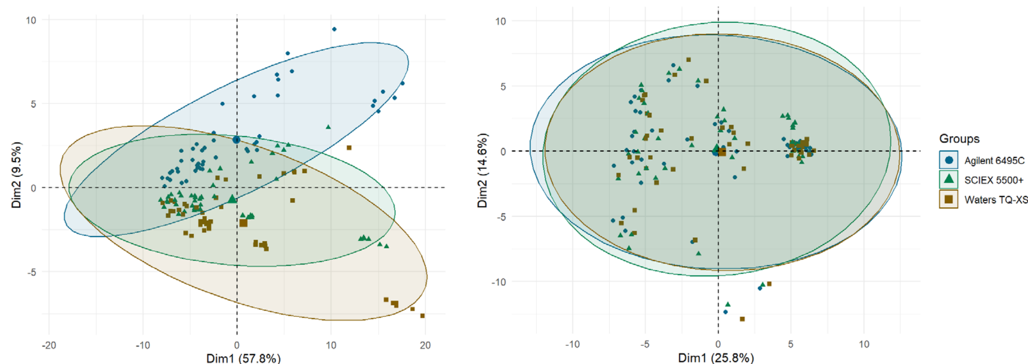

Figure 3: Comparability of concentrations without normalization (left) and normalized to the urine-based QC2 from the urine extension (right).

biocrates
The future of research and health

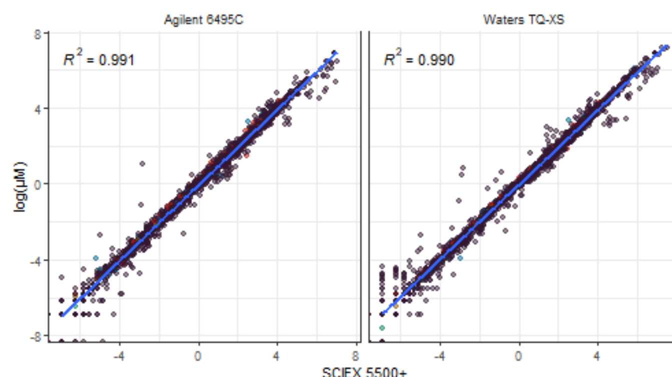

Figure 4: Correlation of urine sample concentrations from SCIEX 5500+ compared to Agilent 6495C and Waters Xevo TQ-XS, respectively.

4 Conclusions

The results showed high reproducibility and strong correlation across all mass spectrometers used. All instruments displayed comparable coverage with the majority of the small molecules measured in the LC-MS/MS part of the kit detected with a CV < 5%. In the FIA-MS/MS part, the main acylcarnitines were well detected, but as expected in this matrix, most of the lipids were measured below LOD in most samples. Taken together, the MxP Quant 500 kit with urine extension (and by extension the AbsoluteIDQ p180 kit) demonstrated a robust and reproducible method for quantifying biologically relevant metabolites in urine.

5 References

1. Bouatra et al. The human urine metabolome. PLoS One 2013; 8(9):e73076
2. Waikar et al. Normalization of urinary biomarkers to creatinine during changes in glomerular filtration rate. Kidney Int 2010; 78(5):486-94

6 Appendix: Overview of metabolite detectability in human urine

An overview of the metabolite detectability across the 31 human samples is provided in the tables below. The metabolites of the MxP Quant 500 kit were categorized by class (small molecules and acylcarnitines) and divided into four color-coded groups according to the percentage of metabolites detected above LOD with CV <30%:
Green >75%,
Blue 50-75%,
Yellow 25-50%
Red <25%.
Metabolites marked with an asterisk are covered by the AbsoluteIDQ p180 kit.

This overview is for orientation purposes only. The detectability for each analyte will depend on the instrument condition in addition to the nature and the quality of the sample. The lipid classes are not shown due to general low detectability in urine samples as described above.

Alkaloids (1)
Trigonelline

Amine oxides (1)
TMAO

Amino acids (20)			
Ala*	Glu*	Leu*	Ser*
Arg*	Gln*	Lys*	Thr*
Asn*	Gly*	Met*	Trp*
Asp*	His*	Phe*	Tyr*
Cys	Ile*	Pro*	Val*

Amino acid related (30)			
alpha-AAA*	Carnosine*	t4-OH-Pro*	PheAlaBetaine
AABA	Cit*	Kynurenine*	ProBetaine
Ac-Orn*	Creatinine*	Met-SO*	Sarcosine*
ADMA*	Cystine	1-Met-His	SDMA*
Anserine	DOPA*	3-Met-His	Taurine*
5-AVA	HArg	Nitro-Tyr*	TrpBetaine
BABA	HCys	Orn*	
Betaine	c4-OH-Pro*	PAG	

Bile acids (14)			
CA	GDCA	GUDCA	TLCA
CDCA	GCDCA	TCA	TMCA
DCA	GLCA	TCDCA	
GCA	GLCAS	TDCA	

Biogenic amines (9)			
beta-Ala	Histamine*	Putrescine*	Spermidine*
GABA	PEA*	Serotonin*	Spermine*
Dopamine*			

Carbohydrates and related (1)
Hexoses (including glucose)*

Carboxylic acids (7)			
AconAcid	DiCA(14:0)	OH-GlutAcid	Suc
DiCA(12:0)	HipAcid	Lac	

Cresols (1)
p-Cresol-SO_4

Fatty acids (12)			
FA(12:0)	FA(18:0)	FA(20:1)	AA
FA(14:0)	FA(18:1)	FA(20:2)	EPA
FA(16:0)	FA(18:2)	FA(20:3)	DHA

Hormones and related (4)			
AbsAcid	Cortisol	Cortisone	DHEAS

Indoles and derivatives (4)			
Indole	3-IAA	3-IPA	Ind-SO_4

Nucleobases and related (2)	
Hypoxanthine	Xanthine

Vitamins and cofactors (1)
Choline

Acylcarnitines (40)			
C0*	C5-M-DC*	C10:1*	C16*
C2*	C5-OH (C3-DC-M)*	C10:2*	C16-OH*
C3*	C5:1*	C12*	C16:1*
C3-DC (C4-OH)*	C5:1-DC*	C12-DC*	C16:1-OH*
C3-OH*	C6 (C4:1-DC)*	C12:1*	C16:2*
C3:1*	C6:1*	C14*	C16:2-OH*
C4*	C7-DC*	C14:1*	C18*
C4:1*	C8*	C14:1-OH*	C18:1*
C5*	C9*	C14:2*	C18:1-OH*
C5-DC (C6-OH)*	C10*	C14:2-OH*	C18:2*