Standardized Targeted Metabolome Analysis in Plasma Samples Using the Absolute*IDQ*® p180 Kit and Waters® ACQUITY UPLC® I-Class together with Xevo® TQ-S micro MS

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1 Introduction

Metabolic signatures give important information about pathophysiological mechanisms and help improve the understanding of diseases [1-3]. The access to a systemic readout of metabolites in blood enables monitoring of all major pathways. Mass spectrometry is one of the fundamental analytical techniques presently used in metabolic phenotyping [4]. To obtain reliable analytical results and improve interlaboratory comparability, automation and standardization of the metabolomics workflow are of utmost importance. Here we present a standardized quantitative Kit that allows the simultaneous analysis of more than 180 endogenous metabolites in 10 µL as typical sample volume from five different key metabolite classes (Figure 1).

188 Metabolites from five key analyte classes

Acylcarnitines:	40
Amino Acids:	19 proteinogenic + ornithine and citrulline
Biogenic Amines:	21
Hexose:	1 (sum of hexoses)

Phospho- and Sphingolipids:

- Phosphatidylcholines	76 (plasmalogens included)
- Lyso-Phosphatidylcholines	14
- Sphingomyelins	15

Figure 1: Metabolite panel of the AbsoluteIDQ® p180 Kit.

The Kit combines UPLC-MS/MS of amino acids and biogenic amines followed by FIA-MS/MS of acylcarnitines, hexoses, phospholipids and sphingolipids into a single workflow.

The Absolute*IDQ*® p180 Kit was validated on a Xevo® TQ-S micro system for human plasma and rat plasma, considering EMA guidelines.

2 Materials and Method

An easy to use and rapid sample preparation with a specially designed 96-well filter plate allows high throughput analysis with minimal amounts of sample volume (10 µL). Blank and zero samples, 7 calibration standards, 3 levels of quality control samples (QC, human plasma based) and plasma samples were analyzed by UPLC-ESI-MS/MS in multiple reaction monitoring (MRM) mode, followed by FIA-ESI-MS/MS. A Waters® UPLC® I-Class system was coupled to a Xevo® TQ-S micro mass spectrometer (Waters Inc.). A BEH C18 (2.1 x 75 mm, 1.7 µm) UPLC column was used for chromatographic separation of amino acids and biogenic amines. Data analysis of >22 000 MRM chromatograms from >230 metabolites and internal standards analyzed in a single filter plate (96 well format) was achieved. Analysis of LC data was performed with TargetLynx (Waters); quantitation is based on an external 7 point calibration. FIA data was converted and imported using the Biocrates® Met*IDQ*™ software. The multifunctional Met*IDQ*™ software enables a workflow automatization, assisting the user with the sample registration, data processing of FIA and LC data, technical validation and statistical analysis.

3 Results and Discussion

Optimization of injection volumes and extract dilutions were performed. The Acquity I-Class system comprising a Sample Manager with Flow-Through-Needle (SM-FTN) showed best peak capacity for analysis of amino acids and biogenic amines (UPLC-MS/MS based metabolites) with an injection volume of 2 µl and an extract dilution at the ratio of 1:2 (dilution with Milli-Q water).

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A TIC chromatogram of amino acids and biogenic amines in a calibration standard is shown in Figure 2.

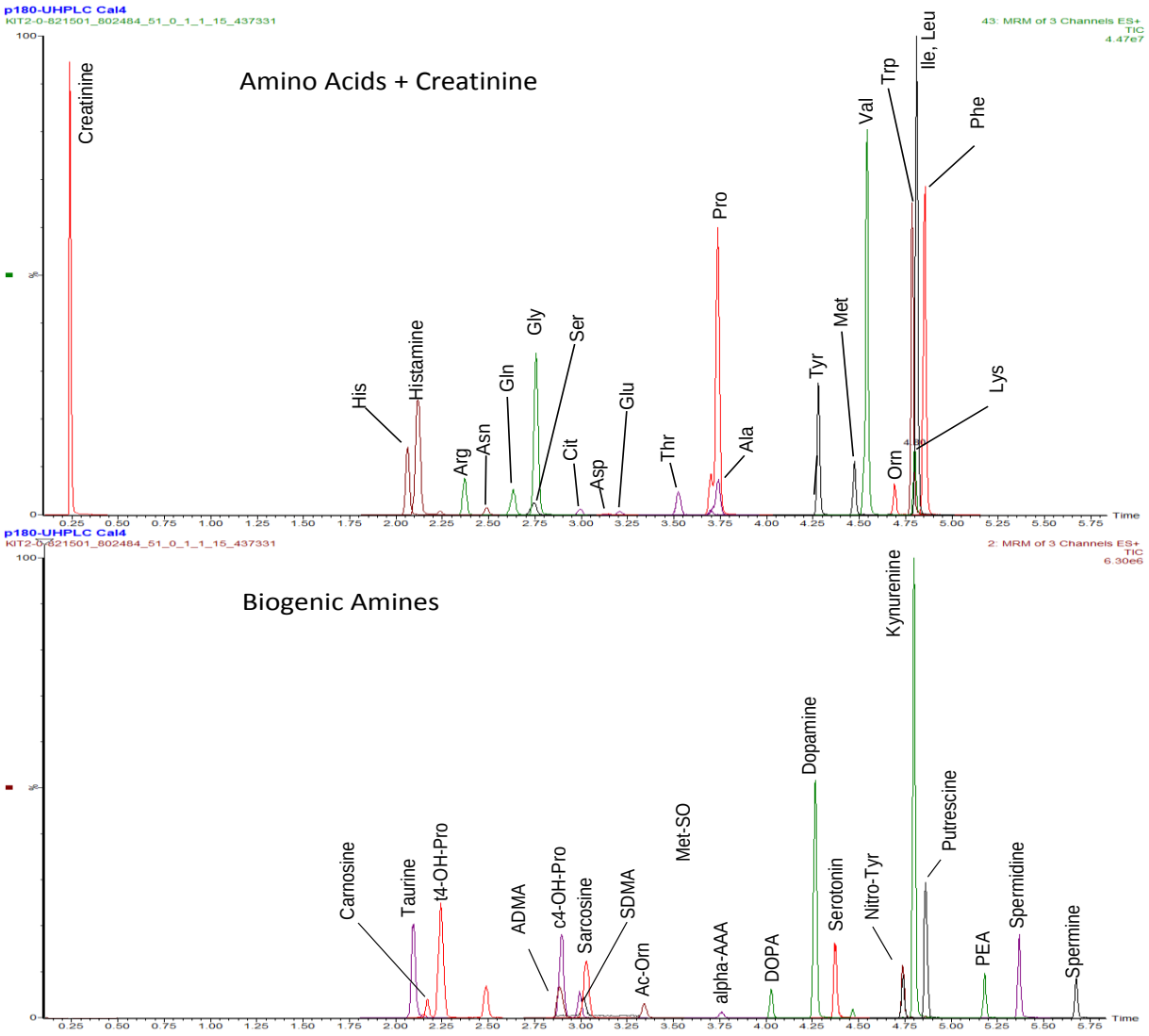

Figure 2: UPLC-MS/MS TIC chromatograms of a calibration standard level 4. Upper chromatogram: amino acids and creatinine; lower chromatogram: biogenic amines

Various extract dilutions were tested for the FIA-MS/MS analysis on the Xevo® TQ-S micro system. For each MS instrument an adequate extract dilution has to be determined to achieve a good compromise between acceptable ion suppression and signal intensity.
A high extract dilution decreases ion suppression that is caused by high abundant metabolites.

Increasing the dilution on the other hand can lead to a loss of signal intensity and thus sensitivity. Different dilutions were tested: extracts were diluted with FIA running solvent B at ratios of 1:3 to 1:25. The high instrument sensitivity of the Xevo® TQ-S micro enabled the optimum extract dilution of 1:20 and an injection volume of 10 µl.

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Validation: Overview

The Absolute*IDQ*® p180 Kit was validated for human and rat plasma on a Xevo® TQ-S micro system. The following tables 1 and 2 give an overview of the validity of amino acids and biogenic amines (UPLC-MS/MS based metabolites) in human plasma and rat plasma.

The validity of the analytes in respective matrix was evaluated in terms of intraday (within batch) and interday (batch-to-batch) precision and accuracy, as well as sensitivity, robustness and selectivity.

Table 1: Analytical validity of amino acids in human plasma and rat plasma

Amino Acid	Validity in human plasma		Validity in rat plasma	
	Valid	Valid with restrictions	Valid	Valid with restrictions
Ala	X		X	
Arg	X		X	
Asn	X		X	
Asp	X		X	
Cit	X		X	
Gln	X		X	
Glu	X		X	
Gly	X		X	
His	X		X	
Ile	X			X[1]
Leu	X			X[1]
Lys	X			
Met	X		X	
Orn	X		X	
Phe	X		X	
Pro	X		X	
Ser	X			X[1]
Thr	X			X[1]
Trp	X		X	
Tyr	X		X	
Val	X		X	

1) Restriction criteria: interday/intraday precision
2) Restriction criteria: interday/intraday accuracy

Table 2: Analytical validity of biogenic amines in human plasma and rat plasma

Biogenic Amine	Validity in human plasma		Validity in rat plasma	
	Valid	Valid with restrictions	Valid	Valid with restrictions
Ac-Orn	X		X	
ADMA	X		X	
SDMA	X		X	
alpha-AAA		X[2]		X[2]
Carnosine	X		X	
Creatinine	X (LLOQ Cal2)			
DOPA	X		X	
Dopamine	X		X	
Histamine		X[2]		X[2]
Kynurenine		X[2]		X[2]
Met-SO	X		X	
Nitro-Tyr		X[1]		X[1]
c4-OH-Pro	X		X	
t4-OH-Pro	X		X	
PEA		X[2]		X[2]
Putrescine	X		X	
Sarcosine	X		X	
Serotonin	X		X	
Spermidine	X		X	
Spermine		X[2]		X[2]
Taurine	X		X	

1) Restriction criteria: interday/intraday precision
2) Restriction criteria: interday/intraday accuracy

The FIA-MS/MS analysis with the p180 Kit enables determination of a broad spectrum of physiological metabolites: lipids, acylcarnitines and sugars. An overview of the validity of acylcarnitines in human and rat plasma is given in Table 3.
Besides acylcarnitines and hexoses, the Absolute*IDQ*® p180 Kit comprises FIA-MS/MS analysis of following lipids:

14 lyso-phosphatidylcholines (lysoPC), 76 phosphatidylcholines (PC) and 15 sphingomyelins (SM).
Since not all FIA metabolites (i.e. lipids and a subset of acylcarnitines) were commercially available as external and internal standards, their accuracy could not be verified. Thus, the validity of these analytes is termed “relative quantitative”. Nevertheless, robust values with high precision were obtained for all metabolite classes.

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Table 3: Analytical validity of acylcarnitines in human plasma and rat plasma

Compound		Validity in human plasma		Validity in rat plasma	
MetIDQ Short Name	Biochemical Name	Valid	Relative quant	Valid	Relative quant
C0	Carnitine	x		x	
C2	Acetylcarnitine	x		x	
C3	Propionylcarnitine*	x		x	
C3:1	Propenoylcarnitine		x		x
C3-OH	Hydroxypropionyl carnitine*		x		x
C4	Butyrylcarnitine*	x		x	
C4:1	Butenylcarnitine		x		x
C4-OH (C3-DC)	Hydroxybutyrylcarnitine*		x		x
C5	Valerylcarnitine*	x		x	
C5:1	Tiglylcarnitine		x		x
C5:1-DC	Glutaconylcarnitine		x		x
C5-DC (C6-OH)	Glutarylcarnitine* (Hydroxyhexanoyl-carnitine)		x		x
C5-M-DC	Methylglutarylcarnitine		x		x
C5-OH (C3-DC-M)	Hydroxyvalerylcarnitine* (Methylmalonylcarnitine)		x		x
C6 (C4:1-DC)	Hexanoylcarnitine* (Fumarylcarnitine)	x		x	
C6:1	Hexenoylcarnitine		x		x
C7-DC	Pimelylcarnitine*		x		x
C8	Octanoylcarnitine*	x		x	
C9	Nonaylcarnitine*		x		x
C10	Decanoylcarnitine*	x		x	
C10:1	Decenoylcarnitine*		x		x
C10:2	Decadienylcarnitine		x		x
C12	Dodecanoylcarnitine*	x		x	
C12:1	Dodecenoylcarnitine*		x		x
C12-DC	Dodecanedioylcarnitine*		x		x
C14	Tetradecanoylcarnitine*	x		x	
C14:1	Tetradecenoylcarnitine*		x		x
C14:1-OH	Hydroxytetradecenoyl-carnitine*		x		x
C14:2	Tetradecadienylcarnitine		x		x
C14:2-OH	Hydroxytetradecadienyl-carnitine		x		x
C16	Hexadecanoylcarnitine*	x		x	
C16:1	Hexadecenoylcarnitine*		x		x
C16:1-OH	Hydroxyhexadecenoyl-carnitine*		x		x
C16:2	Hexadecadienylcarnitine		x		x
C16:2-OH	Hydroxyhexadecadienyl-carnitine		x		x
C16-OH	Hydroxyhexadecanoyl-carnitine*		x		x
C18	Octadecanoylcarnitine*	x		x	
C18:1	Octadecenoylcarnitine*		x		x
C18:1-OH	Hydroxyoctadecenoyl-carnitine		x		x
C18:2	Octadecadienylcarnitine		x		x

* Concentrations are isotope corrected

Performance of the Absolute*IDQ*® p180 Kit on the Xevo® TQ-S micro system was compared to the Kit performance on a Xevo® TQ-MS system coupled to an Acquity UPLC system.
During validation the high sensitivity and robustness of the Xevo® TQ-S micro system was confirmed. Several amino acids and biogenic amines could be quantitated with higher precision and accuracy compared to analysis on the Xevo® TQ-MS. Thus following metabolites are valid for analysis on the Xevo® TQ-S micro, but valid with restriction on the Xevo® TQ-MS: Asp, Cit, Leu, AcOrn, ADMA, SDMA, Creatinine, MetSO and Spermidine.
The higher sensitivity of the Xevo® TQ-S micro also became apparent for sample preparation: For the analysis of amino acids and biogenic amines a lower injection volume was required (2 µl compared to 5 µl). Further, dilution of the extract for FIA analysis was 1:20 for the Xevo® TQ-S micro and 1:4 for the Xevo® TQ-MS.

Validation: UPLC-MS/MS Intraday precision and accuracy (n = 6)

Tables 4 and 5 show the performance at endogenous, diluted and spiked concentration levels of amino acids and biogenic amines in human plasma samples. Excellent intraday precision with values < 15% were obtained for all analytes in human plasma. Excellent accuracies were obtained for most analytes. Accuracy values > ±20% were observed for Lys, Orn, α-AAA, Histamine, Kynurenine, PEA and Spermine.

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Table 4: Intraday precision [%] of amino acids and biogenic amines in human plasma samples (n = 6).

	Human Plasma, Intraday Precision [%]				
Amino Acid	diluted 1:5	diluted 1:2	Endogenous level	Spiked with cal2	Spiked with cal3
Ala	5.8	5.5	4.2	5.8	8.1
Arg	4.4	7.9	3.8	2.7	5.1
Asn	n.d.	3.8	2.9	2.6	3.8
Asp	n.d.	n.d.	n.d.	n.d.	13.2
Cit	11.7	7.3	3.9	5.6	3.7
Gln	3.8	8.3	4.6	4.5	5.1
Glu	6.4	4.4	5.7	4.5	2.9
Gly	4.5	6.9	4.6	2.6	4.4
His	5.0	7.5	4.1	2.9	3.8
Ile	4.5	7.1	5.2	3.2	3.0
Leu	n.d.	n.d.	5.0	6.6	2.7
Lys	7.6	9.4	6.4	4.2	3.5
Met	n.d.	8.9	5.5	3.6	3.0
Orn	5.9	11.0	4.4	2.8	4.6
Phe	3.8	7.4	2.3	1.9	3.3
Pro	6.0	6.8	2.5	1.5	4.8
Ser	8.2	10.5	3.9	3.6	4.7
Thr	6.2	5.1	7.3	3.4	4.0
Trp	6.1	7.2	3.8	1.6	3.2
Tyr	4.1	5.8	4.0	1.4	3.4
Val	5.9	8.4	4.7	4.1	4.1

n.d. … not determined, value < LLOQ

	Human Plasma, Intraday Precision [%]				
Biogenic Amine	diluted 1:5	diluted 1:2	Endogenous level	Spiked with cal2	Spiked with cal3
Ac-Orn	n.d.	n.d.	n.d.	4.1	4.2
ADMA	n.d.	n.d.	7.6	5.0	5.9
SDMA	n.d.	6.5	6.8	6.8	5.5
alpha-AAA	n.d.	n.d.	n.d.	7.3	3.4
Carnosine	n.d.	n.d.	n.d.	7.7	5.3
Creatinine	3.1	3.1	2.5	3.4	2.7
DOPA	n.d.	n.d.	n.d.	3.9	1.5
Dopamine	n.d.	n.d.	n.d.	4.1	2.8
Histamine	n.d.	n.d.	n.d.	4.2	4.0
Kynurenine	n.d.	n.d.	10.3	4.6	7.4
Met-SO	n.d.	n.d.	n.d.	8.6	3.7
Nitro-Tyr	n.d.	n.d.	n.d.	3.0	3.8
c4-OH-Pro	n.d.	n.d.	n.d.	9.2	6.1
t4-OH-Pro	4.0	6.7	3.0	2.0	5.1
PEA	n.d.	n.d.	n.d.	5.9	4.1
Putrescine	n.d.	n.d.	n.d.	10.5	6.0
Sarcosine	n.d.	n.d.	10.6	3.6	2.8
Serotonin	n.d.	n.d.	n.d.	5.0	7.4
Spermidine	n.d.	n.d.	n.d.	14.3	5.1
Spermine	n.d.	n.d.	n.d.	11.2	10.3
Taurine	6.3	3.0	3.0	3.2	1.3

n.d. … not determined, value < LLOQ

Table 5: Intraday accuracy [%] of amino acids and biogenic amines in human plasma samples (n = 6).

	Human Plasma, Intraday Accuracy [%]			
Amino Acid	diluted 1:5	diluted 1:2	spiked with cal2	spiked with cal3
Ala	102.8	103.0	97.3	98.6
Arg	94.2	100.0	110.7	111.0
Asn	n.d.	96.6	100.3	105.3
Asp	n.d.	n.d.	n.d.	109.3
Cit	104.8	102.6	101.7	104.3
Gln	96.3	98.3	112.9	115.7
Glu	96.0	97.0	101.3	105.9
Gly	96.5	99.5	109.2	112.9
His	95.5	99.4	108.6	112.6
Ile	95.0	98.9	110.0	114.0
Leu	n.d.	n.d.	113.5	115.4
Lys	95.5	96.6	119.4	133.0
Met	n.d.	97.8	106.7	111.5
Orn	92.7	93.8	117.9	124.0
Phe	95.0	101.6	107.2	111.0
Pro	96.5	104.5	109.0	109.4
Ser	91.1	97.2	113.1	115.0
Thr	89.5	98.9	103.9	101.0
Trp	93.4	100.3	108.1	107.4
Tyr	94.1	99.6	108.9	109.7
Val	93.5	96.8	108.7	112.4

n.d. … not determined, value < LLOQ

	Human Plasma, Intraday Accuracy [%]			
Biogenic Amine	diluted 1:5	diluted 1:2	spiked with cal2	spiked with cal3
Ac-Orn	n.d.	n.d.	100.7	101.0
ADMA	n.d.	n.d.	100.2	98.4
SDMA	n.d.	95.4	101.1	96.8
alpha-AAA	n.d.	n.d.	83.6	76.6
Carnosine	n.d.	n.d.	94.3	103.1
Creatinine	121.6	109.0	97.8	98.5
DOPA	n.d.	n.d.	92.9	100.3
Dopamine	n.d.	n.d.	100.0	101.4
Histamine	n.d.	n.d.	70.5	69.5
Kynurenine	n.d.	n.d.	122.0	124.6
Met-SO	n.d.	n.d.	114.2	104.5
Nitro-Tyr	n.d.	n.d.	91.9	87.1
c4-OH-Pro	n.d.	n.d.	107.9	99.2
t4-OH-Pro	109.5	100.4	106.9	104.6
PEA	n.d.	n.d.	131.3	86.7
Putrescine	n.d.	n.d.	102.0	90.0
Sarcosine	n.d.	n.d.	97.6	98.4
Serotonin	n.d.	n.d.	101.6	101.1
Spermidine	n.d.	n.d.	112.6	105.3
Spermine	n.d.	n.d.	136.4	127.5
Taurine	97.5	99.4	101.3	100.1

n.d. … not determined, value < LLOQ

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Validation:

FIA-MS/MS Interday Precision (n = 4)

Interday precision of the FIA-MS/MS metabolites was determined in 3 levels of quality controls (low, medium and high concentration), human plasma samples (5 individual, 1 pooled) and rat plasma samples (4 individual, 1 pooled) on 4 different days. Excellent average precision was observed for all metabolite classes with values < 14% (Figure 3).

Reference to Standard Reference Material (SRM) 1950: analysis of amino acids, creatinine and glucose

The excellent performance of the Absolute*IDQ*® p180 Kit was further demonstrated by analyzing the standard reference material NIST 1950 with the Xevo® TQ-S micro system: Excellent accuracy and precision were obtained for all SRM specified amino acids, creatinine and glucose (Figure 4).

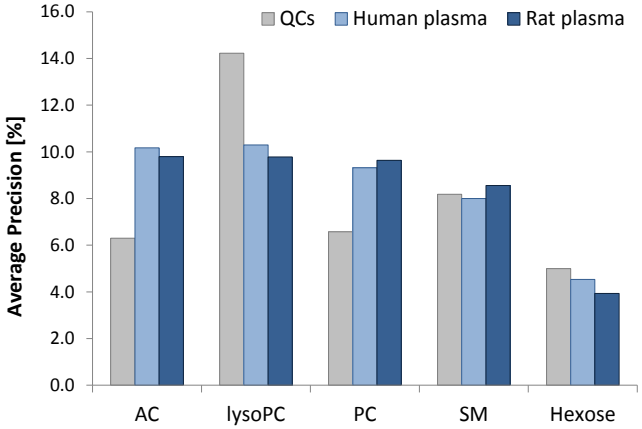

Figure 3: Average interday precision of FIA metabolite classes in quality controls (QCs), human and rat plasma samples.

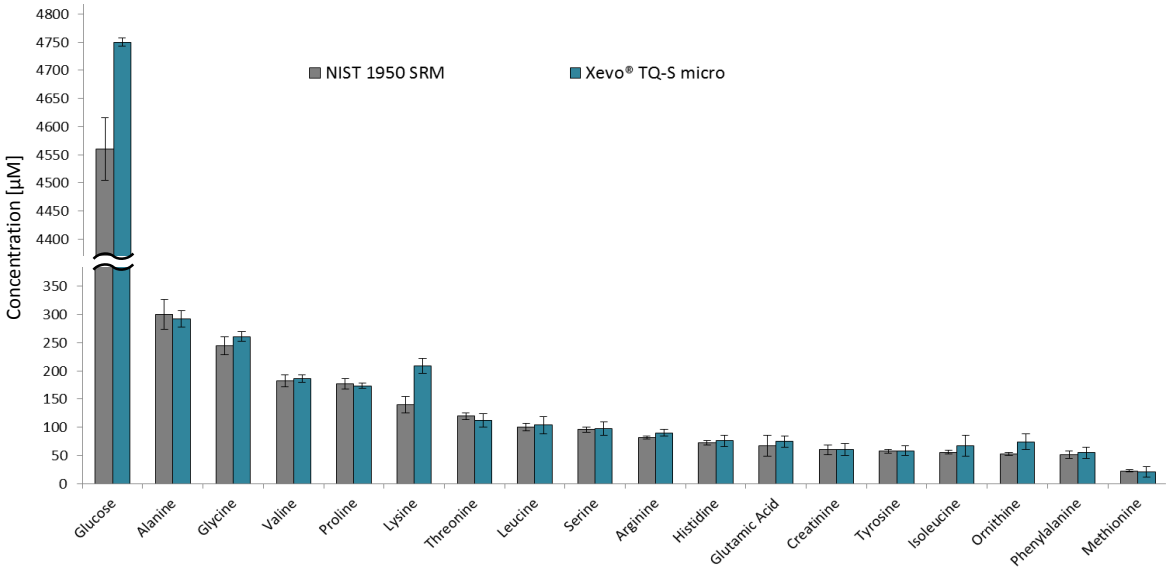

Figure 4: UPLC® Xevo® TQ-S micro results for the NIST standard reference material 1950 in comparison to the target concentration values.

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4 Conclusions

The data presented in this application note reveal that accurate and precise results are obtained for a broad range of metabolites with the Absolute*IDQ*® p180 Kit in human and rat plasma using the Waters® ACQUITY UPLC® I-Class Xevo® TQ-S micro system. The whole process from sample thawing to the final technically validated concentration table was performed within 25 hours for 82 samples, hence, enabling high sample throughput.

Metabolite analysis with the Absolute*IDQ*® p180 Kit demonstrates reliable analytical results, automation and standardization, which is of utmost importance to enable inter-laboratory comparability and inter-instrument robustness.

In summary, the developed and validated Kit on a Waters® UPLC® Xevo® TQ-S micro instrument is a powerful tool for the targeted quantitative analysis of the blood metabolome.

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