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Standardized targeted metabolomics using the biocrates MxP® Quant 500 kit on the Agilent 6495C triple quadrupole LC/MS system

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1 Introduction

Metabolic signatures can provide crucial insights into the physiological mechanisms as well as a better understanding of diseases. For comprehensive metabolomics and lipidomics analyses, analytical reliability, inter-laboratory comparability, automation, and standardization are of utmost importance.

Here, we present the validation of the quantitative MxP® Quant 500 kit on an Agilent 1290 Infinity II – 6495C TQ LC/MS system. The assay offers multiplexed MS/MS analysis of up to 630 metabolites and lipids from 26 analyte classes in only 10 µL sample volume. In addition, 234 pre-defined metabolism indicators, which are biologically meaningful sums and ratios of metabolites and lipids, can be calculated by biocrates' MetIDQ™ software tool MetaboINDICATOR™. Combined, these are 854 metabolic features that can be determined in one experiment with the MxP® Quant 500 kit. The kit allows standardized analysis in a variety of biological sample matrices (e.g. blood plasma/serum, feces, tissue) and species, including studies of the gut microbiome. The MxP® Quant 500 kit combines LC-MS/MS of 13 compound classes, including small molecules, bile acids, and free fatty acids, followed by FIA-MS/MS of 12 lipid classes (including acylcarnitines) and hexoses, into a single workflow (Figure 1).

The Agilent 6495C TQ mass spectrometer offers a very broad dynamic range and excellent sensitivity. Analytical validation was performed on this platform coupled with an Agilent 1290 Infinity II UHPLC frontend for human plasma, following EMA and FDA guidelines.

2 Materials and method

High throughput analysis with a minimal sample volume (10 µL) was achieved by an easy and rapid sample preparation using a patented 96-well filter plate as described in the MxP® Quant 500 kit user manual. Blank and zero samples, 7 calibration standards, 3 levels of quality control samples (human plasma-based QCs) and a variety of plasma samples were subjected to two UHPLC-ESI-MS/MS analyses in multiple reaction monitoring (MRM) mode, followed by three FIA-MS/MS runs. Figure 2 shows two representative chromatograms (positive & negative ion modes) for a human plasma sample. For quantitation, both LC and FIA data were imported directly into the biocrates software MetIDQ™ Oxygen. MetIDQ™ includes an automated target normalization procedure based on QC or sample pool for batch-to-batch and kit plate-to-plate correction for sample cohort across several kit plates.

LC-MS/MS (13 small molecule classes)	FIA-MS/MS (hexoses & 12 lipid classes)
– Alkaloids (1) – Amine oxides (1) – Amino acids (20) – Amino acid related (30) – Bile acids (14) – Biogenic amines (9) – Carboxylic acids (7) – Cresols (1) – Fatty acids (12) – Hormones and related (4) – Indoles and derivatives (4) – Nucleobases and related (2) – Vitamins and cofactors (1)	– Carbohydrates and related (1) – Acylcarnitines (40) – Phosphatidylcholines (74) – Lysophosphatidylcholines (14) – Cholesteryl esters (22) – Sphingomyelins (14) – Ceramides (28) – Dihydroceramides (8) – Hexosylceramides (20) – Dihexosylceramides (9) – Trihexosylceramides (6) – Diacylglycerols (44) – Triacylglycerols (242)

Figure 1: The MxP® Quant 500 kit metabolite panel.

3 Results and discussion

The adaption of the ready-to-use MxP® Quant 500 kit to the Agilent 6495C TQ LC/MS system comprised the optimization of instrumental parameters from sample preparation setup to mass spectrometric features. In sum, combining the LC-MS/MS part and FIA-MS/MS part, the total analysis run time was 40 hours for 80 samples plus quality control samples, calibration standards, a blank, and zero samples on a 96-well plate. Thus, up to three kits can be measured per week and MS instrument.

A comprehensive set of defined human plasma samples was used for the validation of the MxP® Quant 500 kit on the Agilent 6495C TQ mass spectrometer coupled with an Agilent 1290 Infinity II UHPLC. To determine the validity of the analytes, intra-day (within batch) and inter-day (batch-to-batch) analyses were evaluated in terms of precision and accuracy, as well as detection sensitivity, selectivity, and matrix effects.

Excellent intra- and inter-batch accuracy (between 85-115%) and coefficient of variation as a measure for precision (CV <15%) were obtained for all 7-point calibrated analytes and their calibrants above the limit of detection (LOD) in the course of the validation, therefore classified as “quantitative”. LC analytes which rely on a one-point internal calibration were accepted at a precision <20% and an accuracy between 80-120%, therefore classified as “quantitative with restriction”.

The NIST standard reference material (SRM) 1950 was analyzed and the measured concentration values were compared to the certified values to demonstrate the performance of the MxP® Quant 500 kit across three separate 6495C TQ LC/MS systems in different laboratories. Excellent accuracy between 85-115% were obtained for specified analytes in the reference material, amino acids, creatinine, and hexoses (Figure 3). These findings are important in the context of inter-laboratory comparability.

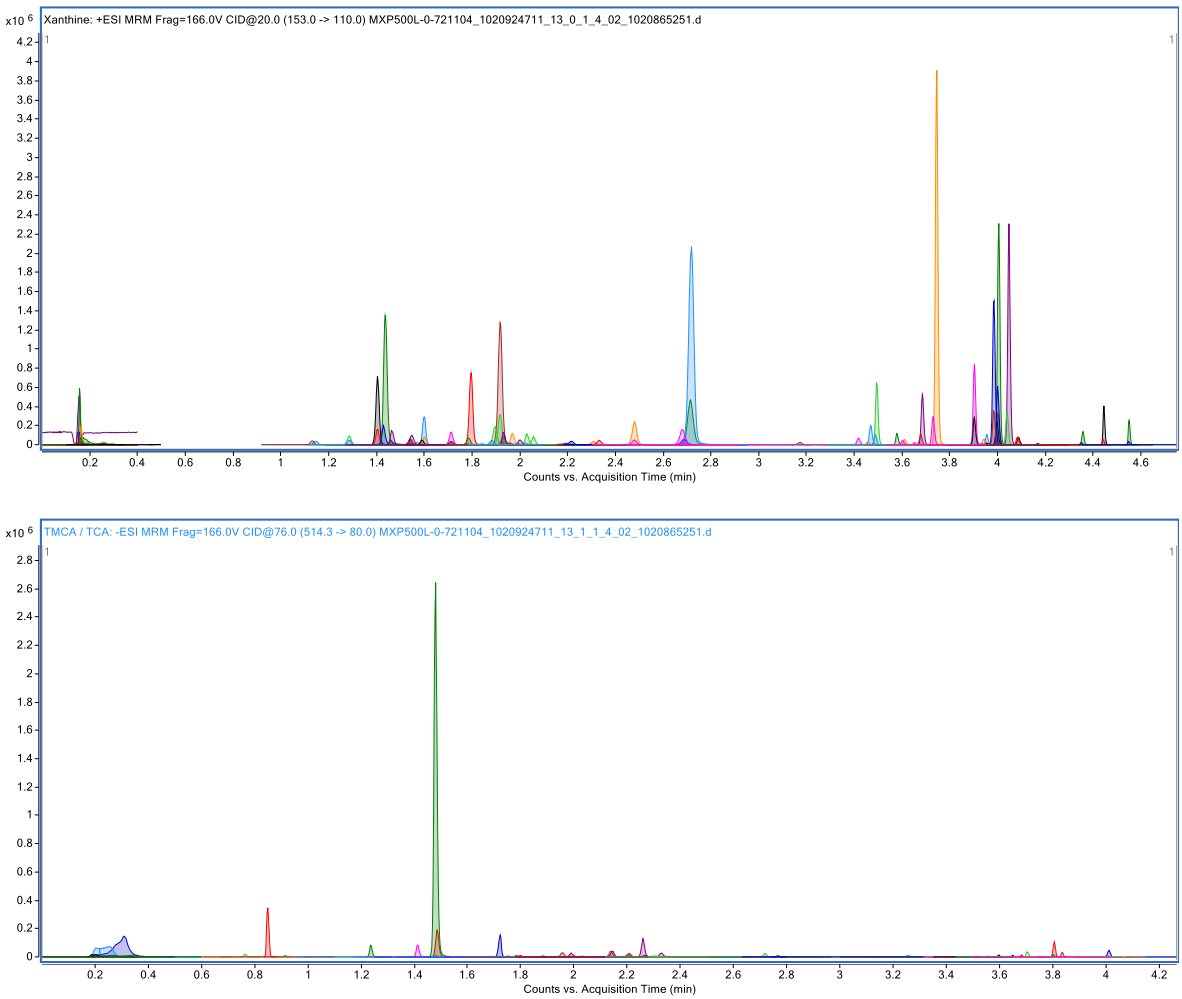

Figure 2: LC extracted ion chromatograms derived from a human plasma sample in positive (upper panel) and negative (lower panel) ion modes, measured with the biocrates MxP® Quant 500 kit on the Agilent 1290 Infinity II UHPLC – 6495C TQ LC/MS system.

Furthermore, a broad spectrum of metabolites present in biological specimens can be determined by FIA-MS/MS analysis with the MxP® Quant 500 kit. These metabolites belong to key analyte classes, such as acylcarnitines (including carnitine), carbohydrates (hexoses), and many highly relevant lipid classes (Figure 1). For FIA-MS/MS, the label quantitative implies analytical precision <15% and accuracy between 80-120%. Since not all FIA-MS/MS metabolites are commercially available as external and internal standards, the validity of these lipids and a subset of acylcarnitines is classified as “relative quantitative”.

In general, the data comparability is given for all kit analytes due to software-based automatic target-normalization.

In a typical human plasma sample, approximately 490-500 small molecules and lipids can be routinely analyzed (>LOD) and quantified with the MxP® Quant 500 kit. Table 1 shows the representative number of metabolites and lipids that were quantified in human plasma, human feces, rat plasma, and mouse liver homogenate. Particularly, the analysis of blood plasma and fecal samples is highly relevant today for microbiome-host-nutrition interaction research. Note that there is a separate

biocrates application note (document number 35039) available detailing a recommended sample preparation protocol for the analysis of fecal samples with the MxP® Quant 500 kit.

4 Conclusions

The MxP® Quant 500 kit is highly suitable for comparative quantitative metabolomics studies of biological samples across different sample cohorts and species with excellent lab-to-lab data comparability following the FAIR principles for scientific data, i.e. findable, accessible, interoperable, and reusable.

The data shown in this application note supports the high reliability and analytical performance of the MxP® Quant 500 kit on the Agilent UHPLC-6495C triple quadrupole LC/MS system, delivering high level of global standardization in metabolomics with excellent lab-to-lab comparability.

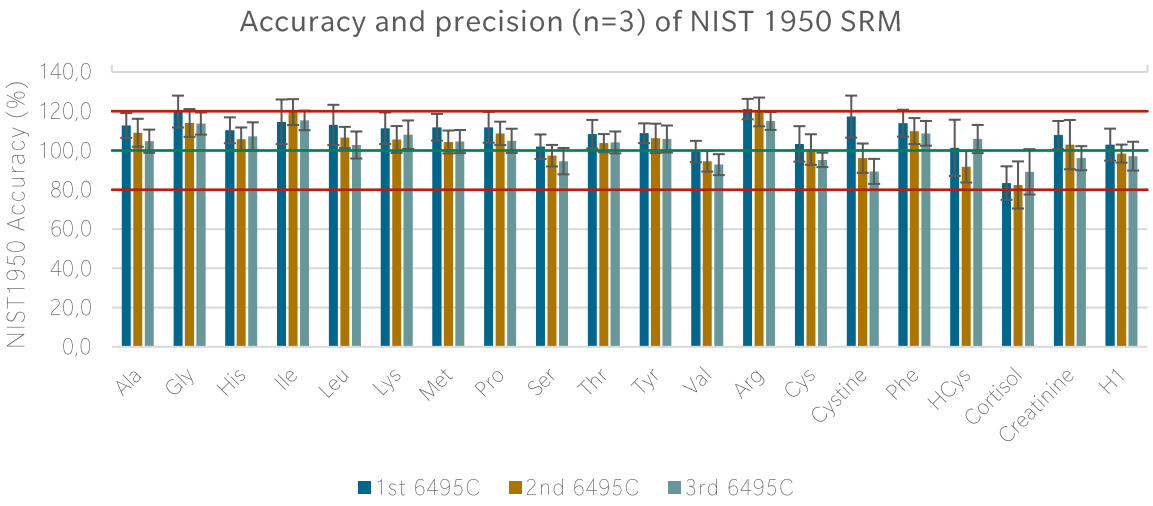

Figure 3: Accuracy and precision of measurements across three different 6495C instruments compared to reference and/or certified NIST 1950 SRM values (*n* = 3). The red lines indicate the accuracy range between 80% and 120%.

Table 1: Number of detectable (>LOD) and quantifiable (>LOD with CV <20%, in parentheses) analytes in each metabolite and lipid class, using the MxP® Quant 500 kit on the Agilent 6495C TQ LC/MS system for the analysis of different sample matrices.

Analyte class	Human plasma	Human feces	Rat plasma	Mouse liver
LC-MS/MS				
Alkaloids	1 (1)	0 (0)	1 (1)	1 (1)
Amine oxides	1 (1)	0 (0)	1 (1)	1 (1)
Amino acids	20 (20)	20 (19)	20 (20)	20 (19)
Amino acid related	23 (20)	21 (20)	23 (23)	23 (21)
Bile acids	12 (11)	13 (13)	10 (8)	9 (9)
Biogenic amines	3 (2)	6 (6)	6 (6)	7 (7)
Carboxylic acids	2 (2)	1 (1)	4 (4)	3 (3)
Cresols	1 (1)	1 (1)	1 (1)	1 (1)
Fatty acids	6 (6)	12 (10)	6 (5)	4 (4)
Hormones and related	3 (2)	1 (1)	0 (0)	1 (0)
Indoles and derivatives	3 (3)	3 (3)	3 (3)	1 (0)
Nucleobases and related	2 (2)	2 (2)	0 (0)	2 (2)
Vitamins and cofactors	1 (1)	1 (1)	1 (1)	1 (1)
Sub-total LC-MS/MS	78 (72)	81 (77)	76 (73)	74 (69)
FIA-MS/MS				
Carbohydrates and related	1 (1)	0 (0)	1 (1)	1 (1)
Acylcarnitines	15 (14)	8 (7)	12 (9)	26 (25)
Lysophosphatidylcholines	13 (12)	7 (6)	14 (14)	13 (12)
Phosphatidylcholines	72 (70)	25 (15)	69 (67)	71 (69)
Sphingomyelins	14 (14)	4 (2)	14 (13)	12 (11)
Ceramides	23 (23)	17 (17)	18 (16)	14 (14)
Dihydroceramides	3 (3)	2 (2)	3 (2)	0 (0)
Hexosylceramides	18 (14)	14 (9)	12 (9)	8 (6)
Dihexosylceramides	9 (8)	5 (2)	8 (5)	3 (2)
Trihexosylceramides	5 (4)	4 (1)	4 (2)	3 (0)
Cholesteryl esters	22 (19)	3 (2)	21 (18)	2 (1)
Diacylglycerols	27 (19)	31 (24)	34 (24)	22 (16)
Triacylglycerols	240 (233)	74 (24)	241 (238)	112 (47)
Sub-total FIA-MS/MS	462 (434)	194 (111)	451 (418)	287 (204)
Total	**540 (506)**	**275 (188)**	**527 (491)**	**361 (273)**

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