Microbiome Analysis Using the MxP® Quant 500 Kit with Human Fecal Samples

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Introduction

The realization that the microbiome is involved in the development, progression, and potentially cure of multiple diseases has led to an increased research interest in the microbiome and related topics such as the microbiota over the last two decades (1) (Figure 1). While plasma, serum, tissue, and urine are typical biological sample matrices for metabolome analysis, in the context of microbiome research, it is very attractive to analyze the metabolome of fecal matter as it is made up of a multitude of small molecules implicated in gut-microbiome activity. The MxP® Quant 500 was developed and validated close to the guidelines for bioanalytical method validation by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) for its use with human plasma. Within the validation procedure we evaluated the kit's applicability to the analysis of other matrices of interest including human feces. In this application note, we report on the successful outcome of this undertaking.

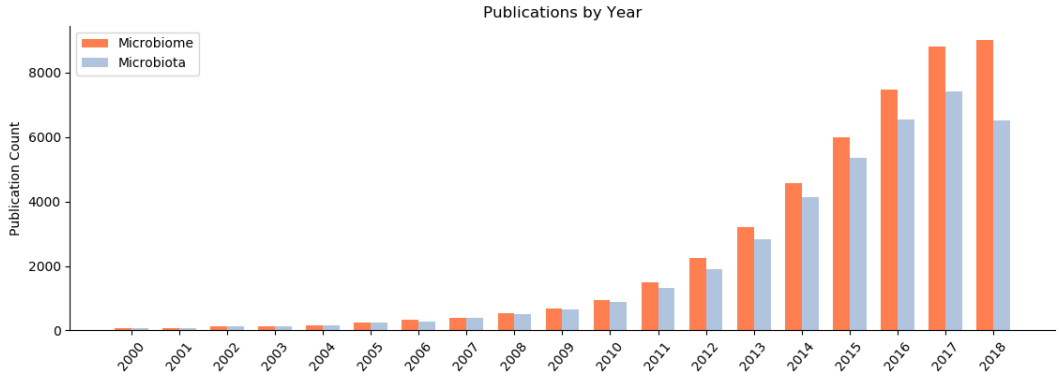

Figure 1: Trends in publications listed in PubMed under the search terms "Microbiome" and "Microbiota" from January 2000 to October 2018.

Materials and Methods

Pooled human fecal homogenate was prepared according to a Biocrates® in-house protocol (available upon request). Briefly, weighted aliquots were diluted 1:3 with extraction buffer (ethanol phosphate buffer) and homogenized by sonication. After centrifugation, supernatants were collected and pooled. A sample volume of 10 µL was used per well. Samples were processed according to the MxP® Quant 500 user manual.

Sample extracts were analyzed using a SCIEX 5500 QTRAP® triple quadrupole mass spectrometer in combination with ultra high performance liquid chromatography (UHPLC) for the analysis of small molecules and with flow injection analysis (FIA) for the analysis of lipids, acylcarnitines, and hexoses. Multiple reaction monitoring (MRM) in combination with the use of internal standards served quantification of metabolites using Biocrates' Met*IDQ*™ software included with the kit.
The pooled sample was analyzed in triplicate in three independent experiments to demonstrate analytical precision of the kit for human fecal samples (Table 1 and 2). Analytes delivering three out of three concentrations above the limit of detection (LOD) in each batch were considered for calculation of mean concentrations. Values above LOD, but below the lower limit of quantitation (LLOQ), as listed in table 1 and 2, were yielded by extrapolation of the calibration curve by the Met*IDQ*™ software.

Pre-analytical Aspects

It is possible that fecal samples are contaminated with blood. Visual inspection to differentiate between occult blood (low levels, present under specific conditions such as colorectal cancer) and gross blood (high

levels, present under conditions such as hemorrhoids) before sampling is crucial to obtaining meaningful results. As with other matrices, differences within samples from one individual exist (food supply, digestion) and may be balanced by collection and pooling of samples from multiple days. Human feces have pH values between 6 and 8 with an average pH of ~6.6. Differences in the pH values of samples will affect compound solubility and extraction efficiency, therefore a correction of pH may be essential for specific studies. Human feces contain 60-85% water. While differences are not as dramatic as seen between urine samples, the variability can bias results. Several approaches for correction of water content exist; freeze-drying of fecal homogenates is one option (1).

Exposure to air and temperature changes can significantly affect fecal samples due to microbial fermentation at room temperature (1, 2). Sample storage at cooler temperatures and, if possible, under anaerobic conditions, as well as consistency in collection procedures are crucial parameters in study execution to avoid flawed results. Freeze-thaw cycles can also impact the metabolic profile of fecal samples; besides breakdown and recovery of fecal metabolites, the release of intracellular microbial contents such as branched-chain and aromatic amino acids is likely to reflect in the results (1). A study on sample handling for metabolic profiling including an optimized protocol for handling of human fecal samples was recently published by Gratton et al. (2). However, other approaches to sample preparation than the one presented in this study may be appropriate to answer a specific research question and supplement a certain study design. We recommend to thoroughly review the literature and consider different approaches, such as freeze-drying of samples, to select the sample selection, collection, and preparation protocol that best serves your needs.

To determine analyte concentrations above the upper limit of quantification (ULOQ), an appropriate dilution of the sample prior to sample preparation and subsequent re-analysis is required. As the kit was developed and validated for use with human plasma, an adjustment of the extraction procedure (e.g. addition of a dilution step) for measurement of specific analytes may be necessary when working with other matrices.

Results and Discussion

The MxP® Quant 500, developed for the use with human plasma, was successfully applied to the analysis of human fecal samples, mouse liver homogenate, and rat plasma (for information on applicability with non-fecal matrices, please see Application Note 35036: Metabolome Analysis in Different Sample Types Using the MxP® Quant 500 Kit). When using the kit with matrices other than human plasma, several points such as the impact of matrix effects and choice of an appropriate normalization procedure have to be considered.

Quality Control / Normalization

For analysis of plasma samples, data normalization using quality control (QC) level 2 (or another appropriate reference) is obligatory and easily performed in the Met*IDQ*™ software. The QC samples provided with the kit are based on human plasma. Therefore, we cannot guarantee that data normalization using the included QCs will improve data quality if the kit is used for the analysis of matrices other than human plasma (such as human feces). For normalization purposes it is recommend to use your own QC sample, e.g. a pool of representative samples of the respective matrix.

Metabolome of Fecal Samples

Metabolite concentrations in human fecal matter can vary considerably as factors such as gender, age, life style, and medical conditions may dramatically influence the metabolome. To test applicability of the MxP® Quant 500 kit for the analysis of fecal matter, human samples of unknown origin were used. The number of subjects used to create the pooled sample was very limited, therefore, our results (Table 1 and 2) should be taken as a representation of what may be expected.

In human fecal homogenate, 117 analytes were above the LOD (Table 1, 2, and 3). Most amino and fatty acids that were above the LOD were in the µM range. Most bile acids showed nM concentrations. Biogenic amines, amino acid-related compounds, and analytes assigned to less extensive compound classes ranged between nM and µM concentrations with the carboxylic acid succinate showing a very high concentration of 460 µM. Several metabolite concentrations were above the ULOQ - marked with * in table 1 and 2, i.e. DCA, putrescine, beta-ala, FA(12:0), and Cer(d18:0/18:0(OH)); for the accurate analysis of these metabolites, sample dilution will be inevitable. As concentrations of other analytes were close to the ULOQ, e.g. CA, FA(18:2), for their analysis, sample dilution may also be advisable. We recommend a test run

evaluating different dilutions of a representative sample or even several samples per condition prior to conducting the assay.

In consideration of total analyte coverage, relatively high numbers of amino acids, amino acid-related compounds, bile acids, biogenic amines, and fatty acids, as well as ceramides were assessed in human feces (Table 3). In contrast to human plasma, the compound counts were particularly high for biogenic amines and fatty acids, but low for glycerophospholipids, sphingolipids, cholesterol esters, glycosylceramides, and especially triacylglycerols. One reason for the low numbers of detected lipids may be the choice of homogenization buffer (ethanol phosphate buffer). Use of different homogenization protocols prior to using the kit may yield higher numbers of detected metabolites and lipids.

While in the LC part many metabolites are common to plasma and feces, in the FIA part most metabolites are unique to plasma (Figure 2). Combined, 40 out of 441 metabolites assessible in plasma were also measurable in fecal samples, while 23 compounds were unique to fecal samples, namely spermidine, carnosine, serotonin, xanthine, Cer(d18:1/14:0), Cer(d16:1/18:0), succinate, anserine, putrescine, DG(18:1_18:3), C14:1-OH, Cer(d16:1/22:0), FA(12:0), Cer(d18:1/18:1), DG(16:1_18:0), BABA, trigonelline, FA(20:1), FA(14:0), C14:1, Cer(d18:0/18:0(OH)), Ac-Orn, and 5-AVA.

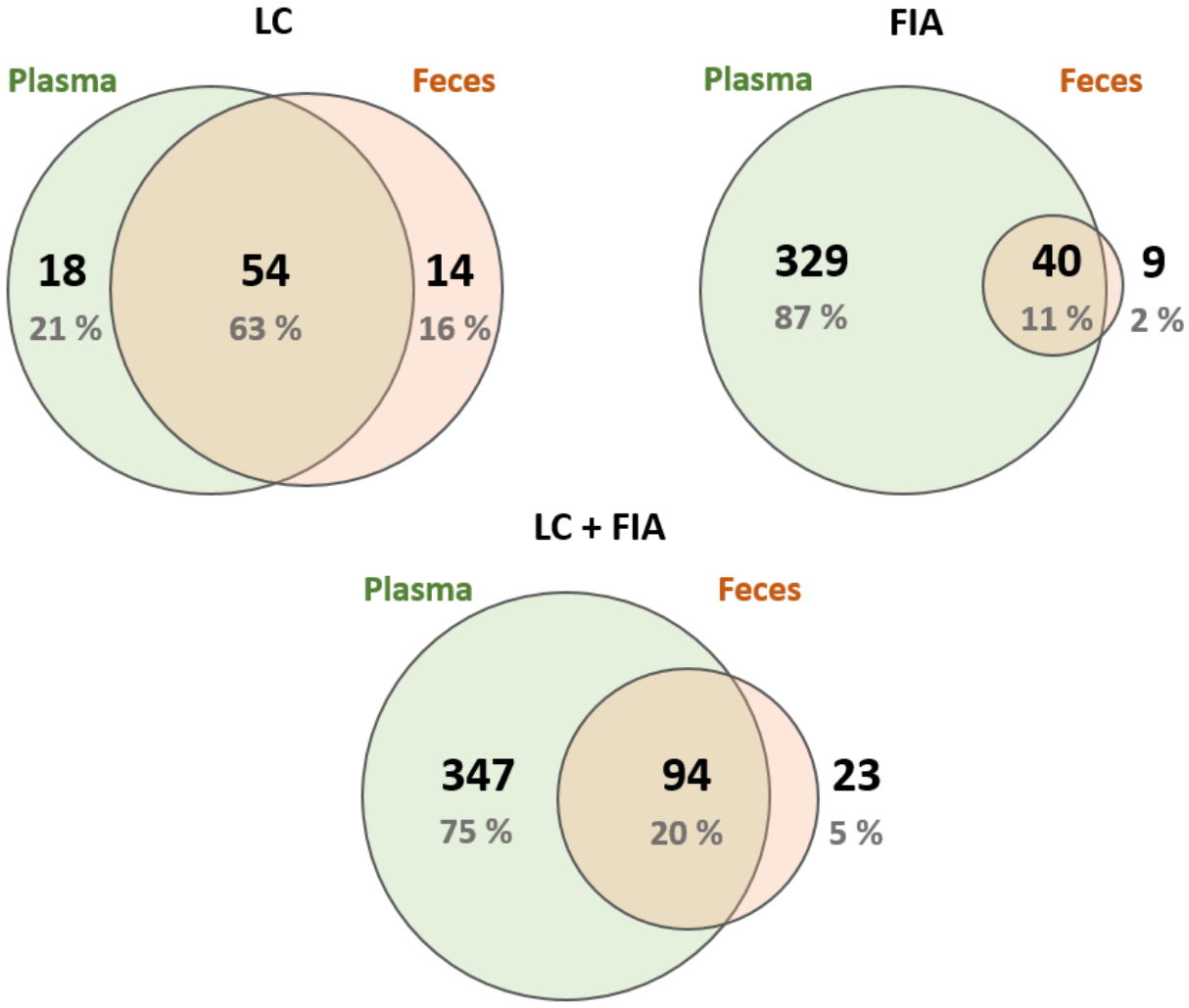

Figure 2: Overlap of analytes in pooled human plasma and pooled human fecal homogenate (data based on three independent experiments). Only analytes with a precision of CV <20 % (LC) and <30 % (FIA) were taken into account. Please note: Use of different homogenization protocols prior to using the kit may yield higher numbers of detected metabolites and lipids. The applied homogenization protocol (ethanol phosphate buffer-based) was optimized for small molecule analysis.

Conclusions

Using the MxP® Quant 500 kit we were able to assess concentrations of 117 metabolites. For the majority of analytes, the precision (CV in %) was within an appropriate analytical range. Thus, the MxP® Quant 500 kit can reliably be used for the analysis of human fecal samples. Please note that the Absolute*IDQ*® p180 kit and the Biocrates® Bile Acid kit have been successfully used in the analysis of the fecal metabolome previously (1, 3–7).

Table 1: Analytical performance of the MxP® Quant 500 kit: LC-MS/MS analysis of pooled human fecal homogenate. Data are based on three independent experiments. Analytes below the LOD are not reported. Please note: Use of different homogenization protocols prior to using the kit may yield higher numbers of detected metabolites and lipids. The applied homogenization protocol (ethanol phosphate buffer-based) was optimized for small molecule analysis.

Analyte	LOD [µM]	LLOQ [µM]	ULOQ [µM]	Conc. [µM]	CV [%]
Alkaloids					
Trigonelline	0.203	1	49	1.20	13.0
Amino Acids					
Ala	11.19	20	1600	124	5.0
Arg	2.727	5	400	22.8	7.9
Asp	1.997	5	400	72.7	23.4 **
Cys	0.087	1	41	2.43	10.2
Gln	5.207	20	1600	13.4	8.7
Glu	3.077	10	800	192	4.9
Gly	6.401	25	2000	67.7	3.3
His	1.425	5	400	4.84	6.4
Ile	2	5	400	85.0	5.5
Leu	2.18	5	400	147	5.8
Lys	2.17	10	800	58.1	3.0
Met	2.503	5	400	24.9	2.7
Phe	2.137	5	400	47.3	6.0
Pro	5.177	10	800	36.3	5.0
Ser	2.773	5	400	28.2	3.8
Thr	1.953	5	400	23.3	4.3
Trp	2.087	5	400	7.06	9.1
Tyr	2.32	5	400	48.6	7.3
Val	4.827	10	800	81.2	7.6
Amino Acid-Related					
alpha-AAA	0.453	1	80	0.65	6.2
AABA	0.46	2.8	64	6.93	7.8
Ac-Orn	0.124	0.5	40	0.65	5.6
Anserine	0.011	0.6	6	0.25	5.4
5-AVA	0.028	0.1	26	18.7	6.3
BABA	0.017	0.7	5	0.31	8.8
Carnosine	0.186	0.5	40	0.30	9.7
Cit	1.887	5	400	24.2	5.4
Cystine	0.012	1	41	0.05	5.3
HArg	0.006	0.4	7	0.03	14.8

HCys	0.318	3.1	137	1.22	25.9 ***
t4-OH-Pro	0.449	1	80	1.12	7.6
Met-SO	0.099	1	80	6.99	17.8
1-Met-His	0.008	1.2	23	0.13	5.4
3-Met-His	0.005	2.4	29	0.36	8.2
Orn	1.743	5	400	5.88	9.4
SDMA	0.019	0.1	8	0.18	4.1
Taurine	0.973	2.5	200	9.37	5.3
Bile Acids					
CA	0.012	0.05	6.3	6.26	6.7
CDCA	0.009	0.01	3.8	1.58	5.7
DCA	0.005	0.08	2.4	57.4 *	6.1
GCA	0.005	0.04	2.1	0.20	7.3
GCDCA	0.001	0.1	2.1	0.27	11.3
GDCA	0.001	0.1	3	0.15	11.7
GLCA	0.005	0.01	1.1	0.02	5.6
GLCAS	0.001	0.03	0.7	0.03	10.6
GUDCA	0.001	0.03	0.7	0.02	13.8
TCA	0.004	0.01	2.2	0.08	11.6
TCDCA	0.008	0.03	0.6	0.11	5.3
TDCA	0.003	0.02	1.3	0.04	10.3
TLCA	0.002	0.01	1.2	0.02	7.6
TMCA	0.002	0.01	4.5	0.01	30.5 ***
Biogenic Amines					
beta-Ala	0.129	0.25	20	215 *	10.3
GABA	0.043	0.07	15	2.73	5.0
PEA	0.001	0.1	8	0.02	49.5 ***
Putrescine	0.07	0.1	8	10.5 *	0.9
Serotonin	0.053	0.1	8	0.11	4.9
Spermidine	0.375	0.5	20	2.34	6.3
Carboxylic Acids					
Suc	12.5	20	763	460	5.5
Cresols					
p-Cresol-SO4	0.007	6	51	0.10	7.3
Fatty Acids					
AA	0.197	5	66	9.68	5.3
DHA	0.021	1	16	7.12	8.2
EPA	0.05	0.5	18	0.71	11.2
FA(12:0)	13.277	n.a.	n.a.	83.3	7.6
FA(14:0)	20.567	n.a.	n.a.	171	7.4
FA(18:1)	28.133	89	221	588 *	3.0
FA(18:2)	4.3	168	276	232 *	13.0
FA(20:1)	6.79	18	23	18.1	9.9
FA(20:2)	0.624	1	39	2.72	36.9 ***
Hormones and Related					

AbsAcid	0.011	2	15	0.03	23.3 ***
Indoles and Derivatives					
3-IAA	0.048	0.3	16	0.99	9.5
3-IPA	0.023	0.1	15	1.04	7.2
Nucleobases and Related					
Hypoxanthine	0.425	1	53	17.9	15.4
Xanthine	0.402	7	55	25.0	16.8
Vitamins and Cofactors					
Choline	0.162	2	39	2.79	8.8

* value > ULOQ, dilution of the sample prior to extraction may be necessary to quantify the respective metabolite
** RSD > 20% (LC) for aspartate concentrations, due to known poor autosampler stability of the analyte
*** RSD > 20% (LC), due to analyte concentration being below or close to the LLOQ

Table 2: Analytical performance of the MxP® Quant 500 kit: FIA-MS/MS analysis of pooled human fecal homogenate. Data are based on three independent experiments. Analytes below the LOD are not reported. Please note: Use of different homogenization protocols prior to using the kit may yield higher numbers of detected metabolites and lipids. The applied homogenization protocol (ethanol phosphate buffer-based) was optimized for small molecule analysis.

Analyte	LOD [µM]	LLOQ [µM]	ULOQ [µM]	Conc. [µM]	CV [%]
Acylcarnitines					
C14:1	0.055	n.a.	n.a.	0.11	11.8
C14:1-OH	0.068	n.a.	n.a.	0.31	18.2
C16	0.124	0.4	12	1.22	52.7 ***
Glycerophospholipids					
lysoPC a C16:0	0.389	n.a.	n.a.	1.76	9.2
lysoPC a C18:1	0.316	n.a.	n.a.	1.25	6.1
lysoPC a C18:2	0.231	n.a.	n.a.	0.89	7.9
PC aa C34:3	0.056	n.a.	n.a.	0.08	16.5
PC aa C36:3	0.126	n.a.	n.a.	0.20	8.5
PC aa C36:4	0.193	n.a.	n.a.	0.29	12.8
PC aa C36:5	0.044	n.a.	n.a.	0.09	14.6
PC ae C34:0	0.033	n.a.	n.a.	0.06	7.1
PC ae C34:2	0.033	n.a.	n.a.	0.05	6.1
Sphingomyelins					
SM (OH) C24:1	0.011	n.a.	n.a.	0.05	20.3
Cholesterol Esters					
CE(14:1)	0.01	1	10000	0.99	51.2 ***
CE(15:0)	0.689	2.66	10000	2.24	15.1
CE(17:0)	0.728	2.12	10000	7.42	19.6
CE(17:1)	0.01	1.04	10000	2.77	22.9
CE(18:0)	0.587	1.79	10000	2.02	14.0
CE(22:0)	0.747	1.16	10000	2.26	59.2 ***
Ceramides					
Cer(d16:1/18:0)	0.016	0.09	10	0.11	12.0
Cer(d16:1/22:0)	0.01	n.a.	n.a.	0.10	6.4
Cer(d18:1/14:0)	0.019	0.04	10	0.10	18.2
Cer(d18:1/16:0)	0.037	n.a.	n.a.	0.27	13.2

Cer(d18:1/18:0)	0.01	n.a.	n.a.	0.21	9.8
Cer(d18:1/18:1)	0.031	n.a.	n.a.	0.09	18.9
Cer(d18:1/20:0)	0.01	n.a.	n.a.	0.05	20.7
Cer(d18:1/22:0)	0.01	n.a.	n.a.	0.11	12.4
Cer(d18:1/24:0)	0.04	n.a.	n.a.	0.10	13.6
Cer(d18:1/24:1)	0.01	n.a.	n.a.	0.23	13.0
Cer(d18:2/16:0)	0.01	n.a.	n.a.	0.04	11.4
Cer(d18:2/24:1)	0.01	n.a.	n.a.	0.04	18.2
Dihydroceramides					
Cer(d18:0/18:0(OH))	1.693	2.5	10	11.2 *	14.7
Diglycerides					
DG(16:0_18:1)	0.852	n.a.	n.a.	2.29	13.6
DG(16:0_18:2)	0.097	n.a.	n.a.	1.97	8.1
DG(16:1_18:0)	0.180	0.34	15	1.05	12.9
DG(18:1_18:1)	0.379	n.a.	n.a.	3.45	8.2
DG(18:1_18:2)	0.531	n.a.	n.a.	6.88	8.8
DG(18:1_18:3)	0.583	n.a.	n.a.	1.32	14.3
DG(18:2_18:2)	0.01	n.a.	n.a.	8.28	9.5
DG(18:2_18:3)	0.01	n.a.	n.a.	0.72	31.8
DG(18:3_18:3)	0.160	n.a.	n.a.	1.72	36.7
Glycosylceramides					
Hex2Cer(d18:1/16:0)	0.01	n.a.	n.a.	0.24	19.6
Hex2Cer(d18:1/18:0)	0.01	n.a.	n.a.	0.09	19.6
Hex2Cer(d18:1/24:1)	0.01	n.a.	n.a.	0.09	13.5
HexCer(d18:1/16:0)	0.01	n.a.	n.a.	0.20	18.9
HexCer(d18:1/20:0)	0.021	n.a.	n.a.	0.07	17.8
HexCer(d18:1/24:1)	0.147	n.a.	n.a.	0.58	12.1
Triglycerides					
TG(18:1_36:3)	0.284	0.25	1000	1.16	18.0
TG(18:1_36:4)	0.192	n.a.	n.a.	0.81	16.9
TG(18:2_36:2)	0.01	n.a.	n.a.	0.78	18.8
TG(18:2_36:3)	0.213	n.a.	n.a.	1.31	15.1
TG(18:2_36:4)	0.09	n.a.	n.a.	1.75	9.6
TG(18:2_36:5)	0.01	n.a.	n.a.	0.30	8.8
TG(18:3_36:4)	0.01	n.a.	n.a.	0.53	7.5

* value > ULOQ, dilution of the sample prior to extraction may be necessary to quantify the respective metabolite
*** RSD > 20% (LC), due to analyte concentration being below or close to the LLOQ

Table 3: Average analyte coverage in pooled human plasma and pooled human fecal homogenate analyzed by use of the MxP® Quant 500 kit. Data are based on three independent experiments.

	Compound Class	TOTAL	Human Plasma		Human Feces *	
			RSD < 20%	RSD > 20%	RSD < 20%	RSD > 20%
LC Part	Alkaloids	1	0	0	1	0
	Amine Oxides	1	1	0	0	0
	Amino Acids	20	20	0	18	3
	Amino Acid Related	30	19	0	14	0
	Bile Acids	14	13	0	13	1
	Biogenic Amines	9	3	0	7	1
	Carboxylic Acids	7	3	0	1	0
	Cresols	1	1	0	1	0
	Fatty Acids	12	5	0	8	1
	Hormones and Related	4	2	0	0	1
	Indoles and Derivatives	4	3	0	2	0
	Nucleobases and Related	2	1	0	2	0
	Vitamins and Cofactors	1	1	0	1	0
	LC TOTAL	**106**	**72**	0	**68**	7
			RSD < 30%	RSD > 30%	RSD < 30%	RSD > 30%
FIA Part	Carbohydrates and Related	1	1	0	0	0
	Acylcarnitines	40	3	0	2	1
	Glycerophospholipids	90	76	1	9	0
	Sphingomyelins	15	14	0	1	0
	Cholesteryl Esters	22	18	1	4	2
	Ceramides	28	15	0	12	0
	Dihydroceramides	8	1	0	1	0
	Glycosylceramides	34	19	0	6	0
	Diglycerides	44	8	0	7	2
	Triglycerides	242	214	0	7	0
	FIA TOTAL	**524**	**369**	2	**49**	5
LC+FIA	**TOTAL**	**630**	**441**	2	**117**	12

* Please note: Use of different homogenization protocols prior to using the kit may yield higher numbers of detected metabolites and lipids. The applied homogenization protocol (ethanol phosphate buffer-based) was optimized for small molecule analysis.

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