Metabolome and Lipidome Analysis of Human Fecal Samples Using the MxP® Quant 500 Kit

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1 Introduction

The emerging links between the microbiome and many disorders have put the human gut microbiota (GM) and the nutrition-microbiome-host interactions at the forefront of clinical research aiming to gain a better understanding of the causalities and the implications of such connections. To that extent, feces has become the most commonly used biological matrix for microbiome research, mainly due to its non-invasive availability and its suitability for 16S-rRNA gene sequencing in studying the gut bacterial composition. However, quantitative metabolomic analyses in feces are much more challenging compared to analyses in buffered systems and highly homogenous sample matrices such as blood plasma or serum. Feces is also known to be directly affected by many factors such as daily nutrition, drug intake, drinking behavior, and gut activity. These factors cause the metabolomic analyses of fecal samples to show higher biological variances, even if samples were obtained from the same individual at different time points, and even from the same stool sample at different topographical locations. Such variances dictate the need in the research community to standardize the pre-analytical sample collection and sample preparation for metabolomic analysis of feces, and to develop tools for reproducible and accurate analyses.

This application note describes a recommended sample collection and sample preparation protocol for fecal samples developed for the analysis using the MxP® Quant 500 kit. Furthermore, the expected metabolite and lipid coverage in feces using the MxP® Quant 500 kit, together with an overview of the expected biological variances are described.

2 Methods

The sample collection and sample preparation protocols applied here, including a lyophilization protocol, have been developed and optimized for the MxP® Quant 500 kit (see pages 15-17). Lyophilization of the sample and normalization of the obtained analysis data to the water content help to minimize the effect of a varying water content between samples. However, this will not completely remove the high biological variance. It is highly recommended to pool at least three replicates from different topographical locations of one stool sample, if homogenization of the whole stool sample is not feasible (Karu et al., 2018).

According to Gratton et al. (2016), recommended best practice is to cool fresh samples at 4 °C, and to conduct extractions within 1 hour and maximal 24 hours after collection to avoid an increase of some amino acid levels. Gorzelak et al. (2015)

highly recommend to immediately freeze the samples at -80°C after collection to prevent changes in the microbial metabolism, allowing up to four freeze-thaw cycles. The use of lyophilized samples compared to fresh ones minimizes variations in the metabolite profile due to differences in water content across samples, which typically ranges from 60-85%. On the other side, a decrease in the number of metabolites upon lyophilization has been shown (Phua et al., 2013).

Sample preparation of fecal samples typically applies directly to raw feces, wet or lyophilized, using a wide range of solvents, water, or combinations. We have tested different extraction buffers including combinations using methanol and isopropanol, with the chosen ethanol/phosphate buffer (85:15 v/v) being suited best for the wide range of studied metabolites and lipids for both wet and lyophilized fecal samples stored at -80 °C until extraction.

Table 1: Number of analytes (per analyte class) detectable >LOD in human fecal samples (*n* = 100) using MxP® Quant 500 kit.

Analyte Class (no. of analytes covered)	No. of analytes detected >LOD in > 90% samples	No. of analytes detected >LOD in 50-90% samples
Alkaloids (1)	0	1
Amine oxides (1)	0	0
Amino acids (20)	19	0
Amino acid related (30)	13	11
Bile acids (14)	4	3
Biogenic amines (9)	5	3
Carbohydrates and related (1)	0	0
Carboxylic acids (7)	2	3
Cresols (1)	1	0
Fatty acids (12)	7	4
Hormones and related (4)	0	1
Indoles and derivatives (4)	1	2
Nucleobases and related (2)	2	0
Vitamins and cofactors (1)	1	0
Acylcarnitines (40)	4	29
Glycerophospholipids (90)	7	23
Sphingomyelins (15)	2	10
Ceramides (28)	22	6
Dihydroceramides (8)	6	1
Glycosylceramides (34)	21	12
Cholesteryl esters (22)	2	14
Diglycerides (44)	12	24
Triglycerides (242)	6	84
Total	**137**	**231**

3 Results and Conclusion

Metabolite and Lipid Coverage Using the MxP® Quant 500 Kit

To demonstrate which metabolites and lipids covered by the MxP® Quant 500 kit can typically be detected in human fecal samples, 100 individual fecal samples were analyzed using the above described sample preparation protocol. For the mass spectrometric analysis, a Waters Xevo® TQ-S mass spectrometer was used. Table 1 shows an overview of the number of analytes per class detected in more than 90% as well as additional analytes detected in 50-90% of the tested samples. In total, 137 metabolites and lipids were detected in more than 90% of the samples at levels higher than the limit of detection (LOD). Additional 231 metabolites and lipids were detected in 50-90% of the tested samples. This means that in a typical human fecal sample approximately up to 370 analytes can be expected >LOD.

In human plasma samples, approximately 30% more metabolites and lipids (up to 520) can be detected >LOD using the MxP® Quant 500 kit compared to fecal samples. Around 25% of the metabolites and lipids covered by the kit are detectable in both matrixes.

Table 2 shows all individual analytes detectable from Table 1.

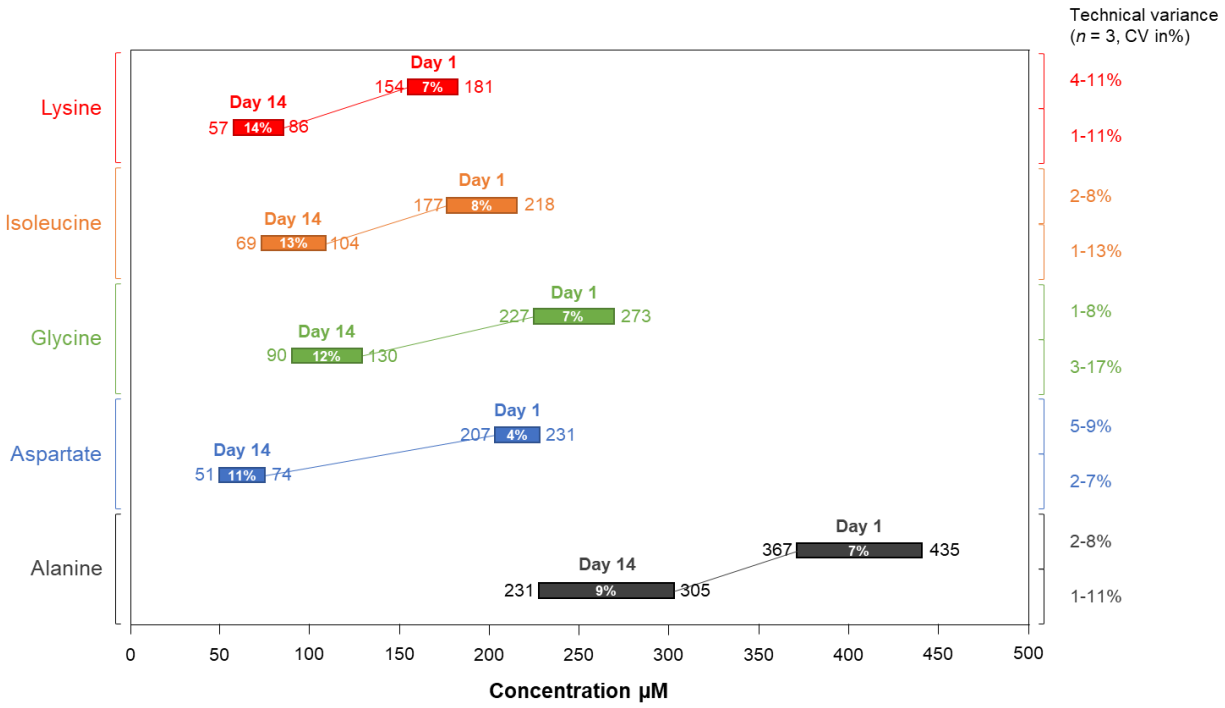

Figure 1: Biological variance of amino acid levels in human fecal samples. Amino acid concentration ranges from different topographical locations (*n* = 6) of one fecal sample at two different time points (day 1 and 14) from the same individuum are depicted. Biological variance in terms of coefficient of variation (CV in %) is indicated in the bars. Technical variance of pipetting replicates (*n* = 3) in terms of CV in % is provided.

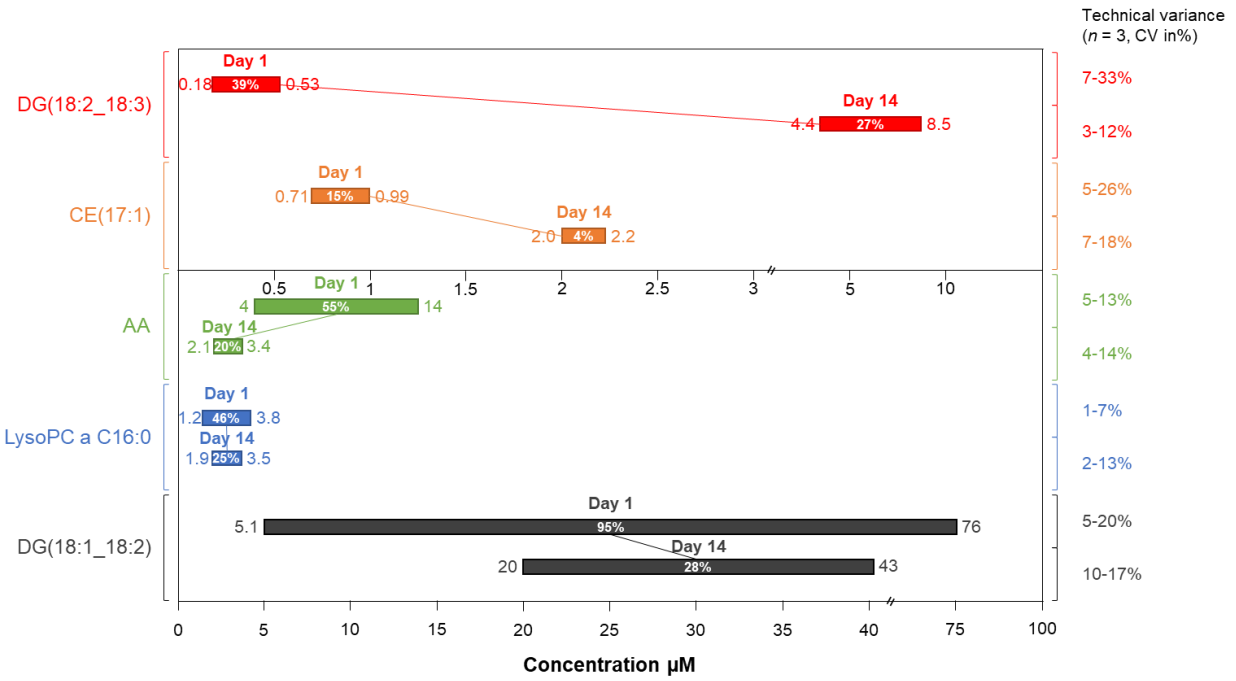

Figure 2: Biological variance of lipid levels in human fecal samples. Diglyceride (DG(18:2_18:3), DG(18:1_18:2)), cholesteryl ester (CE(17:1)), arachidonic acid (AA), and lysophosphatidylcholine (LysoPC a C16:0) concentration ranges from different topographical locations (*n* = 6) of one fecal sample at two different time points (day 1 and 14) from the same individuum are depicted. Biological variance in terms of coefficient of variation (CV in %) is indicated in the bars. Technical variance of pipetting replicates (*n* = 3) in terms of coefficient of variation (CV in %) is provided.

Biological Variance of the Metabolome and Lipidome in Human Fecal Samples

In order to evaluate the heterogeneity of fecal samples, six replicates from the same stool sample were taken as closest proximities as possible and processed as described in the sample preparation paragraph for fresh samples. Three pipetting replicates were performed to calculate technical variance. The same experiment was repeated using samples from the same individuum, but collected two weeks later.

Overall, inter-day variance is higher than the variance of one stool sample. However, remarkable variances within the same stool sample have been noticed. In Figure 1 and 2, some examples for amino acids and fatty acids/lipids, respectively, are depicted.

Alanine, for example, varies between 367 and 435 µM at day 1, and at day 14 between 231 and 305 µM, with excellent coefficients of variation for the technical replicates, demonstrating the heterogeneity of fecal samples even at close topographical regions. Samples taken at day 14 show significant lower concentrations for amino acids compared to day 1. Explanations for these high biological variances could be a combination of effects like different water content of the stool, different food/beverage intake, or bowel activity. Other analytes, like some lipids (Figure 2), show higher or similar concentrations at day 14.

In conclusion, the metabolome of fecal samples shows in part a high biological variance, even in the same topographical region of the sample. Therefore, it is of utmost importance to adhere to the same sample preparation protocol for all fecal samples and take the high biological variances into consideration for metabolomics study design. If homogenization of the whole stool sample is not feasible, pooling of at least three replicates from different topographical locations of one stool sample is highly recommended.

Table 2: List of analytes detectable >LOD in human fecal samples (*n* = 100) using MxP® Quant 500 kit.

Analyte Class	Analyte	Short Name	>LOD in >90% Samples	>LOD in 50-90% Samples
Alkaloids	Trigonelline	Trigonelline		x
Amino acids	Alanine	Ala	x	
Amino acids	Arginine	Arg	x	
Amino acids	Aspartate	Asp	x	
Amino acids	Cysteine	Cys	x	
Amino acids	Glutamate	Glu	x	
Amino acids	Glutamine	Gln	x	
Amino acids	Glycine	Gly	x	
Amino acids	Histidine	His	x	
Amino acids	Isoleucine	Ile	x	
Amino acids	Leucine	Leu	x	
Amino acids	Lysine	Lys	x	
Amino acids	Methionine	Met	x	
Amino acids	Phenylalanine	Phe	x	
Amino acids	Proline	Pro	x	
Amino acids	Serine	Ser	x	
Amino acids	Threonine	Thr	x	
Amino acids	Tryptophan	Trp	x	
Amino acids	Tyrosine	Tyr	x	
Amino acids	Valine	Val	x	
Amino acid related	α-Aminoadipic acid	alpha-AAA	x	
Amino acid related	α-Aminobutyric acid	AABA	x	
Amino acid related	Acetylornithine	Ac-Orn	x	
Amino acid related	Asymmetric dimethylarginine	ADMA		x
Amino acid related	5-Aminovaleric acid	5-AVA	x	
Amino acid related	β-Aminobutyric acid	BABA		x
Amino acid related	Carnosine	Carnosine		x
Amino acid related	Citrulline	Cit	x	
Amino acid related	Creatinine	Creatinine		x
Amino acid related	Cystine	Cystine		x

Analyte Class	Analyte	Short Name	>LOD in >90% Samples	>LOD in 50-90% Samples
Amino acid related	Dihydroxyphenylalanine	DOPA		x
Amino acid related	Homoarginine	HArg		x
Amino acid related	Homocysteine	HCys	x	
Amino acid related	*trans*-4-Hydroxyproline	t4-OH-Pro	x	
Amino acid related	Methionine sulfoxide	Met-SO	x	
Amino acid related	1-Methylhistidine	1-Met-His	x	
Amino acid related	3-Methylhistidine	3-Met-His	x	
Amino acid related	Ornithine	Orn	x	
Amino acid related	Phenylacetylglycine	PAG		x
Amino acid related	Phenylalanine betaine	PheAlaBetaine		x
Amino acid related	Proline betaine	ProBetaine		x
Amino acid related	Sarcosine	Sarcosine	x	
Amino acid related	Symmetric dimethylarginine	SDMA	x	
Amino acid related	Taurine	Taurine		x
Bile acids	Cholic acid	CA	x	
Bile acids	Chenodeoxycholic acid	CDCA		x
Bile acids	Deoxycholic acid	DCA	x	
Bile acids	Glycocholic acid	GCA	x	
Bile acids	Glycochenodeoxycholic acid	GCDCA		x
Bile acids	Taurocholic acid	TCA		x
Bile acids	Taurochenodeoxycholic acid	TCDCA	x	
Biogenic amines	β-Alanine	beta-Ala	x	
Biogenic amines	γ-Aminobutyric acid	GABA	x	
Biogenic amines	Dopamine	Dopamine		x
Biogenic amines	Histamine	Histamine		x
Biogenic amines	Phenylethylamine	PEA	x	
Biogenic amines	Serotonin	Serotonin	x	
Biogenic amines	Spermidine	Spermidine	x	
Biogenic amines	Spermine	Spermine		x
Carboxylic acids	Aconitic acid	AconAcid		x
Carboxylic acids	Hippuric acid	HipAcid		x
Carboxylic acids	3-Hydroxyglutaric acid	OH-GlutAcid	x	
Carboxylic acids	Lactic acid	Lac		x
Carboxylic acids	Succinic acid	Suc	x	
Cresols	p-Cresol sulfate	p-Cresol-SO4	x	
Fatty acids	Lauric acid	FA(12:0)		x
Fatty acids	Myristic acid	FA(14:0)		x
Fatty acids	Palmitic acid	FA(16:0)		x
Fatty acids	Octadecenoic acid	FA(18:1)	x	
Fatty acids	Octadecadienoic acid	FA(18:2)	x	
Fatty acids	Eicosenoic acid	FA(20:1)	x	
Fatty acids	Eicosadienoic acid	FA(20:2)	x	

Analyte Class	Analyte	Short Name	>LOD in >90% Samples	>LOD in 50-90% Samples
Fatty acids	Eicosatrienoic acid	FA(20:3)		x
Fatty acids	Arachidonic acid	AA	x	
Fatty acids	Eicosapentaenoic acid	EPA	x	
Fatty acids	Docosahexaenoid acid	DHA	x	
Hormones and related	Dehydroepiandrosterone sulfate	DHEAS		x
Indoles and derivatives	3-Indoleacetic acid	3-IAA	x	
Indoles and derivatives	3-Indolepropionic acid	3-IPA		x
Indoles and derivatives	Indoxyl sulfate	Ind-SO4		x
Nucleobases and related	Hypoxanthine	Hypoxanthine	x	
Nucleobases and related	Xanthine	Xanthine	x	
Vitamins and cofactors	Choline	Choline	x	
Acylcarnitines	Carnitine	C0		x
Acylcarnitines	Acetylcarnitine	C2		x
Acylcarnitines	Propionylcarnitine	C3		x
Acylcarnitines	Malonylcarnitine (Hydroxybutyrylcarnitine)	C3-DC (C4-OH)	x	
Acylcarnitines	Propenoylcarnitine	C3:1		x
Acylcarnitines	Butyrylcarnitine	C4		x
Acylcarnitines	Valerylcarnitine	C5		x
Acylcarnitines	Glutarylcarnitine (Hydroxyhexanoylcarnitine)	C5-DC (C6-OH)		x
Acylcarnitines	Methylglutarylcarnitine	C5-M-DC		x
Acylcarnitines	Hydroxyvalerylcarnitine (Methylmalonylcarnitine)	C5-OH (C3-DC-M)		x
Acylcarnitines	Tiglylcarnitine	C5:1		x
Acylcarnitines	Glutaconylcarnitine	C5:1-DC		x
Acylcarnitines	Pimeloylcarnitine	C7-DC		x
Acylcarnitines	Nonaylcarnitine	C9		x
Acylcarnitines	Decanoylcarnitine	C10		x
Acylcarnitines	Decadienoylcarnitine	C10:2		x
Acylcarnitines	Dodecanoylcarnitine	C12		x
Acylcarnitines	Dodecanedioylcarnitine	C12-DC		x
Acylcarnitines	Tetradecanoylcarnitine	C14		x
Acylcarnitines	Tetradecenoylcarnitine	C14:1		x
Acylcarnitines	Hydroxytetradecenoylcarnitine	C14:1-OH		x
Acylcarnitines	Tetradecadienoylcarnitine	C14:2		x
Acylcarnitines	Hydroxytetradecadienoylcarnitine	C14:2-OH		x
Acylcarnitines	Hexadecanoylcarnitine	C16		x
Acylcarnitines	Hydroxyhexadecanoylcarnitine	C16-OH	x	
Acylcarnitines	Hexadecenoylcarnitine	C16:1		x
Acylcarnitines	Hydroxyhexadecenoylcarnitine	C16:1-OH		x

Analyte Class	Analyte	Short Name	>LOD in >90% Samples	>LOD in 50-90% Samples
Acylcarnitines	Hexadecadienoylcarnitine	C16:2		x
Acylcarnitines	Hydroxyhexadecadienoylcarnitine	C16:2-OH		x
Acylcarnitines	Octadecanoylcarnitine	C18	x	
Acylcarnitines	Octadecenoylcarnitine	C18:1		x
Acylcarnitines	Hydroxyoctadecenoylcarnitine	C18:1-OH		x
Acylcarnitines	Octadecadienylcarnitine	C18:2	x	
Glycerophospholipids	Lysophosphatidylcholine a C16:0	lysoPC a C16:0	x	
Glycerophospholipids	Lysophosphatidylcholine a C17:0	lysoPC a C17:0	x	
Glycerophospholipids	Lysophosphatidylcholine a C18:0	lysoPC a C18:0	x	
Glycerophospholipids	Lysophosphatidylcholine a C18:1	lysoPC a C18:1	x	
Glycerophospholipids	Lysophosphatidylcholine a C18:2	lysoPC a C18:2		x
Glycerophospholipids	Lysophosphatidylcholine a C20:4	lysoPC a C20:4		x
Glycerophospholipids	Lysophosphatidylcholine a C26:0	lysoPC a C26:0		x
Glycerophospholipids	Lysophosphatidylcholine a C28:1	lysoPC a C28:1		x
Glycerophospholipids	Phosphatidylcholine aa C28:1	PC aa C28:1		x
Glycerophospholipids	Phosphatidylcholine aa C30:0	PC aa C30:0		x
Glycerophospholipids	Phosphatidylcholine aa C32:0	PC aa C32:0	x	
Glycerophospholipids	Phosphatidylcholine aa C32:1	PC aa C32:1		x
Glycerophospholipids	Phosphatidylcholine aa C32:2	PC aa C32:2		x
Glycerophospholipids	Phosphatidylcholine aa C32:3	PC aa C32:3		x
Glycerophospholipids	Phosphatidylcholine aa C34:1	PC aa C34:1		x
Glycerophospholipids	Phosphatidylcholine aa C34:3	PC aa C34:3		x
Glycerophospholipids	Phosphatidylcholine aa C36:1	PC aa C36:1		x
Glycerophospholipids	Phosphatidylcholine aa C36:6	PC aa C36:6		x
Glycerophospholipids	Phosphatidylcholine ae C30:1	PC ae C30:1		x
Glycerophospholipids	Phosphatidylcholine ae C32:1	PC ae C32:1		x
Glycerophospholipids	Phosphatidylcholine ae C32:2	PC ae C32:2		x
Glycerophospholipids	Phosphatidylcholine ae C34:0	PC ae C34:0		x
Glycerophospholipids	Phosphatidylcholine ae C34:1	PC ae C34:1	x	
Glycerophospholipids	Phosphatidylcholine ae C34:2	PC ae C34:2		x
Glycerophospholipids	Phosphatidylcholine ae C36:1	PC ae C36:1		x
Glycerophospholipids	Phosphatidylcholine ae C36:3	PC ae C36:3	x	
Glycerophospholipids	Phosphatidylcholine ae C38:1	PC ae C38:1		x
Glycerophospholipids	Phosphatidylcholine ae C38:2	PC ae C38:2		x
Glycerophospholipids	Phosphatidylcholine ae C38:6	PC ae C38:6		x
Glycerophospholipids	Phosphatidylcholine ae C40:6	PC ae C40:6		x
Sphingomyelins	Sphingomyelin C16:0	SM C16:0	x	
Sphingomyelins	Sphingomyelin C16:1	SM C16:1		x
Sphingomyelins	Sphingomyelin (OH) C16:1	SM (OH) C16:1	x	
Sphingomyelins	Sphingomyelin C18:0	SM C18:0		x
Sphingomyelins	Sphingomyelin C18:1	SM C18:1		x
Sphingomyelins	Sphingomyelin C20:2	SM C20:2		x

Analyte Class	Analyte	Short Name	>LOD in >90% Samples	>LOD in 50-90% Samples
Sphingomyelins	Sphingomyelin (OH) C22:1	SM (OH) C22:1		x
Sphingomyelins	Sphingomyelin C24:0	SM C24:0		x
Sphingomyelins	Sphingomyelin C24:1	SM C24:1		x
Sphingomyelins	Sphingomyelin (OH) C24:1	SM (OH) C24:1		x
Sphingomyelins	Sphingomyelin C26:0	SM C26:0		x
Sphingomyelins	Sphingomyelin C26:1	SM C26:1		x
Ceramides	Ceramide (d16:1/18:0)	Cer(d16:1/18:0)	x	
Ceramides	Ceramide (d16:1/20:0)	Cer(d16:1/20:0)	x	
Ceramides	Ceramide (d16:1/22:0)	Cer(d16:1/22:0)	x	
Ceramides	Ceramide (d16:1/23:0)	Cer(d16:1/23:0)	x	
Ceramides	Ceramide (d16:1/24:0)	Cer(d16:1/24:0)	x	
Ceramides	Ceramide (d18:1/14:0)	Cer(d18:1/14:0)		x
Ceramides	Ceramide (d18:1/16:0)	Cer(d18:1/16:0)	x	
Ceramides	Ceramide (d18:1/18:0(OH))	Cer(d18:1/18:0(OH))	x	
Ceramides	Ceramide (d18:1/18:0)	Cer(d18:1/18:0)	x	
Ceramides	Ceramide (d18:1/18:1)	Cer(d18:1/18:1)	x	
Ceramides	Ceramide (d18:1/20:0(OH))	Cer(d18:1/20:0(OH))	x	
Ceramides	Ceramide (d18:1/20:0)	Cer(d18:1/20:0)	x	
Ceramides	Ceramide (d18:1/22:0)	Cer(d18:1/22:0)	x	
Ceramides	Ceramide (d18:1/23:0)	Cer(d18:1/23:0)	x	
Ceramides	Ceramide (d18:1/24:0)	Cer(d18:1/24:0)	x	
Ceramides	Ceramide (d18:1/24:1)	Cer(d18:1/24:1)	x	
Ceramides	Ceramide (d18:1/25:0)	Cer(d18:1/25:0)		x
Ceramides	Ceramide (d18:1/26:0)	Cer(d18:1/26:0)	x	
Ceramides	Ceramide (d18:1/26:1)	Cer(d18:1/26:1)	x	
Ceramides	Ceramide (d18:2/14:0)	Cer(d18:2/14:0)		x
Ceramides	Ceramide (d18:2/16:0)	Cer(d18:2/16:0)		x
Ceramides	Ceramide (d18:2/18:0)	Cer(d18:2/18:0)	x	
Ceramides	Ceramide (d18:2/18:1)	Cer(d18:2/18:1)	x	
Ceramides	Ceramide (d18:2/20:0)	Cer(d18:2/20:0)		x
Ceramides	Ceramide (d18:2/22:0)	Cer(d18:2/22:0)	x	
Ceramides	Ceramide (d18:2/23:0)	Cer(d18:2/23:0)		x
Ceramides	Ceramide (d18:2/24:0)	Cer(d18:2/24:0)	x	
Ceramides	Ceramide (d18:2/24:1)	Cer(d18:2/24:1)	x	
Dihydroceramides	Dihydroceramide (d18:0/18:0(OH))	Cer(d18:0/18:0(OH))	x	
Dihydroceramides	Dihydroceramide (d18:0/18:0)	Cer(d18:0/18:0)	x	
Dihydroceramides	Dihydroceramide (d18:0/20:0)	Cer(d18:0/20:0)	x	
Dihydroceramides	Dihydroceramide (d18:0/22:0)	Cer(d18:0/22:0)		x
Dihydroceramides	Dihydroceramide (d18:0/24:0)	Cer(d18:0/24:0)	x	
Dihydroceramides	Dihydroceramide (d18:0/24:1)	Cer(d18:0/24:1)	x	
Dihydroceramides	Dihydroceramide (d18:0/26:1(OH))	Cer(d18:0/26:1(OH))	x	
Glycosylceramides	Hexosylceramide (d16:1/24:0)	HexCer(d16:1/24:0)	x	

Analyte Class	Analyte	Short Name	>LOD in >90% Samples	>LOD in 50-90% Samples
Glycosylceramides	Hexosylceramide (d16:1/22:0)	HexCer(d16:1/22:0)		x
Glycosylceramides	Hexosylceramide (d18:1/14:0)	HexCer(d18:1/14:0)	x	
Glycosylceramides	Hexosylceramide (d18:1/16:0)	HexCer(d18:1/16:0)	x	
Glycosylceramides	Hexosylceramide (d18:1/18:0)	HexCer(d18:1/18:0)		x
Glycosylceramides	Hexosylceramide (d18:1/18:1)	HexCer(d18:1/18:1)	x	
Glycosylceramides	Hexosylceramide (d18:1/20:0)	HexCer(d18:1/20:0)	x	
Glycosylceramides	Hexosylceramide (d18:1/22:0)	HexCer(d18:1/22:0)	x	
Glycosylceramides	Hexosylceramide (d18:1/23:0)	HexCer(d18:1/23:0)	x	
Glycosylceramides	Hexosylceramide (d18:1/24:0)	HexCer(d18:1/24:0)	x	
Glycosylceramides	Hexosylceramide (d18:1/24:1)	HexCer(d18:1/24:1)	x	
Glycosylceramides	Hexosylceramide (d18:1/26:0)	HexCer(d18:1/26:0)	x	
Glycosylceramides	Hexosylceramide (d18:1/26:1)	HexCer(d18:1/26:1)	x	
Glycosylceramides	Hexosylceramide (d18:2/16:0)	HexCer(d18:2/16:0)		x
Glycosylceramides	Hexosylceramide (d18:2/18:0)	HexCer(d18:2/18:0)		x
Glycosylceramides	Hexosylceramide (d18:2/20:0)	HexCer(d18:2/20:0)		x
Glycosylceramides	Hexosylceramide (d18:2/22:0)	HexCer(d18:2/22:0)	x	
Glycosylceramides	Hexosylceramide (d18:2/23:0)	HexCer(d18:2/23:0)	x	
Glycosylceramides	Dihexosylceramide (d18:1/14:0)	Hex2Cer(d18:1/14:0)		x
Glycosylceramides	Dihexosylceramide (d18:1/16:0)	Hex2Cer(d18:1/16:0)	x	
Glycosylceramides	Dihexosylceramide (d18:1/18:0)	Hex2Cer(d18:1/18:0)	x	
Glycosylceramides	Dihexosylceramide (d18:1/20:0)	Hex2Cer(d18:1/20:0)		x
Glycosylceramides	Dihexosylceramide (d18:1/22:0)	Hex2Cer(d18:1/22:0)	x	
Glycosylceramides	Dihexosylceramide (d18:1/24:0)	Hex2Cer(d18:1/24:0)	x	
Glycosylceramides	Dihexosylceramide (d18:1/24:1)	Hex2Cer(d18:1/24:1)	x	
Glycosylceramides	Dihexosylceramide (d18:1/26:0)	Hex2Cer(d18:1/26:0)		x
Glycosylceramides	Dihexosylceramide (d18:1/26:1)	Hex2Cer(d18:1/26:1)	x	
Glycosylceramides	Trihexosylceramide (d18:1/16:0)	Hex3Cer(d18:1/16:0)	x	
Glycosylceramides	Trihexosylceramide (d18:1/18:0)	Hex3Cer(d18:1/18:0)		x
Glycosylceramides	Trihexosylceramide (d18:1_20:0)	Hex3Cer(d18:1_20:0)		x
Glycosylceramides	Trihexosylceramide (d18:1_22:0)	Hex3Cer(d18:1_22:0)	x	
Glycosylceramides	Trihexosylceramide (d18:1/24:1)	Hex3Cer(d18:1/24:1)		x
Glycosylceramides	Trihexosylceramide (d18:1/26:1)	Hex3Cer(d18:1/26:1)		x
Cholesteryl esters	Cholesteryl ester (14:0)	CE(14:0)		x
Cholesteryl esters	Cholesteryl ester (15:0)	CE(15:0)	x	
Cholesteryl esters	Cholesteryl ester (15:1)	CE(15:1)		x
Cholesteryl esters	Cholesteryl ester (16:0)	CE(16:0)		x
Cholesteryl esters	Cholesteryl ester (16:1)	CE(16:1)		x
Cholesteryl esters	Cholesteryl ester (17:0)	CE(17:0)		x
Cholesteryl esters	Cholesteryl ester (17:1)	CE(17:1)	x	
Cholesteryl esters	Cholesteryl ester (18:0)	CE(18:0)		x
Cholesteryl esters	Cholesteryl ester (18:1)	CE(18:1)		x
Cholesteryl esters	Cholesteryl ester (18:3)	CE(18:3)		x

Analyte Class	Analyte	Short Name	>LOD in >90% Samples	>LOD in 50-90% Samples
Cholesteryl esters	Cholesteryl ester (20:0)	CE(20:0)		x
Cholesteryl esters	Cholesteryl ester (20:1)	CE(20:1)		x
Cholesteryl esters	Cholesteryl ester (20:5)	CE(20:5)		x
Cholesteryl esters	Cholesteryl ester (22:2)	CE(22:2)		x
Cholesteryl esters	Cholesteryl ester (22:5)	CE(22:5)		x
Cholesteryl esters	Cholesteryl ester (22:6)	CE(22:6)		x
Diglycerides	Diglyceride (14:0_18:1)	DG(14:0_18:1)		x
Diglycerides	Diglyceride (14:0_18:2)	DG(14:0_18:2)		x
Diglycerides	Diglyceride (14:1_18:1)	DG(14:1_18:1)		x
Diglycerides	Diglyceride (16:0_16:1)	DG(16:0_16:1)		x
Diglycerides	Diglyceride (16:0_18:1)	DG(16:0_18:1)		x
Diglycerides	Diglyceride (16:0_18:2)	DG(16:0_18:2)	x	
Diglycerides	Diglyceride (16:0_20:0)	DG(16:0_20:0)		x
Diglycerides	Diglyceride (16:0_20:3)	DG(16:0_20:3)		x
Diglycerides	Diglyceride (16:0_20:4)	DG(16:0_20:4)		x
Diglycerides	Diglyceride (16:1_18:0)	DG(16:1_18:0)	x	
Diglycerides	Diglyceride (16:1_18:2)	DG(16:1_18:2)	x	
Diglycerides	Diglyceride (16:1_20:0)	DG(16:1_20:0)		x
Diglycerides	Diglyceride (17:0_17:1)	DG(17:0_17:1)		x
Diglycerides	Diglyceride (17:0_18:1)	DG(17:0_18:1)		x
Diglycerides	Diglyceride (18:0_20:4)	DG(18:0_20:4)		x
Diglycerides	Diglyceride (18:1_18:1)	DG(18:1_18:1)	x	
Diglycerides	Diglyceride (18:1_18:2)	DG(18:1_18:2)	x	
Diglycerides	Diglyceride (18:1_18:4)	DG(18:1_18:4)		x
Diglycerides	Diglyceride (18:1_20:0)	DG(18:1_20:0)	x	
Diglycerides	Diglyceride (18:1_20:1)	DG(18:1_20:1)	x	
Diglycerides	Diglyceride (18:1_20:2)	DG(18:1_20:2)	x	
Diglycerides	Diglyceride (18:1_20:3)	DG(18:1_20:3)		x
Diglycerides	Diglyceride (18:1_20:4)	DG(18:1_20:4)		x
Diglycerides	Diglyceride (18:1_22:5)	DG(18:1_22:5)		x
Diglycerides	Diglyceride (18:2_18:2)	DG(18:2_18:2)	x	
Diglycerides	Diglyceride (18:2_18:3)	DG(18:2_18:3)	x	
Diglycerides	Diglyceride (18:2_18:4)	DG(18:2_18:4)		x
Diglycerides	Diglyceride (18:2_20:0)	DG(18:2_20:0)	x	
Diglycerides	Diglyceride (18:2_20:4)	DG(18:2_20:4)		x
Diglycerides	Diglyceride (18:3_18:3)	DG(18:3_18:3)	x	
Diglycerides	Diglyceride (18:3_20:2)	DG(18:3_20:2)		x
Diglycerides	Diglyceride (21:0_22:6)	DG(21:0_22:6)		x
Diglycerides	Diglyceride (22:1_22:2)	DG(22:1_22:2)		x
Diglycerides	Diglyceride-O(14:0_18:2)	DG-O(14:0_18:2)		x
Diglycerides	Diglyceride-O(16:0_20:4)	DG-O(16:0_20:4)		x
Diglycerides	Diglyceride-O(18:2_18:2)	DG-O(18:2_18:2)		x

Analyte Class	Analyte	Short Name	>LOD in >90% Samples	>LOD in 50-90% Samples
Triglycerides	Triglyceride (14:0_32:2)	TG(14:0_32:2)		x
Triglycerides	Triglyceride (14:0_35:2)	TG(14:0_35:2)		x
Triglycerides	Triglyceride (16:0_28:2)	TG(16:0_28:2)		x
Triglycerides	Triglyceride (16:0_32:3)	TG(16:0_32:3)		x
Triglycerides	Triglyceride (16:0_33:2)	TG(16:0_33:2)		x
Triglycerides	Triglyceride (16:0_34:4)	TG(16:0_34:4)		x
Triglycerides	Triglyceride (16:0_35:3)	TG(16:0_35:3)		x
Triglycerides	Triglyceride (16:0_36:6)	TG(16:0_36:6)		x
Triglycerides	Triglyceride (16:1_28:0)	TG(16:1_28:0)	x	
Triglycerides	Triglyceride (16:1_30:1)	TG(16:1_30:1)		x
Triglycerides	Triglyceride (16:1_36:5)	TG(16:1_36:5)		x
Triglycerides	Triglyceride (16:1_38:4)	TG(16:1_38:4)		x
Triglycerides	Triglyceride (16:1_38:5)	TG(16:1_38:5)		x
Triglycerides	Triglyceride (17:0_32:1)	TG(17:0_32:1)		x
Triglycerides	Triglyceride (17:0_34:3)	TG(17:0_34:3)		x
Triglycerides	Triglyceride (17:0_36:4)	TG(17:0_36:4)		x
Triglycerides	Triglyceride (17:1_34:3)	TG(17:1_34:3)		x
Triglycerides	Triglyceride (17:1_36:4)	TG(17:1_36:4)		x
Triglycerides	Triglyceride (17:1_36:5)	TG(17:1_36:5)		x
Triglycerides	Triglyceride (17:1_38:5)	TG(17:1_38:5)		x
Triglycerides	Triglyceride (17:1_38:6)	TG(17:1_38:6)		x
Triglycerides	Triglyceride (17:1_38:7)	TG(17:1_38:7)	x	
Triglycerides	Triglyceride (17:2_34:2)	TG(17:2_34:2)		x
Triglycerides	Triglyceride (17:2_34:3)	TG(17:2_34:3)		x
Triglycerides	Triglyceride (17:2_36:2)	TG(17:2_36:2)		x
Triglycerides	Triglyceride (17:2_36:3)	TG(17:2_36:3)		x
Triglycerides	Triglyceride (17:2_36:4)	TG(17:2_36:4)		x
Triglycerides	Triglyceride (17:2_38:5)	TG(17:2_38:5)		x
Triglycerides	Triglyceride (17:2_38:6)	TG(17:2_38:6)		x
Triglycerides	Triglyceride (17:2_38:7)	TG(17:2_38:7)		x
Triglycerides	Triglyceride (18:0_32:2)	TG(18:0_32:2)		x
Triglycerides	Triglyceride (18:0_36:5)	TG(18:0_36:5)		x
Triglycerides	Triglyceride (18:0_38:6)	TG(18:0_38:6)		x
Triglycerides	Triglyceride (18:0_38:7)	TG(18:0_38:7)		x
Triglycerides	Triglyceride (18:1_26:0)	TG(18:1_26:0)		x
Triglycerides	Triglyceride (18:1_30:1)	TG(18:1_30:1)		x
Triglycerides	Triglyceride (18:1_32:3)	TG(18:1_32:3)		x
Triglycerides	Triglyceride (18:1_33:2)	TG(18:1_33:2)		x
Triglycerides	Triglyceride (18:1_33:3)	TG(18:1_33:3)		x
Triglycerides	Triglyceride (18:1_35:3)	TG(18:1_35:3)		x
Triglycerides	Triglyceride (18:1_36:5)	TG(18:1_36:5)		x
Triglycerides	Triglyceride (18:1_36:6)	TG(18:1_36:6)		x

Analyte Class	Analyte	Short Name	>LOD in >90% Samples	>LOD in 50-90% Samples
Triglycerides	Triglyceride (18:1_38:5)	TG(18:1_38:5)		x
Triglycerides	Triglyceride (18:1_38:6)	TG(18:1_38:6)		x
Triglycerides	Triglyceride (18:1_38:7)	TG(18:1_38:7)		x
Triglycerides	Triglyceride (18:2_33:0)	TG(18:2_33:0)		x
Triglycerides	Triglyceride (18:2_33:2)	TG(18:2_33:2)		x
Triglycerides	Triglyceride (18:2_34:4)	TG(18:2_34:4)		x
Triglycerides	Triglyceride (18:2_35:3)	TG(18:2_35:3)		x
Triglycerides	Triglyceride (18:2_38:4)	TG(18:2_38:4)		x
Triglycerides	Triglyceride (18:2_38:5)	TG(18:2_38:5)		x
Triglycerides	Triglyceride (18:2_38:6)	TG(18:2_38:6)		x
Triglycerides	Triglyceride (18:3_33:2)	TG(18:3_33:2)		x
Triglycerides	Triglyceride (18:3_35:2)	TG(18:3_35:2)		x
Triglycerides	Triglyceride (18:3_38:6)	TG(18:3_38:6)		x
Triglycerides	Triglyceride (20:1_26:1)	TG(20:1_26:1)		x
Triglycerides	Triglyceride (20:1_30:1)	TG(20:1_30:1)		x
Triglycerides	Triglyceride (20:1_32:2)	TG(20:1_32:2)		x
Triglycerides	Triglyceride (20:1_34:3)	TG(20:1_34:3)		x
Triglycerides	Triglyceride (20:2_32:1)	TG(20:2_32:1)		x
Triglycerides	Triglyceride (20:2_34:3)	TG(20:2_34:3)	x	
Triglycerides	Triglyceride (20:2_34:4)	TG(20:2_34:4)		x
Triglycerides	Triglyceride (20:2_36:5)	TG(20:2_36:5)		x
Triglycerides	Triglyceride (20:3_32:0)	TG(20:3_32:0)		x
Triglycerides	Triglyceride (20:3_32:1)	TG(20:3_32:1)		x
Triglycerides	Triglyceride (20:3_32:2)	TG(20:3_32:2)	x	
Triglycerides	Triglyceride (20:3_34:0)	TG(20:3_34:0)		x
Triglycerides	Triglyceride (20:3_34:2)	TG(20:3_34:2)		x
Triglycerides	Triglyceride (20:3_34:3)	TG(20:3_34:3)		x
Triglycerides	Triglyceride (20:3_36:3)	TG(20:3_36:3)		x
Triglycerides	Triglyceride (20:3_36:4)	TG(20:3_36:4)		x
Triglycerides	Triglyceride (20:3_36:5)	TG(20:3_36:5)		x
Triglycerides	Triglyceride (20:4_30:0)	TG(20:4_30:0)		x
Triglycerides	Triglyceride (20:4_32:2)	TG(20:4_32:2)		x
Triglycerides	Triglyceride (20:4_33:2)	TG(20:4_33:2)		x
Triglycerides	Triglyceride (20:4_34:3)	TG(20:4_34:3)		x
Triglycerides	Triglyceride (20:4_35:3)	TG(20:4_35:3)		x
Triglycerides	Triglyceride (20:5_34:0)	TG(20:5_34:0)		x
Triglycerides	Triglyceride (20:5_34:2)	TG(20:5_34:2)		x
Triglycerides	Triglyceride (22:0_32:4)	TG(22:0_32:4)	x	
Triglycerides	Triglyceride (22:1_32:5)	TG(22:1_32:5)	x	
Triglycerides	Triglyceride (22:2_32:4)	TG(22:2_32:4)		x
Triglycerides	Triglyceride (22:3_30:2)	TG(22:3_30:2)		x
Triglycerides	Triglyceride (22:4_32:0)	TG(22:4_32:0)		x

Analyte Class	Analyte	Short Name	>LOD in >90% Samples	>LOD in 50-90% Samples
Triglycerides	Triglyceride (22:4_32:2)	TG(22:4_32:2)		x
Triglycerides	Triglyceride (22:4_34:2)	TG(22:4_34:2)		x
Triglycerides	Triglyceride (22:5_34:3)	TG(22:5_34:3)		x
Triglycerides	Triglyceride (22:6_32:0)	TG(22:6_32:0)		x
Triglycerides	Triglyceride (22:6_32:1)	TG(22:6_32:1)		x
Triglycerides	Triglyceride (22:6_34:3)	TG(22:6_34:3)		x
Total			**137**	**231**

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Analysis of Human Fecal Samples with the MxP® Quant 500 Kit

Below you find an extraction protocol to analyze human fecal samples with Biocrates' MxP® Quant 500 kit. Please note that we have carried out feasibility tests only and that the MxP® Quant 500 kit has not been validated with fecal samples. We recommend performing pilot tests with representative fecal samples before starting a larger study. The results may depend on the nature and on the preparation of the samples.

1. Preparation of Feces Extraction Buffer

Total volume: 400 mL

Buffer	Description
Feces extraction buffer	Ethanol/phosphate buffer (85:15 v/v): 340 mL ethanol + 60 mL phosphate buffer (10 mM)[1] Cool the buffer (2-4 °C) before use.

2. Sample Collection and Storage

Step	Do this
1	Take a portion of the stool (0.2-3 g) using the spoon of a vial container like Sarstedt Inc FECES CONTAINER (ideally, pool from three different locations, or use them as separate samples to get an overview about inhomogeneity of individual samples).
2	If possible, put the samples on ice and transport them as soon as possible to the laboratory.
3	Document sample collection and transport time.
4	Weigh fresh samples and note the individual sample weights.
5	If possible, lyophilize samples prior to storage (see section 3).
6	Store the samples as soon as possible at -80 °C.

[1] Recommended: Sigma, Phosphate buffer solution, P5244 (0.1 M, pH = 7.5 at 25 °C); 1:10 diluted

3. Lyophilization Protocol

This protocol is developed for the Beta 2-16 model lyophilization instrument from Martin Christ Gefriertrocknungsanlagen GmbH, Germany for up to 72 fecal samples to be processed in parallel. Table 3 shows the appropriate program to lyophilize the samples.

Step	Do this
1	Place the empty sample rack into the condenser chamber of the instrument and close the lid.
2	Push “prepare” button: condenser will cool down; vacuum pump will get ready in 1 hour.
3	Place frozen samples into the rack and start the lyophilization program (Table 3).
4	Weigh again the dried samples for water content calculation.
5	By using a spatula convert the samples into powder.
6	Freeze the samples at -80 °C until sample extraction.

Table 3: Recommended lyophilization program for fecal samples.

Step	Temperature [°C]	Pressure [mbar]	Time [hh:mm]
1	-40	Atmospheric	-
2	-36	0.12	00:06
3	-35	0.12	08:00
4	-25	0.12	08:00
5	-15	0.12	08:00
6	-5	0.001	08:00
7	5	0.001	02:00
8	15	0.001	02:00
9	25	0.001	02:00
10	30	0.001	02:00
11	30	0.001	00:30
12	defrost and vent		01:00
Total			**41:36**

4. Sample Preparation (Day 1)

Step	Do this
1	Thaw the samples at room temperature.
2	Add 10-fold volume of cold (2-4 °C) feces extraction buffer to each lyophilized sample, or 3-fold volume if samples are not lyophilized.
3	Shake at 2000 rpm for 2 x 2 minutes or vortex for 3 minutes. Shake at 200 rpm for 30 minutes on ice, sonicate at 70 W for 5 minutes on ice, and centrifuge at 800 g for 3 minutes (if possible, at 2-4 °C).
4	Take the supernatant and centrifuge again at 19,000 g for 10 minutes at 2-4 °C.
5	Take the supernatant and store it immediately at -80 °C until analysis.

5. Kit Preparation (Day 2)

Step	Do this
1	Thaw the sample extracts at room temperature.
2	Load 10 µL of the sample extracts onto the 96-well kit plate and follow the protocol as described in the MxP® Quant 500 kit user manual.

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