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Metabolome and lipidome analysis of human fecal samples

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Table of contents

1 Introduction

The emerging links between the microbiome and many disorders have put human gut microbiota (GM) and nutrition-microbiome-host interactions at the forefront of clinical research aiming to explore the causalities and implications of such connections. 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 gut bacterial composition. However, quantitative metabolomic analyses in feces are more challenging than analyses in buffered systems and highly homogenous sample matrices such as blood plasma or serum. Feces is also directly affected by factors such as daily nutrition, drug intake, fluid intake, and gut activity. As a result, metabolomic analyses of fecal samples show higher biological variances than other samples, even if samples were obtained from the same individual at different time points, and even from the same stool sample at different topographical locations. This variability suggests an urgent need to standardize pre-analytical sample collection and preparation for metabolomic analysis of feces, and to develop tools for reproducible and accurate analyses.

The varying water content of feces samples, typically ranging from 60% to 85%, makes the interpretation of metabolomics results particularly challenging. While metabolite concentrations in urine samples can be normalized to creatinine to overcome differences in water content, no such metabolite exists for stool samples.

This application note describes a sample preparation protocol for fresh, frozen, and lyophilized fecal samples developed for analyses using the MxP® Quant 500 kit. It includes advice on improving the comparability of metabolomics results across studies and laboratories. Finally, it describes expected metabolite detectability in feces together with an overview of the expected biological variances. A separate application note covers the advantages and disadvantages of different sampling devices and the effect of storage time and temperature on metabolite stability in fecal samples.[1]

2 Methods

The sample collection and preparation protocols applied here have been developed and optimized for the MxP Quant 500 kit (see section 6). Lyophilization of the sample and normalization of the obtained data to the water content help minimize the effect of varying water content between samples but will not completely eliminate biological variance. It is highly recommended to homogenize the entire stool sample if possible, or to pool at least three replicates from different topographical locations of one stool sample.[2]

The recommended best practice according to Gratton et al.[3] is to cool fresh samples at 4 °C, and conduct extractions within one hour (24 hours at most) after collection to avoid increased amino acid levels. Gorzelak et al.[4] highly recommend immediately freezing the samples at -80 °C after collection to prevent changes in the microbial metabolism, allowing up to four freeze-thaw cycles. Using lyophilized samples instead of fresh samples eliminates differences in water content and minimizes variations in the metabolite profile, although lyophilization has been shown to reduce the number of detectable metabolites.[5]

Preparation of fecal samples typically involves raw feces, either wet or lyophilized. We have tested different extraction solvents and solvent combinations using methanol, ethanol, isopropanol, water and phosphate buffer. For the wide range of polar metabolites and lipids covered by the MxP Quant 500 kit, extraction with pure isopropanol achieved the best results regarding detectability and variance for wet fecal samples (stored at -80 °C until extraction). For lyophilized fecal samples, we recommend an ethanol/phosphate buffer (85:15 volume per volume (v/v)) or 80% isopropanol in water for extraction. The results were slightly better with 80% isopropanol in water, but this was tested in a small number of samples only. If focusing on small molecules rather than

lipids, extraction with ethanol/phosphate buffer (85:15 v/v) is recommended for both wet and lyophilized fecal samples. Sample preparation protocols are detailed in section 6. For an overview of additional extraction methods tested for feces preparation for biocrates kits, refer to the results published by Erben and colleagues.[6]

Table 1: Number of analytes (per analyte class) detectable in human fecal samples using the MxP® Quant 500 kit (detectable = concentration above the limit of detection (LOD)).

Analyte class (no. of analytes covered)	No. of analytes detected >LOD in >80% samples	No. of analytes detected >LOD in 50-80% samples
Alkaloids (1)	1	0
Amine oxides (1)	0	0
Amino acids (20)	18	1
Amino acid-related (30)	18	4
Bile acids (14)	12	2
Biogenic amines (9)	6	1
Carbohydrates and related (1)	1	0
Carboxylic acids (7)	0	1
Cresols (1)	1	0
Fatty acids (12)	9	3
Hormones (4)	0	1
Indoles and derivatives (4)	3	1
Nucleobases and related (2)	2	0
Vitamins and cofactors (1)	1	0
Acylcarnitines (40)	3	7
Ceramides (28)	26	1
Cholesteryl esters (22)	3	6
Diglycerides (44)	32	3
Dihydroceramides (8)	5	3
Glycosylceramides (34)	20	7
Phosphatidylcholines (90)	0	11
Sphingomyelins (15)	0	1
Triglycerides (242)	122	70

3 Results

3.1 Metabolite coverage using the MxP® Quant 500 kit

To demonstrate which polar metabolites and lipids covered by the MxP® Quant 500 kit can typically be detected in human fecal samples, 48 individual freshly frozen fecal samples were analyzed using the sample preparation protocol described above. We used a SCIEX 5500+ system for the mass spectrometric analysis. Table 1 shows an overview of the number of analytes per class detected in more than 80% of the samples, as well as additional analytes detected in 50-80% of the tested samples. In total, 283 metabolites and lipids were detected in more than 80% of the samples at concentrations above the limit of detection (LOD). An additional 123 metabolites and lipids were detected in 50-80% of the tested samples. This means that in a typical human fecal sample up to 406 analytes can be expected >LOD.

In human plasma samples, approximately 25% more metabolites and lipids (up to 520) can be detected >LOD compared to fecal samples. Around 400 of the metabolites and lipids covered by the kit are detectable in both matrices. Table 2 lists the detectability of all individual analytes from Table 1.

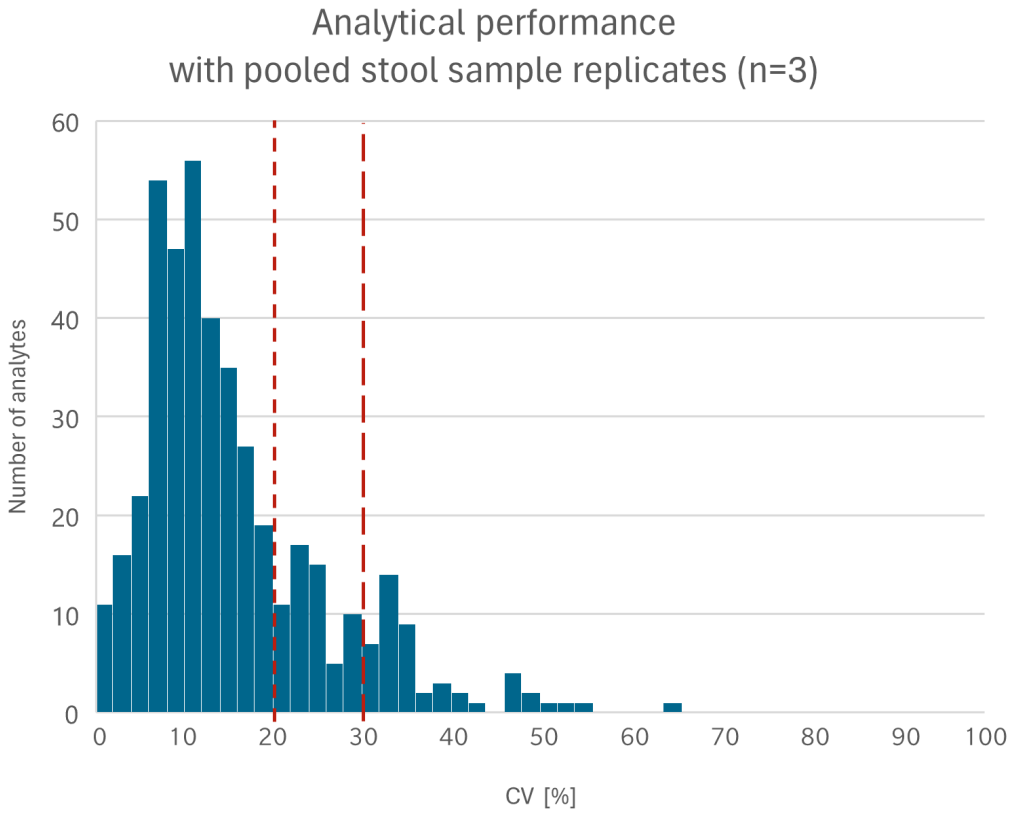

Figure 1: Histogram of all analyte CVs calculated from concentrations of replicates of pooled feces samples (n=3). The acceptable CV thresholds for the LC-MS/MS (20%) and FIA-MS/MS (30%) measurements according to kit specifications are shown as red dashed lines.

The accuracy of the quantification could not be calculated as there was no standard material with known concentrations available at the time this test was conducted. Analytical performance was assessed by calculating the precision based on the coefficient of variance (CV) of three replicates of pooled feces samples (Figure 1). 88% of the detected analytes measured with LC-MS/MS showed a CV <20%, and 87% of the detected analytes measured with FIA-MS/MS showed a CV <30%. The median CV was 12%.

About 40% of the analytes above 20% and 30% CV, respectively, displayed concentrations below the lower limit of quantification (LLOQ) and/or were very close to the LOD, and 4% displayed concentrations above the upper limit of quantification (ULOQ).

3.2 Biological variance of the metabolome and lipidome in human fecal samples

To evaluate the heterogeneity of fecal samples, six aliquots from the same stool sample were taken in close proximity to one another (as close as possible) and processed as described above for fresh samples. Three pipetting replicates of each aliquot were measured. The experiment was repeated using a different sample from the same individual collected two weeks later.

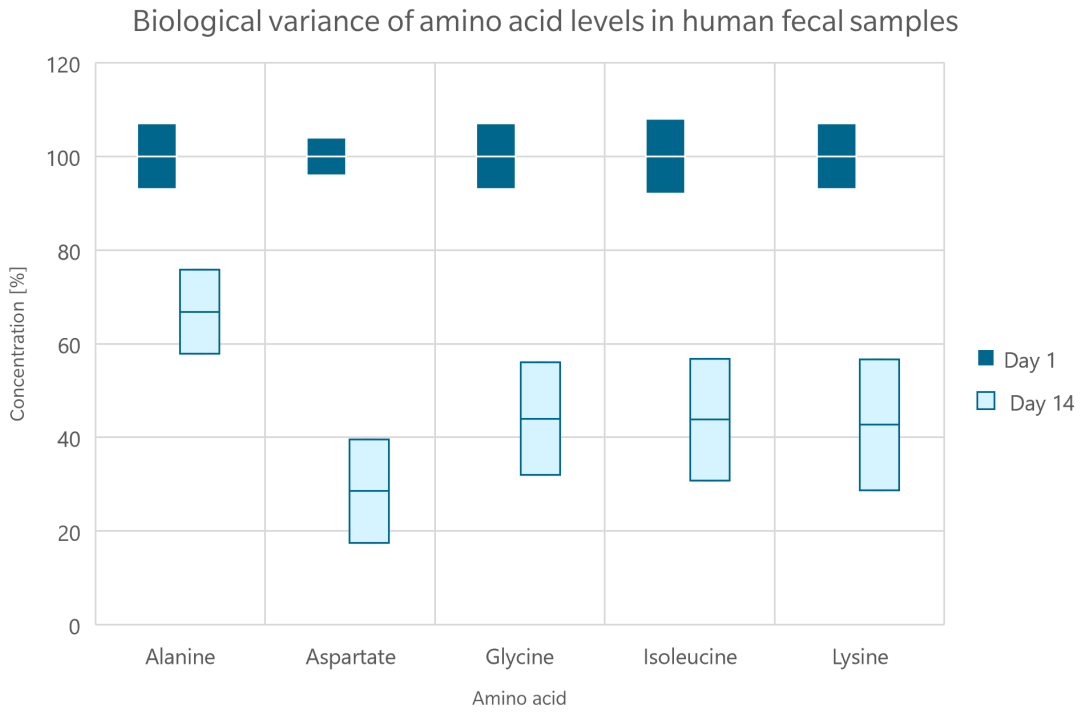

Figure 2: Biological variance of amino acid levels in human fecal samples. Amino acid concentration ranges from different topographical locations (n = 6) of one fecal sample at two different time points (day 1 and 14) from the same individual are depicted.

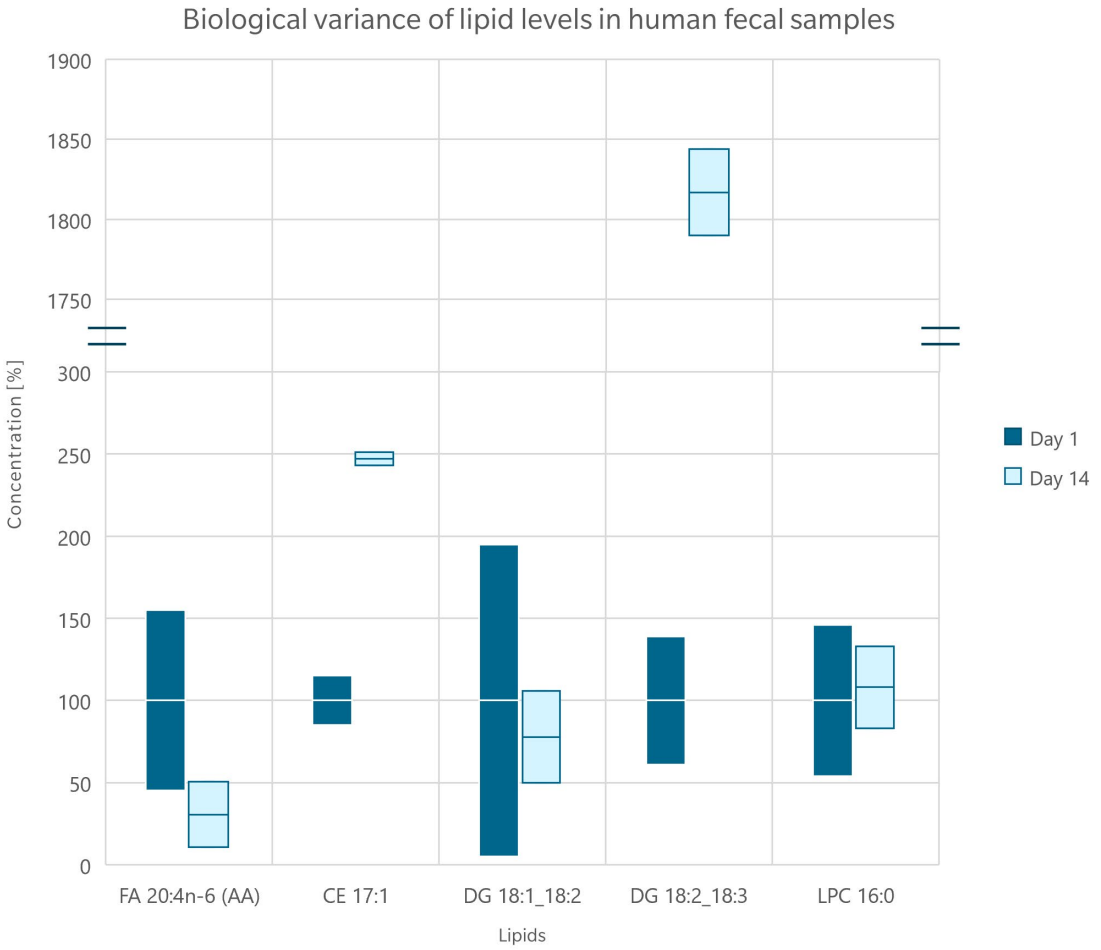

Figure 3: Biological variance of lipid levels in human fecal samples. Diglyceride (DG 18:2_18:3, DG18:1_18:2), cholesteryl ester (CE 17:1), arachidonic acid (AA), and lysophosphatidylcholine (LPC 16:0) concentration ranges from different topographical locations (n = 6) of one fecal sample at two different time points (day 1 and 14) from the same individual are depicted.

As expected, inter-day variance was higher than intra-sample variance, though the latter was significant. Figures 2 and 3 show some examples for amino acids and lipids, respectively. Amino acid concentrations were relatively consistent across different sample locations, but day 14 samples had significantly lower concentrations compared to day 1. For example, alanine concentration varied between 367 and 435 µM at day 1, and between 231 and 305 µM at day 14.

These numbers reveal a pronounced biological heterogeneity of fecal samples over time. This effect was even more prominent among lipids, with DG 18:2_18:3 concentration increasing more than 18-fold between day 1 and 14.

The technical variance, calculated from three technical replicates per sample, was below 15% for all amino acids and below 30% for all lipids shown, meaning they were in the usual specification range for all metabolites. This proves the differences between day 1 and day 14 are due to biological variances, which play a bigger role than in blood-based or tissue samples. These differences could be attributed to factors such as water content of the stool, different food/beverage intake, or bowel activity.

Most lipids exhibited notable concentration variances within aliquots taken from the same stool sample. For example, the DG 18:1_18:2 concentration varied from almost zero to twice the mean value (Figure 3). This underlines how huge the differences between aliquots of the same stool sample can be. Therefore, we highly recommend homogenizing the entire stool sample and taking an aliquot for further sample preparation, rather than simply taking an aliquot of a stool sample, to avoid misleading results.

3.3 Metabolite stability in feces samples and different feces sampling devices

As discussed in section 2, we recommend using freshly frozen or lyophilized stool samples. However, studies involving at-home sampling may require sampling devices that facilitate the collection of a defined amount of stool and stabilize the metabolites without freezing.

In another application note,[1] we compared two sampling devices and investigated metabolite stability using different buffers or solutions for metabolite stabilization and extraction.

4 Conclusions

The MxP Quant 500 kit enables simultaneous quantification of more than 400 different metabolites in human feces. Although designed for quantification of plasma metabolites, the kit meets analytical specifications for fecal samples. Testing highlighted the robustness of the assay for fecal samples, as the majority of small molecules were below 20% CV and lipids below 30% CV.

However, the biological variance of the metabolome in fecal samples was significantly higher than the analytical variance, even when the samples were taken in close proximity within the same stool sample. Therefore, it is important to adhere to the same sample preparation protocol for all fecal samples and take biological variance into consideration during 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 strongly recommended.

For interpretation, we encourage using metabolite ratios (available with the MetaboINDICATOR™ tool in the WebIDQ workflow manager) to reduce variance caused by diverse water contents and sampling locations.

Table 2: List of analytes detectable >LOD in human fecal samples (n = 48) using MxP® Quant 500 kit.

Analyte class	Analyte	Short name	>LOD in >80% samples	>LOD in 50-80% 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	Glutamine	Gln	x	
Amino acids	Glutamic acid	Glu	x	
Amino acids	Glycine	Gly	x	
Amino acids	Histidine	His	x	
Amino acids	Isoleucine	Iso	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	1-Methylhistidine	1-Met-His	x	
Amino acid-related	3-Methylhistidine	3-Met-His	x	
Amino acid-related	5-Aminovaleric acid	5-AVA	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	L-Anserine	Anserine	x	
Amino acid-related	β-Aminobutyric acid	BABA		x
Amino acid-related	cis -4-Hydroxyproline	c4-OH-Proline	x	
Amino acid-related	Citrulline	Cit	x	
Amino acid-related	Cystine	Cystine	x	
Amino acid-related	Homoarginine	HArg	x	
Amino acid-related	Homocysteine	HCys	x	
Amino acid-related	Methionine-sulfoxide	Met-SO	x	
Amino acid-related	Ornithine	Orn		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	trans-4-Hydroxyproline	t4-OH-Pro	x	
Amino acid-related	Taurine	Taurine	x	
Amino acid-related	Tryptophan betaine	TrpBetaine		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	Glycodeoxycholic acid	GDCA	x	
Bile acids	Glycolithocholic acid	GLCA	x	

Bile acids	Glycolithocholic acid sulfate	GLCAS	x	
Bile acids	Glycoursodeoxycholic acid	GUDCA	x	
Bile acids	Taurocholic acid	TCA	x	
Bile acids	Taurochenodeoxycholic acid	TCDCA	x	
Bile acids	Taurodeoxycholic acid	TDCA	x	
Bile acids	Taurolithocholic acid	TLCA	x	
Bile acids	Tauromurocholic acid	TMCA		x
Biogenic amines	β-Alanine	b-Ala	x	
Biogenic amines	γ-Aminobutyric acid	GABA	x	
Biogenic amines	Histamine	Histamine		x
Biogenic amines	Phenylethylamine	PEA	x	
Biogenic amines	Putrescine	Putrescine	x	
Biogenic amines	Serotonin	Serotonin	x	
Biogenic amines	Spermidine	Spermidine	x	
Carboxylic acids	Aconitic acid	AconAcid		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	Stearic acid	FA 18:0		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	
Fatty acids	Eicosatrienoic acid	FA 20:3		x
Fatty acids	Arachidonic acid (FA 20:4ω6)	FA 20:4n-6 (AA)	x	
Fatty acids	Eicosapentaenoic acid (FA 20:5ω3)	FA 20:5n-3 (EPA)	x	
Fatty acids	Docosahexaenoic acid (FA 22:6ω3)	FA 22:6n-3 (DHA)	x	
Hormones	Abscisic acid	AbsAcid		x
Indoles	Indoleacetic acid	3-IAA	x	
Indoles	Indolepropionic acid	3-IPA	x	
Indoles	Indoxyl sulfate	Ind-SO_4		x
Indoles	Indole	Indole	x	
Nucleobases and related	Hypoxanthine	Hypoxanthine	x	
Nucleobases and related	Xanthine	Xanthine	x	
Vitamins and cofactors	Choline	Choline	x	

Acylcarnitines	Malonylcarnitine (Hydroxybutyrylcarnitine)	C3-DC (C4-OH)	x	
Acylcarnitines	Propenoylcarnitine	C3:1	x	
Acylcarnitines	Butyrylcarnitine	C4		x
Acylcarnitines	Hydroxyvalerylcarnitine (Methylmalonylcarnitine)	C5-OH (C3-DC-M)		x
Acylcarnitines	Nonanoylcarnitine	C9		x
Acylcarnitines	Hydroxytetradecadienoyl-carnitine	C14:2-OH	x	
Acylcarnitines	Hexadecanoylcarnitine	C16		x
Acylcarnitines	Hydroxyhexadecanoyl-carnitine	C16-OH		x
Acylcarnitines	Hexadecenoylcarnitine	C16:1		x
Acylcarnitines	Octadecanoylcarnitine	C18		x
Carbohydrates and related	Hexose	Hexose	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	Cer d18:1/18:0	x	
Ceramides	Ceramide d18:1/18:0-OH	Cer d18:1/18:0-OH	x	
Ceramides	Ceramide d18:1/18:1	Cer d18:1/18:1	x	
Ceramides	Ceramide d18:1/20:0	Cer d18:1/20:0	x	
Ceramides	Ceramide d18:1/20:0-OH	Cer d18:1/20:0-OH	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 d18:1/26:0	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	
Cholesteryl esters	Cholesteryl ester 16:1	CE 16:1		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 18:2	CE 18:2	x	
Cholesteryl esters	Cholesteryl ester 18:3	CE 18:3		x
Cholesteryl esters	Cholesteryl ester 20:5	CE 20:5		x
Cholesteryl esters	Cholesteryl ester 22:0	CE 22:0		x
Cholesteryl esters	Cholesteryl ester 22:1	CE 22:1	x	
Diglycerides	Diacylglyceride 14:0_14:0	DG 14:0_14:0	x	
Diglycerides	Diacylglyceride 14:0_18:1	DG 14:0_18:1	x	
Diglycerides	Diacylglyceride 14:0_18:2	DG 14:0_18:2	x	
Diglycerides	Diacylglyceride 14:1_18:1	DG 14:1_18:1	x	
Diglycerides	Diacylglyceride 14:1_20:2	DG 14:1_20:2		x
Diglycerides	Diacylglyceride 16:0_16:1	DG 16:0_16:1	x	
Diglycerides	Diacylglyceride 16:0_18:1	DG 16:0_18:1	x	
Diglycerides	Diacylglyceride 16:0_18:2	DG 16:0_18:2	x	
Diglycerides	Diacylglyceride 16:0_20:0	DG 16:0_20:0		x
Diglycerides	Diacylglyceride16:0_20:3	DG 16:0_20:3	x	
Diglycerides	Diacylglyceride 16:0_20:4	DG 16:0_20:4	x	
Diglycerides	Diacylglyceride 16:1_18:0	DG 16:1_18:0	x	
Diglycerides	Diacylglyceride 16:1_18:1	DG 16:1_18:1	x	
Diglycerides	Diacylglyceride 16:1_18:2	DG 16:1_18:2	x	
Diglycerides	Diacylglyceride 17:0_18:1	DG 17:0_18:1	x	
Diglycerides	Diacylglyceride 18:0_20:4	DG 18:0_20:4	x	
Diglycerides	Diacylglyceride 18:1_18:1	DG 18:1_18:1	x	
Diglycerides	Diacylglyceride 18:1_18:2	DG 18:1_18:2	x	
Diglycerides	Diacylglyceride 18:1_18:3	DG 18:1_18:3	x	
Diglycerides	Diacylglyceride 18:1_18:4	DG 18:1_18:4	x	
Diglycerides	Diacylglyceride 18:1_20:0	DG 18:1_20:0	x	
Diglycerides	Diacylglyceride 18:1_20:1	DG 18:1_20:1	x	
Diglycerides	Diacylglyceride 18:1_20:2	DG 18:1_20:2	x	
Diglycerides	Diacylglyceride 18:1_20:3	DG 18:1_20:3		x
Diglycerides	Diacylglyceride 18:1_20:4	DG 18:1_20:4	x	
Diglycerides	Diacylglyceride 18:1_22:5	DG 18:1_22:5	x	
Diglycerides	Diacylglyceride 18:1_22:6	DG 18:1_22:6	x	
Diglycerides	Diacylglyceride 18:2_18:2	DG 18:2_18:2	x	
Diglycerides	Diacylglyceride 18:2_18:3	DG 18:2_18:3	x	

Diglycerides	Diacylglyceride 18:2_18:4	DG 18:2_18:4	x	
Diglycerides	Diacylglyceride 18:2_20:0	DG 18:2_20:0	x	
Diglycerides	Diacylglyceride 18:2_20:4	DG 18:2_20:4	x	
Diglycerides	Diacylglyceride 18:3_18:3	DG 18:3_18:3	x	
Diglycerides	Diacylglyceride O-14:0_18:2	DG O-14:0_18:2	x	
Diglycerides	Diacylglyceride O-16:0_18:1	DG O-16:0_18:1	x	
Dihydroceramides	Dihydroceramide d18:0/18:0	Cer d18:0/18:0		x
Dihydroceramides	Dihydroceramide d18:0/18:0-OH	Cer d18:0/18:0-OH	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	Cer d18:0/26:1	x	
Dihydroceramides	Dihydroceramide d18:0/26:1-OH	Cer d18:0/26:1-OH	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 18:1/18:0	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	
Glycosylceramides	Hexosylceramide d16:1/22:0	Hex-Cer d16:1/22:0		x
Glycosylceramides	Hexosylceramide d18:1/14:0	Hex-Cer d18:1/14:0		x
Glycosylceramides	Hexosylceramide d18:1/16:0	Hex-Cer d18:1/16:0	x	
Glycosylceramides	Hexosylceramide d18:1/18:0	Hex-Cer d18:1/18:0	x	
Glycosylceramides	Hexosylceramide d18:1/18:1	Hex-Cer d18:1/18:1	x	
Glycosylceramides	Hexosylceramide d18:1/20:0	Hex-Cer d18:1/20:0		x
Glycosylceramides	Hexosylceramide d18:1/22:0	Hex-Cer d18:1/22:0	x	
Glycosylceramides	Hexosylceramide d18:1/23:0	Hex-Cer d18:1/23:0	x	
Glycosylceramides	Hexosylceramide d18:1/24:0	Hex-Cer d18:1/24:0	x	
Glycosylceramides	Hexosylceramide d18:1/24:1	Hex-Cer d18:1/24:1	x	
Glycosylceramides	Hexosylceramide d18:2/16:0	Hex-Cer d18:2/16:0	x	
Glycosylceramides	Hexosylceramide d18:2/18:0	Hex-Cer d18:2/18:0		x
Glycosylceramides	Hexosylceramide d18:2/20:0	Hex-Cer d18:2/20:0		x
Lysophosphatidyl-cholines	Lysophosphatidylcholine 18:1	LPC 18:1		x
Lysophosphatidyl-cholines	Lysophosphatidylcholine 18:2	LPC 18:2		x
Phosphatidylcholines	Phosphatidylcholine 32:2	PC 32:2		x
Phosphatidylcholines	Phosphatidylcholine 32:3	PC 32:3		x
Phosphatidylcholines	Phosphatidylcholine 34:3	PC 34:3		x
Phosphatidylcholines	Phosphatidylcholine 36:3	PC 36:3		x
Phosphatidylcholines	Phosphatidylcholine 36:4	PC 36:4		x
Phosphatidylcholines	Phosphatidylcholine 36:5	PC 36:5		x
Phosphatidylcholines	Phosphatidylcholine 38:3	PC 38:0		x
Phosphatidylcholines	Phosphatidylcholine 34:2	PC O-34:2		x
Phosphatidylcholines	Phosphatidylcholine 34:3	PC O-34:3		x
Phosphatidylcholines	Phosphatidylcholine 36:3	PC O-36:3		x

Sphingomyelins	Sphingomyelin 38:3	SM 38:3		x
Triglycerides	Triacylglyceride 14:0_32:2	TG 14:0_32:2		x
Triglycerides	Triacylglyceride 14:0_34:1	TG 14:0_34:1		x
Triglycerides	Triacylglyceride 14:0_34:2	TG 14:0_34:2	x	
Triglycerides	Triacylglyceride 14:0_36:1	TG 14:0_36:1		x
Triglycerides	Triacylglyceride 14:0_36:2	TG 14:0_36:2	x	
Triglycerides	Triacylglyceride 14:0_36:3	TG 14:0_36:3	x	
Triglycerides	Triacylglyceride 14:0_36:4	TG 14:0_36:4	x	
Triglycerides	Triacylglyceride 16:0_28:1	TG 16:0_28:1		x
Triglycerides	Triacylglyceride 16:0_28:2	TG 16:0_28:2		x
Triglycerides	Triacylglyceride 16:0_30:2	TG 16:0_30:2		x
Triglycerides	Triacylglyceride 16:0_32:0	TG 16:0_32:0		x
Triglycerides	Triacylglyceride 16:0_32:1	TG 16:0_32:1	x	
Triglycerides	Triacylglyceride 16:0_32:2	TG 16:0_32:2	x	
Triglycerides	Triacylglyceride 16:0_33:1	TG 16:0_33:1		x
Triglycerides	Triacylglyceride 16:0_33:2	TG 16:0_33:2		x
Triglycerides	Triacylglyceride 16:0_34:0	TG 16:0_34:0	x	
Triglycerides	Triacylglyceride 16:0_34:1	TG 16:0_34:1	x	
Triglycerides	Triacylglyceride 16:0_34:2	TG 16:0_34:2	x	
Triglycerides	Triacylglyceride 16:0_34:3	TG 16:0_34:3	x	
Triglycerides	Triacylglyceride 16:0_34:4	TG 16:0_34:4		x
Triglycerides	Triacylglyceride 16:0_35:1	TG 16:0_35:1		x
Triglycerides	Triacylglyceride 16:0_35:2	TG 16:0_35:2	x	
Triglycerides	Triacylglyceride 16:0_35:3	TG 16:0_35:3		x
Triglycerides	Triacylglyceride 16:0_36:2	TG 16:0_36:2	x	
Triglycerides	Triacylglyceride 16:0_36:3	TG 16:0_36:3	x	
Triglycerides	Triacylglyceride 16:0_36:4	TG 16:0_36:4	x	
Triglycerides	Triacylglyceride 16:0_36:5	TG 16:0_36:5	x	
Triglycerides	Triacylglyceride 16:0_36:6	TG 16:0_36:6	x	
Triglycerides	Triacylglyceride 16:0_37:3	TG 16:0_37:3	x	
Triglycerides	Triacylglyceride 16:0_38:1	TG 16:0_38:1		x
Triglycerides	Triacylglyceride 16:0_38:2	TG 16:0_38:2	x	
Triglycerides	Triacylglyceride 16:0_38:3	TG 16:0_38:3	x	
Triglycerides	Triacylglyceride 16:0_38:4	TG 16:0_38:4	x	
Triglycerides	Triacylglyceride 16:0_38:6	TG 16:0_38:6		x
Triglycerides	Triacylglyceride 16:0_38:7	TG 16:0_38:7		x
Triglycerides	Triacylglyceride 16:1_32:1	TG 16:1_32:1		x
Triglycerides	Triacylglyceride 16:1_32:2	TG 16:1_32:2		x
Triglycerides	Triacylglyceride 16:1_34:0	TG 16:1_34:0		x
Triglycerides	Triacylglyceride 16:1_34:1	TG 16:1_34:1	x	
Triglycerides	Triacylglyceride 16:1_34:2	TG 16:1_34:2	x	

Triglycerides	Triacylglyceride 16:1_34:3	TG 16:1_34:3		x
Triglycerides	Triacylglyceride 16:1_36:1	TG 16:1_36:1	x	
Triglycerides	Triacylglyceride 16:1_36:2	TG 16:1_36:2	x	
Triglycerides	Triacylglyceride 16:1_36:3	TG 16:1_36:3	x	
Triglycerides	Triacylglyceride 16:1_36:4	TG 16:1_36:4	x	
Triglycerides	Triacylglyceride 16:1_36:5	TG 16:1_36:5		x
Triglycerides	Triacylglyceride 16:1_38:3	TG 16:1_38:3	x	
Triglycerides	Triacylglyceride 16:1_38:4	TG 16:1_38:4	x	
Triglycerides	Triacylglyceride 16:1_38:5	TG 16:1_38:5		x
Triglycerides	Triacylglyceride 17:0_34:1	TG 17:0_34:1	x	
Triglycerides	Triacylglyceride 17:0_34:2	TG 17:0_34:2	x	
Triglycerides	Triacylglyceride 17:0_36:3	TG 17:0_36:3	x	
Triglycerides	Triacylglyceride 17:0_36:4	TG 17:0_36:4	x	
Triglycerides	Triacylglyceride 17:1_34:1	TG 17:1_34:1	x	
Triglycerides	Triacylglyceride 17:1_34:2	TG 17:1_34:2		x
Triglycerides	Triacylglyceride 17:1_34:3	TG 17:1_34:3		x
Triglycerides	Triacylglyceride 17:1_36:3	TG 17:1_36:3	x	
Triglycerides	Triacylglyceride 17:1_36:4	TG 17:1_36:4		x
Triglycerides	Triacylglyceride 17:1_36:5	TG 17:1_36:5		x
Triglycerides	Triacylglyceride 17:1_38:5	TG 17:1_38:5		x
Triglycerides	Triacylglyceride 17:1_38:6	TG 17:1_38:6		x
Triglycerides	Triacylglyceride 17:2_34:2	TG 17:2_34:2		x
Triglycerides	Triacylglyceride 17:2_36:2	TG 17:2_36:2		x
Triglycerides	Triacylglyceride 17:2_36:3	TG 17:2_36:3		x
Triglycerides	Triacylglyceride 17:2_36:4	TG 17:2_36:4	x	
Triglycerides	Triacylglyceride 17:2_38:5	TG 17:2_38:5	x	
Triglycerides	Triacylglyceride 17:2_38:6	TG 17:2_38:6		x
Triglycerides	Triacylglyceride 17:2_38:7	TG 17:2_38:7		x
Triglycerides	Triacylglyceride 18:0_32:1	TG 18:0_32:1	x	
Triglycerides	Triacylglyceride 18:0_32:2	TG 18:0_32:2		x
Triglycerides	Triacylglyceride 18:0_34:2	TG 18:0_34:2	x	
Triglycerides	Triacylglyceride 18:0_34:3	TG 18:0_34:3	x	
Triglycerides	Triacylglyceride 18:0_36:1	TG 18:0_36:1	x	
Triglycerides	Triacylglyceride 18:0_36:2	TG 18:0_36:2	x	
Triglycerides	Triacylglyceride 18:0_36:3	TG 18:0_36:3	x	
Triglycerides	Triacylglyceride 18:0_36:4	TG 18:0_36:4	x	
Triglycerides	Triacylglyceride 18:0_36:5	TG 18:0_36:5	x	
Triglycerides	Triacylglyceride 18:0_38:6	TG 18:0_38:6		x
Triglycerides	Triacylglyceride 18:0_38:7	TG 18:0_38:7		x
Triglycerides	Triacylglyceride 18:1_26:0	TG 18:1_26:0	x	
Triglycerides	Triacylglyceride 18:1_28:1	TG 18:1_28:1	x	

Triglycerides	Triacylglyceride 18:1_30:0	TG 18:1_30:0	x	
Triglycerides	Triacylglyceride 18:1_30:1	TG 18:1_30:1		x
Triglycerides	Triacylglyceride 18:1_30:2	TG 18:1_30:2		x
Triglycerides	Triacylglyceride 18:1_31:0	TG 18:1_31:0		x
Triglycerides	Triacylglyceride 18:1_32:0	TG 18:1_32:0	x	
Triglycerides	Triacylglyceride 18:1_32:1	TG 18:1_32:1	x	
Triglycerides	Triacylglyceride 18:1_32:2	TG 18:1_32:2	x	
Triglycerides	Triacylglyceride 18:1_32:3	TG 18:1_32:3		x
Triglycerides	Triacylglyceride 18:1_33:0	TG 18:1_33:0		x
Triglycerides	Triacylglyceride 18:1_33:1	TG 18:1_33:1	x	
Triglycerides	Triacylglyceride 18:1_33:2	TG 18:1_33:2	x	
Triglycerides	Triacylglyceride 18:1_33:3	TG 18:1_33:3		x
Triglycerides	Triacylglyceride 18:1_34:1	TG 18:1_34:1	x	
Triglycerides	Triacylglyceride 18:1_34:2	TG 18:1_34:2	x	
Triglycerides	Triacylglyceride 18:1_34:3	TG 18:1_34:3	x	
Triglycerides	Triacylglyceride 18:1_34:4	TG 18:1_34:4	x	
Triglycerides	Triacylglyceride 18:1_35:2	TG 18:1_35:2	x	
Triglycerides	Triacylglyceride 18:1_35:3	TG 18:1_35:3	x	
Triglycerides	Triacylglyceride 18:1_36:0	TG 18:1_36:0	x	
Triglycerides	Triacylglyceride 18:1_36:1	TG 18:1_36:1	x	
Triglycerides	Triacylglyceride 18:1_36:2	TG 18:1_36:2	x	
Triglycerides	Triacylglyceride 18:1_36:3	TG 18:1_36:3	x	
Triglycerides	Triacylglyceride 18:1_36:4	TG 18:1_36:4	x	
Triglycerides	Triacylglyceride 18:1_36:5	TG 18:1_36:5	x	
Triglycerides	Triacylglyceride 18:1_36:6	TG 18:1_36:6	x	
Triglycerides	Triacylglyceride 18:1_38:5	TG 18:1_38:5	x	
Triglycerides	Triacylglyceride 18:1_38:6	TG 18:1_38:6	x	
Triglycerides	Triacylglyceride 18:1_38:7	TG 18:1_38:7	x	
Triglycerides	Triacylglyceride 18:2_28:0	TG 18:2_28:0		x
Triglycerides	Triacylglyceride 18:2_30:0	TG 18:2_30:0	x	
Triglycerides	Triacylglyceride 18:2_30:1	TG 18:2_30:1		x
Triglycerides	Triacylglyceride 18:2_32:0	TG 18:2_32:0	x	
Triglycerides	Triacylglyceride 18:2_32:1	TG 18:2_32:1	x	
Triglycerides	Triacylglyceride 18:2_32:2	TG 18:2_32:2	x	
Triglycerides	Triacylglyceride 18:2_33:0	TG 18:2_33:0		x
Triglycerides	Triacylglyceride 18:2_33:1	TG 18:2_33:1	x	
Triglycerides	Triacylglyceride 18:2_33:2	TG 18:2_33:2	x	
Triglycerides	Triacylglyceride 18:2_34:0	TG 18:2_34:0	x	
Triglycerides	Triacylglyceride 18:2_34:1	TG 18:2_34:1	x	
Triglycerides	Triacylglyceride 18:2_34:2	TG 18:2_34:2	x	
Triglycerides	Triacylglyceride 18:2_34:3	TG 18:2_34:3	x	

Triglycerides	Triacylglyceride 18:2_34:4	TG 18:2_34:4	x	
Triglycerides	Triacylglyceride 18:2_35:1	TG 18:2_35:1	x	
Triglycerides	Triacylglyceride 18:2_35:2	TG 18:2_35:2	x	
Triglycerides	Triacylglyceride 18:2_35:3	TG 18:2_35:3	x	
Triglycerides	Triacylglyceride 18:2_36:0	TG 18:2_36:0	x	
Triglycerides	Triacylglyceride 18:2_36:1	TG 18:2_36:1	x	
Triglycerides	Triacylglyceride 18:2_36:2	TG 18:2_36:2	x	
Triglycerides	Triacylglyceride 18:2_36:3	TG 18:2_36:3	x	
Triglycerides	Triacylglyceride 18:2_36:4	TG 18:2_36:4	x	
Triglycerides	Triacylglyceride 18:2_36:5	TG 18:2_36:5	x	
Triglycerides	Triacylglyceride 18:2_38:4	TG 18:2_38:4	x	
Triglycerides	Triacylglyceride 18:2_38:5	TG 18:2_38:5	x	
Triglycerides	Triacylglyceride 18:2_38:6	TG 18:2_38:6		x
Triglycerides	Triacylglyceride 18:3_32:0	TG 18:3_32:0		x
Triglycerides	Triacylglyceride 18:3_32:1	TG 18:3_32:1		x
Triglycerides	Triacylglyceride 18:3_33:2	TG 18:3_33:2		x
Triglycerides	Triacylglyceride 18:3_34:0	TG 18:3_34:0	x	
Triglycerides	Triacylglyceride 18:3_34:1	TG 18:3_34:1	x	
Triglycerides	Triacylglyceride 18:3_34:2	TG 18:3_34:2	x	
Triglycerides	Triacylglyceride 18:3_34:3	TG 18:3_34:3	x	
Triglycerides	Triacylglyceride 18:3_35:2	TG 18:3_35:2	x	
Triglycerides	Triacylglyceride 18:3_36:1	TG 18:3_36:1	x	
Triglycerides	Triacylglyceride 18:3_36:2	TG 18:3_36:2	x	
Triglycerides	Triacylglyceride 18:3_36:3	TG 18:3_36:3	x	
Triglycerides	Triacylglyceride 18:3_36:4	TG 18:3_36:4	x	
Triglycerides	Triacylglyceride 18:3_38:5	TG 18:3_38:5	x	
Triglycerides	Triacylglyceride 18:3_38:6	TG 18:3_38:6	x	
Triglycerides	Triacylglyceride 20:0_32:3	TG 20:0_32:3	x	
Triglycerides	Triacylglyceride 20:0_32:4	TG 20:0_32:4	x	
Triglycerides	Triacylglyceride 20:0_34:1	TG 20:0_34:1	x	
Triglycerides	Triacylglyceride 20:1_32:1	TG 20:1_32:1		x
Triglycerides	Triacylglyceride 20:1_32:2	TG 20:1_32:2		x
Triglycerides	Triacylglyceride 20:1_32:3	TG 20:1_32:3		x
Triglycerides	Triacylglyceride 20:1_34:0	TG 20:1_34:0		x
Triglycerides	Triacylglyceride 20:1_34:1	TG 20:1_34:1	x	
Triglycerides	Triacylglyceride 20:1_34:2	TG 20:1_34:2	x	
Triglycerides	Triacylglyceride 20:1_34:3	TG 20:1_34:3	x	
Triglycerides	Triacylglyceride 20:2_32:1	TG 20:2_32:1		x
Triglycerides	Triacylglyceride 20:2_34:1	TG 20:2_34:1		x
Triglycerides	Triacylglyceride 20:2_34:2	TG 20:2_34:2	x	
Triglycerides	Triacylglyceride 20:2_34:3	TG 20:2_34:3	x	

Triglycerides	Triacylglyceride 20:2_34:4	TG 20:2_34:4	x	
Triglycerides	Triacylglyceride 20:2_36:5	TG 20:2_36:5	x	
Triglycerides	Triacylglyceride 20:3_32:1	TG 20:3_32:1		x
Triglycerides	Triacylglyceride 20:3_34:2	TG 20:3_34:2		x
Triglycerides	Triacylglyceride 20:3_34:3	TG 20:3_34:3	x	
Triglycerides	Triacylglyceride 20:3_36:3	TG 20:3_36:3	x	
Triglycerides	Triacylglyceride 20:3_36:4	TG 20:3_36:4	x	
Triglycerides	Triacylglyceride 20:3_36:5	TG 20:3_36:5	x	
Triglycerides	Triacylglyceride 20:4_35:3	TG 20:4_35:3		x
Triglycerides	Triacylglyceride 20:4_36:2	TG 20:4_36:2		x
Triglycerides	Triacylglyceride 20:4_36:3	TG 20:4_36:3		x
Triglycerides	Triacylglyceride 20:4_36:4	TG 20:4_36:4		x
Triglycerides	Triacylglyceride 20:4_36:5	TG 20:4_36:5		x
Triglycerides	Triacylglyceride 20:5_34:0	TG 20:5_34:0		x
Triglycerides	Triacylglyceride 20:5_34:1	TG 20:5_34:1	x	
Triglycerides	Triacylglyceride 20:5_34:2	TG 20:5_34:2		x
Triglycerides	Triacylglyceride 20:5_36:2	TG 20:5_36:2		x
Triglycerides	Triacylglyceride 20:5_36:3	TG 20:5_36:3	x	
Triglycerides	Triacylglyceride 22:0_32:4	TG 22:0_32:4	x	
Triglycerides	Triacylglyceride 22:1_32:5	TG 22:1_32:5	x	
Triglycerides	Triacylglyceride 22:2_32:4	TG 22:2_32:4	x	
Triglycerides	Triacylglyceride 22:4_32:0	TG 22:4_32:0		x
Triglycerides	Triacylglyceride 22:4_32:2	TG 22:4_32:2	x	
Triglycerides	Triacylglyceride 22:5_32:1	TG 22:5_32:1		x
Triglycerides	Triacylglyceride 22:5_34:1	TG 22:5_34:1		x
Triglycerides	Triacylglyceride 22:5_34:3	TG 22:5_34:3		x
Triglycerides	Triacylglyceride 22:6_32:0	TG 22:6_32:0		x
Triglycerides	Triacylglyceride 22:6_32:1	TG 22:6_32:1		x
Triglycerides	Triacylglyceride 22:6_34:1	TG 22:6_34:1		x
Triglycerides	Triacylglyceride 22:6_34:2	TG 22:6_34:2		x
Triglycerides	Triacylglyceride 22:6_34:3	TG 22:6_34:3	x	
Total			283	123

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6 Standard operating procedure (SOP) for the preparation of human fecal samples

6.1 Introduction

Below are extraction protocols for analyzing human fecal samples (both fresh/wet and dried/lyophilized) with the following kits:

- MxP® Quant 500 XL
- MxP® Quant 500
- AbsoluteIDQ® p180
- AbsoluteIDQ® p400 HR
- MxP® Quant HR Xpress™
- AbsoluteIDQ® Bile Acids

We recommend using a Precellys® homogenizer for best results. If this is not available, please use the protocol without homogenizer in section 6.8. The protocols are recommendations based on our experience and can be modified according to your needs or ideas. Please note that we have only carried out feasibility tests, and the kits have 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, quality and preparation of the samples.

6.2 References

For additional protocols and impressions, please refer to findings published by Erben et al.: https://nutrition.bmj.com/content/4/2/374

This study compared eight different stool extraction methods of varying complexity. Among the protocols found to be least time-consuming and most suitable for routine use, isopropanol proved to be the most efficient solvent, yielding the highest number and broadest range of metabolites. We confirmed this in independent tests at biocrates using fresh (wet) fecal samples. When using lyophilized samples, we found that ethanol/phosphate buffer gave a better metabolite coverage compared to isopropanol.

6.3 Sample collection

Step	Instruction
i	– Due to variation in metabolite concentrations across a stool sample, we recommend collecting and pooling three aliquots from different positions in each stool sample (3-spot sampling). – Ideally, the collected stool samples should be processed or frozen as soon as possible after passage.
1	Collect fresh feces in airtight containers to prevent desiccation and put them immediately on ice until further processing.
2	Transfer three aliquots from different positions in each stool sample (approx. 200-500 mg each, 600-1500 mg in total) into a standard cryogenic or feces collection vial and record the weight.
3	Snap-freeze the samples in liquid nitrogen (-196 °C) if possible, or freeze at -80 °C.
4	Store the cryogenic vials at lowest available temperature (preferably at -80 °C) until use.

6.4 Preparing extraction solvents

Extraction solvent	Description
Isopropanol for fresh/wet fecal samples	Pure isopropanol, LC-MS grade
Ethanol/phosphate buffer for dried/lyophilized fecal samples	Ethanol/phosphate buffer* (85:15 v/v), combine – 85 mL of HPLC grade ethanol with – 15 mL of phosphate buffer, 0.01 M*
Alternative: Isopropanol/H_2O for dried/lyophilized fecal samples	80% isopropanol buffer (80:20 v/v), combine – 80 mL of pure isopropanol, LC-MS grade with – 20 mL of distilled or Milli-Q water

* Recommended: Sigma, P5244 (0.1 M, pH = 7.5 at 25 °C); 1:10 diluted

6.5 WebIDQ software and workflow differences

The table below describes the steps that are different to the regular workflow. All steps not mentioned here are unchanged and performed according to the user manual for the relevant kit.

Step	Instruction
1	Select the material "feces" (or similar that applies) when registering fecal samples in the LIMS module of WebIDQ.
2	Use the extraction solvent as zero sample. In the **Zeros** tab of the **Worklist generation** window, link the used extraction solvent.

6.6 Homogenizer equipment

Item	Description
Homogenizer	Homogenizer Precellys 24 with Cryolys
Precellys lysing kits (tubes and beads) – Tubes should not be filled with more than 2/3 of total volume For further information visit: https://www.bertin-technologies.com/replay-webinar-power-up-your-sample-homogenization-by-selecting-the-best-precellys-lysing-kit/	Option 1 for dried/lyophilized feces: – 50-100 mg sample amount: 2 mL standard tubes with 1.4 mm ceramic beads – Sample amounts larger than 100 mg: 7 mL or 15 mL standard tubes with 2.8 mm ceramic beads **Option 2 for fresh/wet feces:** – 200-300 mg sample amount: 2 mL standard tubes with 1.4 mm ceramic beads – 300-1000 mg sample amount: 7 mL standard tubes with 2.8 mm ceramic beads – 1-2 g sample amount: 15 mL standard tubes with 2.8 mm ceramic beads
Nitrogen	Liquid nitrogen

6.7 Sample preparation using a Precellys homogenizer

6.7.1 Fresh/wet fecal samples

Step	Instruction
1	Prepare Precellys: Fill liquid nitrogen into Cryolys unit and make sure it is attached to the Precellys. Set the nitrogen flow to max 2 bar. Adjust Precellys temperature to 0-4 °C.
2	Prepare an Excel sheet for recording the weight of the samples.
3	Prepare a box with ice and place the extraction solvent in an appropriate container on ice.
4	Put the original sample vials in a sample rack on ice.
5	Prepare the Precellys standard tubes from section 4 and label them.
6	Transfer the full original sample (if samples were collected according to section 5 of this document) or transfer an aliquot of approx. 200-500 mg to the prepared Precellys tubes with the ceramic beads and record the weight.
7	Add the 3-fold volume of extraction solvent to each fecal sample, e.g. add 3 mL iso-propanol to 1 g feces.
8	Make sure the Precellys temperature is at 0-4 °C.
9	Homogenize samples 3 times for 30 sec at 5,800 rpm, 30 sec pause in between.
10	Centrifuge at 2,000 g (rcf) for 2 min at 2-4 °C. If this speed is not available, or if the centrifuge cannot be cooled, centrifuge at 800 g (rcf) for 2 min.
11	Transfer the supernatant to a new and labeled vial.
12	Centrifuge at 10,000 g (rcf) for 5 min at 2-4 °C.
13	Transfer the supernatant to a new and labeled vial.
14	Keep the extract on ice for immediate kit preparation or store at –80 °C.
15	For kit preparation, add 10 µL of the extract to the kit plate and follow the regular kit user manual.

6.7.2 Dried/lyophilized fecal samples

Step	Instruction
1	Prepare Precellys: Fill liquid nitrogen into Cryolys unit and make sure it is attached to the Precellys. Set the nitrogen flow to max 2 bar. Adjust Precellys temperature to 0-4 °C.
2	Prepare an Excel sheet for recording the weight of the samples.
3	Prepare a box with ice and place the extraction solvent in an appropriate container on ice.
4	Put the original sample vials in a sample rack on ice.
5	Prepare the Precellys standard tubes from section 4 and label them.
6	Transfer the full original sample or an aliquot of approx. 50-100 mg to the prepared Precellys tubes with the ceramic beads and record the weight.
7	Add the 10-fold volume of extraction solvent to each fecal sample, e.g. add 500 µL ethanol/phosphate buffer (85:15 v/v) to 50 mg feces.
8	Make sure the Precellys temperature is at 0-4 °C.
9	Homogenize samples 3 times for 30 sec at 5,800 rpm, 30 sec pause in between.
10	Centrifuge at 2,000 g (rcf) for 2 min at 2-4 °C. If this speed is not available, or if the centrifuge cannot be cooled, centrifuge at 800 g (rcf) for 2 min.
11	Transfer the supernatant to a new and labeled vial.
12	Centrifuge at 10,000 g (rcf) for 5 min at 2-4 °C.
13	Transfer the supernatant to a new and labeled vial.
14	Keep the extract on ice for immediate kit preparation or store at –80 °C.
15	For kit preparation, add 10 µL of the extract to the kit plate and follow the regular kit user manual.

6.8 Sample preparation without homogenizer

6.8.1 Fresh/wet fecal samples

Step	Instruction
1	Prepare Excel sheet for recording the weight of the samples.
2	Prepare a box with ice and place the extraction solvent in an appropriate container on ice.
3	Put the original sample vials in a sample rack on ice.
4	Prepare appropriate vials and label them.
5	Transfer the full original sample (if samples were collected according to section 5 of this document) or transfer an aliquot of approx. 200-500 mg to the prepared vials and record the weight.
6	Add the 3-fold volume of extraction solvent to each fecal sample, e.g. add 3 mL iso-propanol to 1 g feces, and vortex thoroughly.
7	Shake samples at 450 rpm for 30 min at 2-4 °C.
8	Sonicate samples for 5 min at approx. 2-10 °C.
9	Centrifuge at 2,000 g (rcf) for 2 min at 2-4 °C. If this speed is not available, or if the centrifuge cannot be cooled, centrifuge at 800 g (rcf) for 2 min.
10	Transfer the supernatant to a new and labeled vial.
11	Centrifuge at 10,000 g (rcf) for 5 min at 2-4 °C.
12	Transfer the supernatant to a new and labeled vial.
13	Keep the extract on ice for immediate kit preparation or store at –80 °C.
14	For kit preparation, add 10 µL of the extract to the kit plate and follow the regular kit user manual.

6.8.2 Dried/lyophilized fecal samples

Step	Instruction
1	Prepare Excel sheet for recording the weight of the samples.
2	Prepare a box with ice and place extraction solvent in an appropriate container on ice.
3	Put the original sample vials in a sample rack on ice.
4	Prepare appropriate vials and label them.
5	Transfer the full original sample or an aliquot of approx. 50-100 mg to the prepared vials and record the weight.
6	Add the 10-fold volume of extraction solvent to each fecal sample, e.g. add 500 µL ethanol/phosphate buffer (85:15 v/v) to 50 mg feces and vortex thoroughly.
7	Shake samples at 450 rpm for 30 min at 2-4 °C.
8	Sonicate samples for 5 min at approx. 2-10 °C.
9	Centrifuge at 2,000 g (rcf) for 2 min at 2-4 °C. If this speed is not available, or if the centrifuge cannot be cooled, centrifuge at 800 g (rcf) for 2 min.
10	Transfer the supernatant to a new and labeled vial.
11	Centrifuge at 10,000 g (rcf) for 5 min at 2-4 °C.
12	Transfer the supernatant to a new and labeled vial.
13	Keep the extract on ice for immediate kit preparation or store at –80 °C.
14	For kit preparation, add 10 µL of the extract to the kit plate and follow the regular kit user manual.

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