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Feces sampling with OmicSnap® devices and metabolite stability

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1 Introduction

The gut microbiome is considered one of the most important factors contributing to the regulation of host health. Changes and perturbations of the microbiome have been associated with a range of diseases, which has led to a growing interest in the utilization of the microbiome for therapeutic, diagnostic, and prognostic purposes.

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 are 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 with other sample types, even from the same stool sample at different topographical locations. This variability calls for standardized pre-analytical sample collection and preparation for metabolomics analysis of feces.

For best results, it is recommended to immediately freeze fresh feces samples at -80° for metabolomics. The corresponding standard operating procedure and the resulting metabolite detectability are described in a separate application note[1]. The present application note describes feces sampling with the OmicSnap® device that can facilitate fecal sampling and stabilize the samples when freezing at -80°C right away is not possible, for example in remote sampling or at-home sampling studies.
It elucidates the advantages and disadvantages of the device and the effect of different stabilization buffers, storage time, and temperature on metabolite stability.

Feces metabolomics studies were conducted with these devices using the biocrates SMartIDQ alpha kit. The kit quantifies more than 100 polar metabolites that provide information on the gut microbiome composition and host health via high throughput LC-MS/MS measurement.

2 Devices for feces sampling

Most feces sampling devices are optimized for subsequent RNA sequencing or metagenomics. One of the most frequently used tools is the DNA/RNA Shield Fecal Collection Kit from Zymo Research.
It contains feces sampling tubes with DNA/RNA Shield buffer that stabilizes DNA and RNA. We have tested this buffer and found that while many metabolites can be detected, they are highly unstable at room temperature. Consequently, we consider this device not suitable for metabolomics analyses.

It is strongly recommended to use other devices instead, for example the OmicSnap® or the OMNImet™·GUT devices.

The OMNImet™·GUT feces sampling device is supplied by DNA Genotek and specifically designed for feces metabolomics analysis. This device will soon be discussed in a separate application note.

Immundiagnostik AG offers the OmicSnap® Meta tube as an all-in-one device for sampling, stabilizing, and homogenizing a defined amount of fecal material for subsequent metabolomic analyses. The OmicSnap® Meta tube is prefilled with 1.7 mL of a premixed buffer solution for metabolite stabilization. The OmicSnap® device can also be ordered without buffer, enabling the use of other buffers better suited for the target analytes. The device sample collection rod can hold up to 100 mg (±10%) of fecal material.

3 Methods

3.1 Sampling

The OmicSnap® devices come with an instruction manual that describes how the sampling should be carried out. Sampling should always be performed with fresh feces.

The OmicSnap® devices were ordered without buffer and the suitability of 3 different buffered extraction/stabilization solutions were tested: a citrate buffer (1.5 mL; 0.1 M, pH 3), an ethanol solution (1.5 mL EtOH, 100 %) and an isopropanol/ methanol solution (750 µL IPA + 750 µL MeOH). The device contains a rod ("dipstick") that is pushed into the feces to collect the sample. We pushed the rod in 3 different locations of the feces to mitigate the lack of sample homogeneity. The rod was then inserted into the buffer-filled device. According to our experience, an initial immediate homogenization is key, which is why we added ceramic beads (Precellys Tissue homogenizing CKMix, not included in the device) to the tubes and shook vigorously and extensively for homogenization.

While homogenization of feces with the buffers brings metabolism to a halt, long-term metabolite stability requires the sample to be frozen at or below -80°C. In the case of remote sampling, this should occur as soon as the samples have been shipped to the laboratory.

To investigate the stability of metabolites over time, several samples from 3 individuals were taken and either frozen at -80°C right away or kept at room temperature for up to 7 days before analysis.

3.2 Sample preparation for metabolomics

Fecal samples collected with the OmicSnap® devices from three donors were measured in triplicate with the biocrates SMartIDQ alpha kit.

In preparation for metabolomics, samples were thawed on ice. Despite the initial homogenization by shaking right after sampling, samples were homogenized mechanically once more before measurement as undissolved particles remained with all devices tested after the initial vigorous shaking. Importantly, 100 µL of phosphate solution (0.1 M) were added to the samples with ethanol and isopropanol/methanol solutions directly before the mechanical extraction procedure to enhance extraction of polar analytes. To maintain consistent sample volumes, an additional 100 µL of citrate buffer was added to the samples in citrate-based solution. Phosphate buffer was added at this point in the workflow as adding it prior to sample storage would compromise metabolite stability at room temperature.

For the mechanical homogenization before measurement, we recommend using Precellys Evolution or Precellys 24 homogenization devices. In the present set

of experiments all samples underwent a mechanical second homogenization before measurement.

The suspension was then transferred to another tube and centrifuged (2 min at 10000 rpm at 2°C), and the supernatant was used for LC-MS/MS analysis according to the SMartIDQ alpha kit specifications.

3.3 Measures to handle differences in water content

The varying water content of feces samples, typically ranging from 60% to 85%, makes 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.

When working with feces sample suspensions, a fraction of the sample can be separated after homogenization, followed by drying and weighing with a special accuracy scale. By normalizing concentrations to the dry weight, differences in water content can be partially mitigated.

4 Results

4.1 Influence of extraction buffer on metabolite detectability and stability

Figure 1 provides a count of the metabolites detected in each buffer preparation. Table 1 provides an overview of the metabolites detected from each class with a deviation of ≤20% from the day 0 concentration after 7 days at room temperature.

The ethanol buffer provided the highest detectability, with 74 metabolites detected above the limit of detection (LOD) in at least two donors, and only 16 metabolites undetected (Figure 1). This buffer also showed the best stability of the three buffers tested (Table 1).

The detectability with the IPA/MeOH buffer was lower than with the ethanol buffer, with 63 metabolites quantified in at least two donors, and 22 undetected. The IPA/MeOH buffer performed almost as well as the ethanol buffer for metabolite stability, yielding better results for a few selected metabolites (homoarginine, homocysteine, alpha-aminoadipic acid, GUDCA, aconitic acid, and fatty acid 20:3).

During homogenization, feces samples dissolved more readily in the citrate buffer. However, using the citrate buffer resulted in the lowest number of metabolites detected (58 detected in at least two donors) and 40 metabolites not detected at all, see Figure 1. Amino acids, amino acid-related metabolites, and bile acids were particularly affected, with both poor detection and poor stability (Table 1). The citrate buffer was the only one enabling detection of succinate, though it was only stable for up to 5 days.

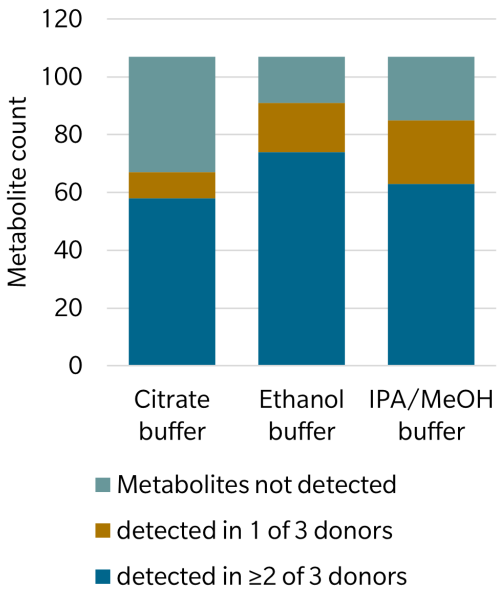

Figure 1: Detectability of the 107 SMartIDQ alpha kit metabolites > LOD in OmicSnap® fecal samples in the same three individuals, depending on the buffer used for stabilization and extraction.

Table 1: Detectability and 7-day stability at room temperature of the 107 SMartIDQ alpha kit metabolites by class and buffer used for stabilization and extraction in OmicSnap® fecal samples. Metabolites were counted as stable when concentration deviated ≤20% from day 0 concentration after 7 days at room temperature.

Metabolite class (no. of metabolites in class)	No. of metabolites detected and stable after 7 days, in buffer		
	Citrate	Ethanol (+PP)	IPA/MeOH (+PP)
Alkaloids (1)	1	1	1
Amine oxides (1)	0	0	0
Amino acids (20)	15	18	17
Amino acid-related (31)	15	22	18
Bile acids (14)	3	13	13
Biogenic amines (9)	2	7	4
Carboxylic acids (7)	1	1	2
Cresols (1)	1	1	1
Fatty acids (12)	1	11	10
Hormones (4)	1	1	1
Indoles and derivatives (4)	2	3	2
Nucleobases and related (2)	2	2	2
Vitamins and cofactors (1)	0	1	1

Some metabolites like cysteine and glutamine were readily detected but lacked stability in all three buffers.

These findings highlight that while an ethanol-based buffer may be suitable to yield a broad panel of metabolites and stabilize them, other buffers can be considered if a specific metabolite is of crucial relevance. Metabolite stability when sampling with OmicSnap® devices with ethanol-based buffer is discussed at the single metabolite level in section 4.3.

Figure 2 showcases the concentrations of anserine, an amino acid-related metabolite, and cholic acid, a bile acid, by buffer in feces samples taken with the OmicSnap® device in three different donors over time. The plots show that stability does not only vary depending on the buffer used and the time at room temperature, but also that there are inter-individual stability differences. Of note, the same metabolite in a sample with the same buffer appeared to be stable in samples from one donor but repeatedly exceed the acceptance thresholds for stability in samples from another donor. These plots exemplify that feces are a matrix with inherently high variance, a challenge that remains independent of sampling devices and extraction buffers used.

4.2 Metabolite detectability

Since the combination of OmicSnap® tubes with ethanol buffer turned out superior in the buffer comparison, this approach was in focus for metabolite detectability evaluation.

Figure 3 displays the summed-up concentrations per class for the three donors in comparison. These results were obtained from sampling with devices stored at –80°C right after homogenization until measurement (i.e., 0 days at room temperature). In all individuals tested, the metabolite class contributing the most to the total metabolite content are fatty acids, followed by amino acids and amino acid-related metabolites. The visualization demonstrates the interindividual differences and the variance in total

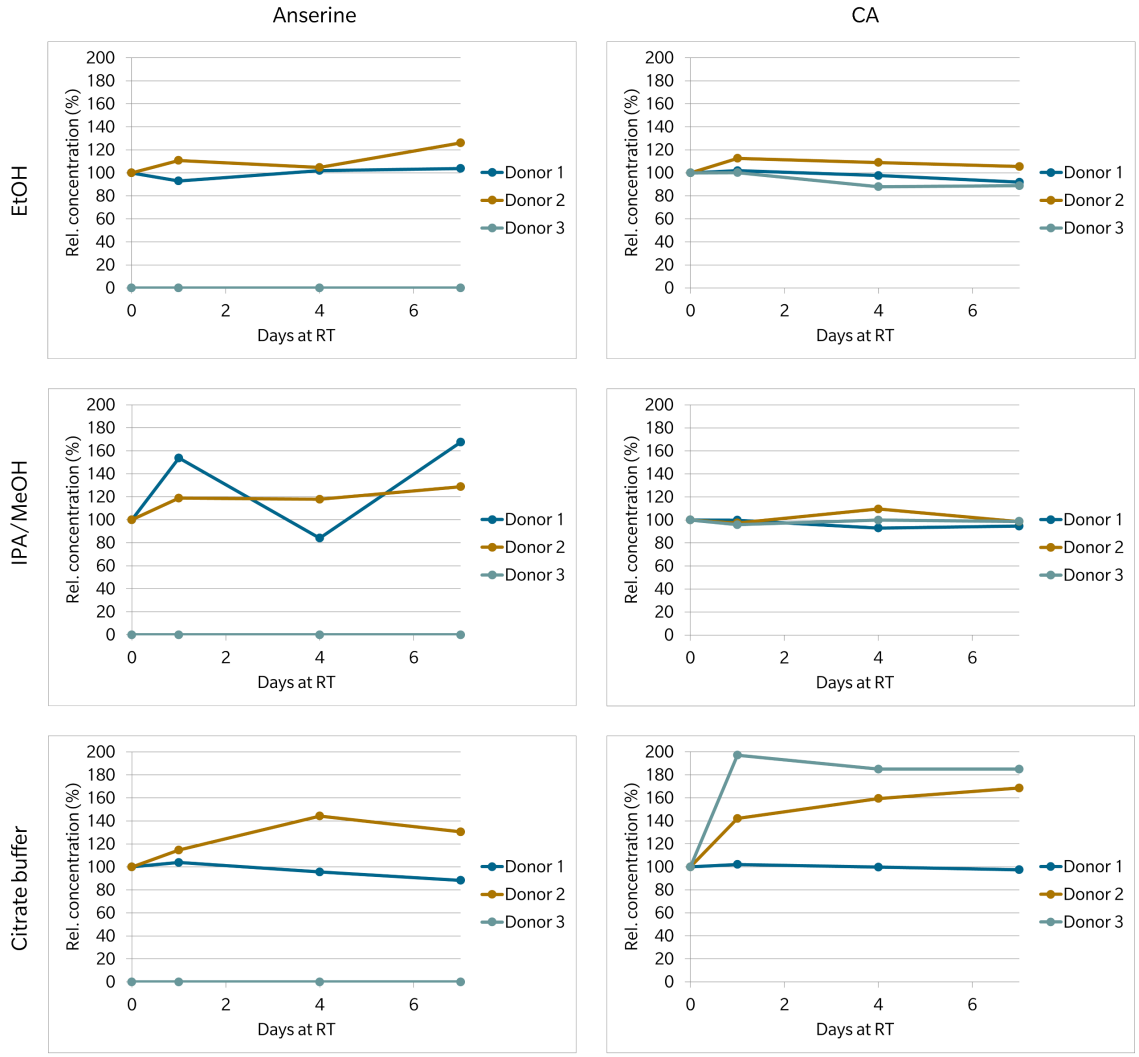

Figure 2: Stability of anserine and cholic acid (CA) in EtOH, IPA/MeOH and citrate buffer buffer for 1, 4 or 7 days at room temperature relative to the metabolite concentrations at day 0. RT, room temperature.

metabolite content due to different fatty acid content (Figure 3).

The individual detectability of small molecules included in the SMartIDQ alpha kit is shown in Table 2. Most metabolite classes were well detectable in feces samples. Of the 107 small molecules assessed with the kit, 74 were detectable above the limit of detection (LOD) in at least 2 of the 3 individuals using the OmicSnap® device. Only 16 metabolites were not detected in any of the three individuals at levels above the LOD threshold.

Despite usually being detectable in feces when applying the biocrates standard protocol for measuring feces samples, several metabolites were not detected in the present experiment, probably because of the high dilution (1:16) compared to the biocrates standard procedure for fresh feces (1:4).

This may be partially mitigated by using a lower buffer volume for the devices or pipetting a larger sample volume on the kit plate for measurement, i.e., 20 or 30 µL instead of 10 µL.

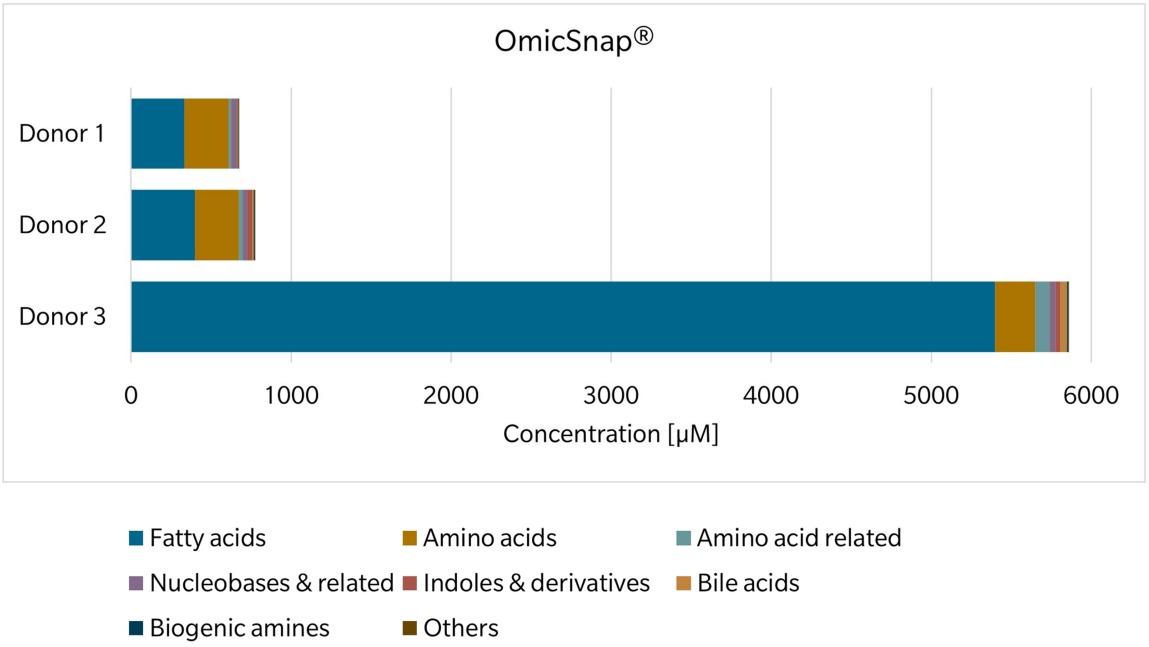

Figure 3: Total metabolite content and attribution to different ontology classes of fecal samples collected with OmicSnap® tubes (with phosphate-buffered EtOH) from three donors

Table 2: Detectability of the 107 SMartIDQ alpha kit metabolites in OmicSnap® fecal samples from three donors.

Analyte class	Metabolite	OmicSnap®	
		>LOD in ≥2 of 3 donors	>LOD in 1 of 3 donors
Alkaloids	Trigonelline	✓	
Amine oxides	Trimethylamine N-oxide		
Amino acids	Alanine	✓	
Amino acids	Arginine	✓	
Amino acids	Asparagine		✓
Amino acids	Aspartic acid	✓	
Amino acids	Cysteine	✓	
Amino acids	Glutamine	✓	
Amino acids	Glutamic acid	✓	
Amino acids	Glycine	✓	
Amino acids	Histidine	✓	
Amino acids	Isoleucine	✓	
Amino acids	Leucine	✓	
Amino acids	Lysine	✓	
Amino acids	Methionine	✓	
Amino acids	Phenylalanine	✓	

Analyte class	Metabolite	OmicSnap®	
		>LOD in ≥2 of 3 donors	>LOD in 1 of 3 donors
Amino acids	Proline	✓	
Amino acids	Serine	✓	
Amino acids	Threonine	✓	
Amino acids	Tryptophan	✓	
Amino acids	Tyrosine	✓	
Amino acids	Valine	✓	
Amino acid-related	1-Methylhistidine	✓	
Amino acid-related	3-Methylhistidine	✓	
Amino acid-related	5-Aminovaleric acid	✓	
Amino acid-related	α-Aminoadipic acid		✓
Amino acid-related	α-Aminobutyric acid	✓	
Amino acid-related	Acetylornithine	✓	
Amino acid-related	Asymmetric dimethylarginine		✓
Amino acid-related	L-Anserine	✓	
Amino acid-related	β-Aminobutyric acid	✓	
Amino acid-related	Betaine		
Amino acid-related	Carnitine		
Amino acid-related	Carnosine		✓
Amino acid-related	cis-4-Hydroxyproline		
Amino acid-related	Citrulline	✓	
Amino acid-related	Creatinine	✓	
Amino acid-related	Cystine	✓	
Amino acid-related	3,4-Dihydroxyphenylalanine		
Amino acid-related	Homoarginine	✓	
Amino acid-related	Homocysteine	✓	
Amino acid-related	Kynurenine		
Amino acid-related	Methionine-sulfoxide	✓	
Amino acid-related	Nitrotyrosine		
Amino acid-related	Ornithine	✓	
Amino acid-related	Phenylacetylglycin		✓
Amino acid-related	Phenylalanine betaine		✓
Amino acid-related	Proline betaine	✓	
Amino acid-related	Sarcosine		✓
Amino acid-related	Symmetric dimethylarginine	✓	
Amino acid-related	trans-4-Hydroxyproline	✓	

Analyte class	Metabolite	OmicSnap®	
		>LOD in ≥2 of 3 donors	>LOD in 1 of 3 donors
Amino acid-related	Taurine	✓	
Amino acid-related	Tryptophan betaine	✓	
Bile acids	Cholic acid	✓	
Bile acids	Chenodeoxycholic acid	✓	
Bile acids	Deoxycholic acid	✓	
Bile acids	Glycocholic acid	✓	
Bile acids	Glycochenodeoxycholic acid	✓	
Bile acids	Glycodeoxycholic acid	✓	
Bile acids	Glycolithocholic acid		✓
Bile acids	Glycolithocholic acid sulfate	✓	
Bile acids	Glycoursodeoxycholic acid	✓	
Bile acids	Taurocholic acid		✓
Bile acids	Taurochenodeoxycholic acid	✓	
Bile acids	Taurodeoxycholic acid	✓	
Bile acids	Taurolithocholic acid	✓	
Bile acids	Tauromurocholic acid		✓
Biogenic amines	β-Alanine	✓	
Biogenic amines	γ-Aminobutyric acid	✓	
Biogenic amines	Dopamine		
Biogenic amines	Histamine		✓
Biogenic amines	Phenylethylamine		✓
Biogenic amines	Putrescine	✓	
Biogenic amines	Serotonin	✓	
Biogenic amines	Spermidine	✓	
Biogenic amines	Spermine		
Carboxylic acids	Aconitic acid		✓
Carboxylic acids	Dodecanedioic acid	✓	
Carboxylic acids	Tetradecanedioic acid		
Carboxylic acids	Hippuric acid		
Carboxylic acids	3-Hydroxyglutaric acid		
Carboxylic acids	Lactic acid		
Carboxylic acids	Succinic acid		
Cresols	p-Cresol sulfate	✓	
Fatty acids	Lauric acid		✓
Fatty acids	Myristic acid		✓
Fatty acids	Palmitic acid		✓

Analyte class	Metabolite	OmicSnap®	
		>LOD in ≥2 of 3 donors	>LOD in 1 of 3 donors
Fatty acids	Stearic acid		✓
Fatty acids	Octadecenoic acid	✓	
Fatty acids	Octadecadienoic acid	✓	
Fatty acids	Eicosenoic acid	✓	
Fatty acids	Eicosadienoic acid	✓	
Fatty acids	Eicosatrienoic acid	✓	
Fatty acids	Arachidonic acid (FA 20:4ω6)	✓	
Fatty acids	Eicosapentaenoic acid (FA 20:5ω3)	✓	
Fatty acids	Docosahexaenoic acid (FA 22:6ω3)	✓	
Hormones	Abscisic acid	✓	
Hormones	Cortisol		
Hormones	Cortisone		
Hormones	Dihydroepiandrosterone sulfate	✓	
Indoles	Indoleacetic acid	✓	
Indoles	Indolepropionic acid	✓	
Indoles	Indoxyl sulfate	✓	
Indoles	Indole	✓	
Nucleobases and related	Hypoxanthine	✓	
Nucleobases and related	Xanthine	✓	
Vitamins and cofactors	Choline	✓	
Total		74	17

4.3 Metabolite stability

As mentioned in section 4.1, a metabolite was categorized as stable for a given storage time when the measured concentration was between 80% and 120% of the concentration measured after 0 days of storage at room temperature. Of the 91 metabolites detected, 11 metabolites exceeded these acceptance thresholds on day 1 already. However, several of these were within limits again in the samples stored for 4 or 7 days. This may have biological reasons, for example another metabolite may be unstable and converted into the measured metabolite, leading to a concentration increase after day 1, while the measured metabolite itself is only mildly unstable, leading to a slow decrease over the next days. This could explain the observations for cysteine, for example.

Another possible explanation is the inhomogeneous nature of the matrix feces. The different samples taken from the same feces with the devices may have had a slightly different composition, leading to deviating concentrations in single samples (independent of storage time) without the

stability being negatively affected. Our studies have shown this positional variance is usually between 10% and 30% and may thus lead to concentrations outside the accepted range. This could be the case for GLCAS, for example.

With 16 of the 91 metabolites displaying a concentration outside the acceptance range at one time point or more, the results indicate that at least 75 metabolites are stable in OmicSnap® devices at room temperature for at least 7 days.

Table 3: Metabolite concentration of the 91 detectable SMartIDQ alpha metabolites after 1 to 7 days of OmicSnap® sample storage at room temperature (RT) in percent of day 0 concentrations. Concentrations more than 20% above or below the 0-day concentration are considered unstable and highlighted in red.

Analyte class	Short name	RT for 1 day [%]	RT for 4 days [%]	RT for 7 days [%]
Alkaloids	Trigonelline	102	95	87
Amino acids	Ala	101	99	97
Amino acids	Arg	94	95	92
Amino acids	Asn	85	85	86
Amino acids	Asp	99	101	93
Amino acids	Cys	200	122	68
Amino acids	Gln	83	72	56
Amino acids	Glu	99	96	91
Amino acids	Gly	98	98	93
Amino acids	His	103	101	97
Amino acids	Ile	102	99	96
Amino acids	Leu	105	96	94
Amino acids	Lys	94	92	85
Amino acids	Met	98	93	94
Amino acids	Phe	102	98	95
Amino acids	Pro	103	98	94
Amino acids	Ser	96	97	88
Amino acids	Thr	97	91	85
Amino acids	Trp	100	96	93
Amino acids	Tyr	102	96	92
Amino acids	Val	102	98	93
Amino acid-related	1-Met-His	128	108	106
Amino acid-related	3-Met-His	99	95	97
Amino acid-related	5-AVA	101	95	93
Amino acid-related	AABA	102	97	96
Amino acid-related	Ac-Orn	100	95	91

Analyte class	Short name	RT for 1 day [%]	RT for 4 days [%]	RT for 7 days [%]
Amino acid-related	ADMA	86	95	88
Amino acid-related	alpha-AAA	0	0	0
Amino acid-related	Anserine	102	103	115
Amino acid-related	BABA	98	91	95
Amino acid-related	Carnosine	108	102	98
Amino acid-related	Cit	101	94	92
Amino acid-related	Creatinine	92	112	80
Amino acid-related	Cystine	122	100	84
Amino acid-related	HArg	58	66	46
Amino acid-related	HCys	115	94	84
Amino acid-related	Met-SO	107	108	90
Amino acid-related	Orn	92	94	83
Amino acid-related	PAG	14	7	7
Amino acid-related	PheAlaBetaine	101	93	95
Amino acid-related	ProBetaine	98	100	89
Amino acid-related	Sarcosine	105	92	100
Amino acid-related	SDMA	97	91	92
Amino acid-related	t4-OH-Pro	103	98	103
Amino acid-related	Taurine	100	95	93
Amino acid-related	TrpBetaine	104	94	111
Bile acids	CA	105	98	95
Bile acids	CDCA	102	101	99
Bile acids	DCA	104	99	98
Bile acids	GCA	119	102	104
Bile acids	GCDCA	110	105	103

Analyte class	Short name	RT for 1 day [%]	RT for 4 days [%]	RT for 7 days [%]
Bile acids	GDCA	112	105	104
Bile acids	GLCA	105	101	97
Bile acids	GLCAS	131	104	105
Bile acids	GUDCA	133	117	125
Bile acids	TCA	105	102	97
Bile acids	TCDCA	113	101	98
Bile acids	TDCA	103	97	108
Bile acids	TLCA	109	85	93
Bile acids	TMCA	148	143	109
Biogenic amines	beta-Ala	108	102	102
Biogenic amines	GABA	112	115	112
Biogenic amines	Histamine	98	93	93
Biogenic amines	PEA	96	88	88
Biogenic amines	Putrescine	94	85	80
Biogenic amines	Serotonin	100	94	92
Biogenic amines	Spermidine	114	118	100
Carboxylic acids	AconAcid	71	89	72
Carboxylic acids	DiCA(12:0)	104	96	95
Cresols	p-Cresol-SO4	105	100	98
Fatty acids	FA 12:0	103	101	94
Fatty acids	FA 14:0	105	90	84
Fatty acids	FA 16:0	99	85	85
Fatty acids	FA 18:0	98	90	89
Fatty acids	FA 18:1	104	95	97
Fatty acids	FA 18:2	103	91	88
Fatty acids	FA 20:1	101	110	94
Fatty acids	FA 20:2	111	96	88
Fatty acids	FA 20:3	106	84	65
Fatty acids	FA 20:4n-6 (AA)	100	100	91
Fatty acids	FA 20:5n-3 (EPA)	98	78	87
Fatty acids	FA 22:6n-3 (DHA)	109	108	103
Hormones	AbsAcid	103	139	128
Hormones	DHEAS	105	100	94
Indoles	3-IAA	101	98	97
Indoles	3-IPA	107	99	102
Indoles	Ind-SO4	75	56	85
Indoles	Indole	82	87	77
Nucleobases and related	Hypoxanthine	105	101	98

Analyte class	Short name	RT for 1 day [%]	RT for 4 days [%]	RT for 7 days [%]
Nucleobases and related	Xanthine	103	98	96
Vitamins and cofactors	Choline	104	103	102
Deviation ≤20%		96	98	97

5 Conclusions

One challenge with the Immundiagnostik OmicSnap® device is the difficulty of accurately filling the hollow sampling rod, particularly with samples that are too hard or too watery. Furthermore, instant homogenization is only possible when ceramic beads are separately obtained and added to the OmicSnap® tubes; and a second, mechanical homogenization is still required before measurement.

The 1:16 dilution is another disadvantage. This relatively high dilution may contribute to the absence of certain metabolites typically found in fecal samples using biocrates standard protocol (dilution 1:4).

Despite these challenges, **OmicSnap® showed very good overall coverage and stability of the SMartIDQ alpha kit metabolites**. OmicSnap® enabled the detection of 91 out of 107 SMartIDQ® alpha kit metabolites in at least one of three donors, with 74 metabolites detected above the limit of detection (LOD) in at least two donors. Moreover, **75 of the detected metabolites remained stable for 7 days at room temperature.**

The OmicSnap® device, when combined with ethanol or IPA/MeOH solution for stabilization and extraction, emerges as a suitable device for broader profiling of several different metabolite classes. The option to add a preferred custom buffer depending on the metabolites of interest offers flexibility. Notably, Immundiagnostik also offers OmicSnap® Meta tubes prefilled with a stabilizing buffer that is supposed to stabilize metabolites for diagnostic measurements. As this product was not tested, we cannot comment on its suitability.

In conclusion, the OmicSnap® device offers robust metabolite coverage and stability for fecal metabolomics and is well suited for most metabolite classes when combined with an ethanol buffer. The devices facilitate remote sampling and are, in principle, suitable for at-home sampling – an advantage that holds significant potential for both clinical studies and commercial consumer testing. The integration of fecal metabolomics with microbiome sequencing offers novel options to approach the challenges of personalized medicine with multiomics.

6 References

1. biocrates application note, 2024: Metabolome and lipidome analysis of human fecal samples.