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# Towards routine high-throughput analysis of fecal bile acids: validation of an enzymatic cycling method for the quantification of total bile acids in human stool samples on fully automated clinical chemistry analyzers

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## Abstract

**Objectives:** Bile acid diarrhea is a common but under-diagnosed condition. Because the gold standard test (<sup>75</sup>SeHCAT) is time-consuming and not widely available, fecal bile acid excretion is typically assessed by chromatography and mass spectrometry. Although enzymatic cycling assays are well established for the rapid and cost-effective analysis of total bile acids (TBA) in serum or plasma, their full potential has yet not been extended to stool samples in clinical routine.

**Methods:** The performance of the ‘Total bile acids 21 FS’ reagent (DiaSys) was evaluated in fecal matrix according to CLSI guidelines and EU-IVD Regulations (2017/745), and compared to an established microplate-based kit (IDK<sup>®</sup>) by measuring patient stool samples (n=122). Method agreement was assessed by Passing-Bablok and Bland-Altman analysis.

The quantification of eight individual BAs was assessed using HPLC-MS/MS as reference method.

**Results:** The DiaSys assay showed linearity between 3.5 and 130 μmol/L, good repeatability, total precision, and reproducibility with CVs of 1.7 %, 3.5 %, and 3.0 %. Limit of blank (LoB), detection (LoD), and quantitation (LoQ) were ≤0.17, ≤0.3, and 3.5 μmol/L, respectively. No significant interference from endogenous substances was observed. The methods showed good correlation up to 140 μmol/L (r=0.988), despite differences in the quantification of individual BAs, with mean deviations of 7 % (DiaSys) and 31 % (IDK<sup>®</sup>), respectively.

**Conclusions:** The advantages of enzymatic TBA analysis on fully automated clinical chemistry platforms can be exploited for the routine analysis of stool samples. However, cycling assays may benefit from reference standards that take into account the composition of the fecal BA pool.

**Keywords:** bile acids; stool; chronic diarrhea; enzymatic assay; clinical laboratory test; assay validation

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## Introduction

Bile acids (BAs) are 3α-hydroxylated amphipathic steroids derived from cholesterol [1, 2]. After being synthesized in the liver, the two primary BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA) are conjugated with glycine (G) or taurine (T) before excretion into the bile (Figure 1A). In response to dietary fat, the conjugated primary BAs are secreted into the duodenum, facilitating digestion and absorption of fatty acids, cholesterol, and fat-soluble vitamins [1, 2]. About 95 % are actively reabsorbed in the terminal ileum, transported back to the liver, and re-secreted into the bile (enterohepatic circulation) [1, 2]. The remaining primary BAs enter the colon, where they are deconjugated and metabolized by resident bacteria into the secondary BAs, namely deoxycholic acid (DCA) and lithocholic acid (LCA). In addition, CDCA can undergo epimerization to form

ursodeoxycholic acid (UDCA). Bile acids that are not passively reabsorbed in the colon (approx. 200–600 mg/day) are excreted via the feces (Figure 1A).

Excess delivery of BAs to the colon can stimulate colonic motility and water secretion, leading to bile acid diarrhea (BAD). BAD can be classified into type 1, in patients with bile acid malabsorption (BAM) due to ileal inflammation such as Crohn’s disease or ulcerative colitis, and type 2, also known as primary or idiopathic BAD [2]. A systematic review suggests that 25–30 % of patients with chronic diarrhea or diarrhea-predominant irritable bowel syndrome (IBS-D) have idiopathic BAD [3].

Although the 7-day <sup>75</sup>seleno-taurohomocholic acid retention test (<sup>75</sup>SeHCAT) is considered the diagnostic gold standard for BAD, it is relatively expensive and time-consuming. Furthermore, <sup>75</sup>SeHCAT has limited availability and is not approved in several countries, including the United States [4–6]. Instead, the fecal excretion of total bile acids (TBA) is quantified over 48 h, mainly using high-performance liquid chromatography (HPLC), which is expensive and time-consuming, making it less suitable for high-throughput diagnostics [7, 8].

In this regard, enzymatic cycling methods based on 3α-hydroxysteroid dehydrogenase (3α-HSD) offer significant advantages by combining simplicity, accessibility, and low costs [7, 8]. 3α-HSD catalyzes the reversible oxidation of 3α-hydroxylated steroids [7, 8]. Current 5th-generation TBA routine assays utilize thionicotinamide adenine dinucleotide (Thio-NAD<sup>+</sup>) as a coenzyme [8–10]. In the presence of

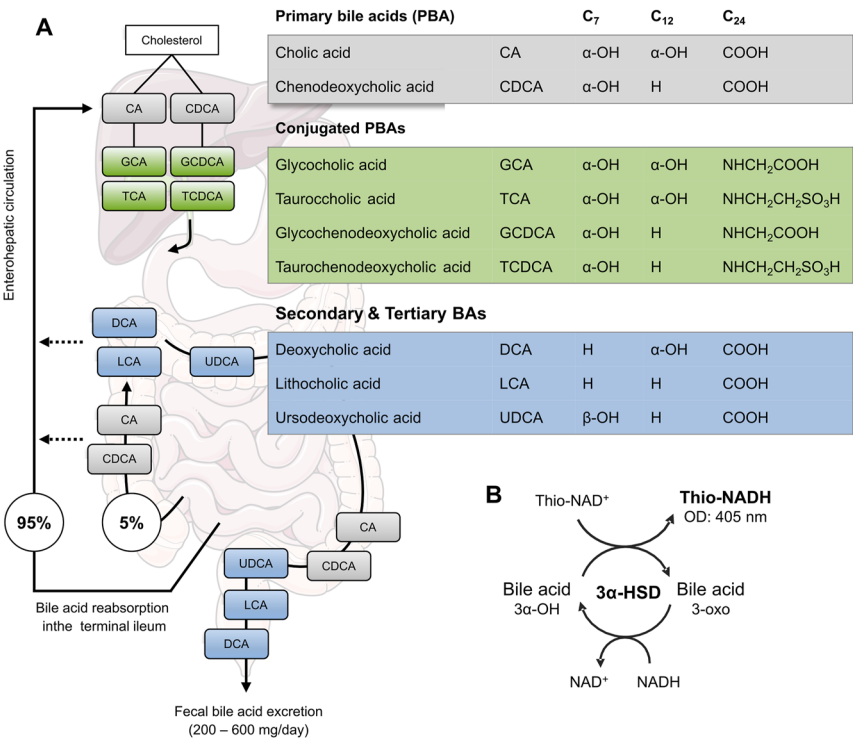
excess NADH, the amplified generation of Thio-NADH can be detected as a linear increase in optical density at 405 nm (Figure 1B) [7]. Recently, a photometric kit has been developed (IDK<sup>®</sup> Bile Acids) for the enzymatic quantification of fecal TBA on manual photometers or microplate readers. Using this method, Kumar et al. (2022) reported that TBA measurement in a single random stool sample may have diagnostic utility for severe BAD [11].

Here, we evaluated the ‘Total bile acids 21 FS’ assay (DiaSys), originally developed for the routine TBA analysis in serum, for its performance in human stool samples. The reagent was validated for the new intended use on a respo<sup>®</sup> 910 analyzer according to Clinical and Laboratory Standards Institute (CLSI) guidelines and the European *In Vitro* Diagnostics Regulation (EU-IVD 2017/745). The application was compared to the IDK<sup>®</sup> Bile Acids kit by measuring 122 patient stool extracts. In addition, the quantification of eight individual BAs (CA, CDCA, DCA, UDCA, TCA, GCA, TCDCA, and GCDCA) was assessed, using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) as non-enzymatic reference method.

Materials and methods

Sample preparation

Stool extracts were prepared from anonymous left-over samples provided by Biovis Diagnostik MVZ GmbH in IDK



**Figure 1:** Bile acids, enterohepatic circulation, and TBA cycling assay principle. (A) Primary BAs (CA and CDCA) are synthesized in the liver and conjugated to glycine (G) or taurine (T) before secretion into the duodenum. BAs reaching the colon are deconjugated and metabolized into secondary and tertiary BAs (DCA, LCA, and UDCA) by intestinal bacteria. In healthy individuals, approx. 95 % are reabsorbed in the terminal ileum. Malabsorption or overproduction can lead to excess BAs within the colon and bile acid diarrhea. (B) Enzymatic TBA cycling assays utilize 3α-hydroxysteroid dehydrogenase (3α-HSD), which catalyzes the reversible oxidation of bile acids. In the presence of excess NADH, the linear increase in Thio-NADH can be detected at 405 nm.

Extract<sup>®</sup> specimen tubes (Immundiagnostik AG, Bensheim, Germany). The samples were thawed, vortexed, and centrifuged for 10 min at 2.300 *g*. The supernatant containing the extracted fecal bile acids (resulting dilution: 1:100) was transferred to Eppendorf tubes and stored at –80 °C until measurement.

## Total bile acid measurement

For method validation and comparison, the Total bile acids 21 FS reagent (DiaSys Diagnostic Systems GmbH, Holzheim, Germany) was performed on the automated responders<sup>®</sup> 910 analyzer (DiaSys Diagnostic Systems, Holzheim, Germany) according to the manufacturer's instructions. The linear increase in Thio-NADH was detected at 405 nm (primary wavelength) and 600 nm (secondary wavelength) [7]. Assay performance was confirmed on two clinical chemistry analyzers responders<sup>®</sup> 940 (DiaSys Diagnostic Systems, Holzheim, Germany) and BioMajesty<sup>®</sup> JCA-BM6010/C (JEOL Ltd, Akishima, Japan). See Supplementary Tables S1–S4 for further details.

The photometric IDK<sup>®</sup> Bile Acids reagent (Immundiagnostik AG, Bensheim, Germany) was performed on a Sunrise<sup>™</sup> microplate reader (Tecan Group Ltd., Männedorf, Switzerland) according to the manufacturer's instructions at 405/620 nm wavelength. Samples exceeding the highest IDK<sup>®</sup> standard (>90 µmol/L) were diluted 1:1 with IDK Extract<sup>®</sup> buffer as recommended by the manufacturer. For method comparison, patient stool extracts (n=122) were measured in duplicate. See Supplementary Tables S1–S3 for further details.

For the measurement of individual bile acids, the two reagents were adapted for use on a BioMajesty<sup>®</sup> JCA-BM6010/C analyzer (JEOL Ltd, Akishima, Japan). After calibration using the assay-specific standards, eight individual bile acid solutions were measured in single determination. Thio-NADH formation was detected at 410/596 nm wavelength. See Supplementary Tables S1–S3 for further details. The preparation of aqueous bile acid solutions (weigh in: 50 µmol/L) was described previously [10]. Bile acid salts (≥95 % purity) and bile acid solutions in methanol (≥98 % purity) for calibration were purchased from AppliChem (Darmstadt, Germany), Merck (Darmstadt, Germany), and Cambridge Isotope Laboratories (Tewksbury, USA), respectively.

## Linearity

The linear range was assessed according to CLSI guidelines (EP06, 2nd Edition) for three different reagent lots by

measuring serial dilutions of two different stool extracts with low (52 µmol/L) and high (170 µmol/L) total bile acid concentrations [12]. To assess lower and upper linearity, both extracts were serially diluted with IDK<sup>®</sup> extraction buffer (dilutions: 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10) and measured in four replicates. The theoretical value for each dilution was calculated by linear regression analysis and the mean recovery was assessed. Acceptance criteria were ± 15 % deviation from the theoretical value for lower linearity (between 0 and 15 µmol/L) and ± 10 % deviation for upper linearity (between 15 and 130 µmol/L).

## Precision

Within-run precision (repeatability) and within-laboratory precision (total precision) were assessed according to CLSI guidelines (EP05, 3rd Edition) for three reagent lots and three stool samples with analyte concentrations in the lower normal range (<20 µmol/L), near the decision cut-off (60–80 µmol/L), and in the upper measuring range (>100 µmol/L) [13]. The stool extracts were pooled and aliquots were frozen at –80 °C until measurement. The repeatability was assessed by 20 sequential measurements of one aliquot for each concentration (n=20). The total precision was assessed over 20 days (two runs per day with two replicates each) on the same analyzer (n=80), using a fresh aliquot for each run. The order of samples and controls was changed in every run, and the time of measurement varied daily. For the reproducibility studies, the samples were analyzed over five days (one run with five replicates per day) on three different analyzers (n=75). The final results were reported as coefficient of variation (CV).

## Detection capability

The limit of blank (LoB), limit of detection (LoD), and limit of quantitation (LoQ) were assessed according to CLSI guidelines (EP17, 2nd Edition) for three different reagent lots [14]. To determine the LoB, four blank samples (IDK<sup>®</sup> Extraction buffer) were measured over three days with five replicates per day (n=60). The LoB was calculated using the following equation:  $LoB = \text{mean}_{\text{blank}} + 1.645 \times SD_{\text{blank sample}}$ . To determine the LoD, five low-level samples (0.2, 0.3, 0.4, 0.5, and 0.7 µmol/L) were prepared from stool extract by dilution with IDK<sup>®</sup> Extraction buffer and measured in five-fold determination over three days (n=75). The LoD was calculated using the following equation:  $LoD = LoB + 1.645 \times SD_{\text{low-level sample}}$ . To determine the LoQ, nine samples (1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 6.0 µmol/L) were prepared from stool extract

and measured over 20 days (two runs per day with two replicates each) on the same analyzer (n=80). The LoQ is given as the lowest concentration measured with a within-laboratory CV of <20 %.

## Interference

The interference from endogenous substances was assessed according to CLSI guidelines (EP07, 3rd Edition) using two pooled stool extracts with final analyte concentrations of <40 µmol/L and >80 µmol/L [15]. Each dilution step was measured in five-fold determination.

## HPLC-MS/MS method

HPLC-MS/MS measurement of aqueous bile acid solutions was performed as described previously [10], using a 3200 QTRAP® LC-MS/MS System (AB Sciex Pte Ltd., Framingham, USA), Series 200 Micro Pumps and Autosampler (PerkinElmer, Waltham, USA), CORTECS T3 Column (120 Å, 2.7 µm, 2.1 mm × 50 mm) and CORTECS T3 VanGuard Cartridge (120 Å, 2.7 µm, 2.1 mm × 5 mm; Waters Corporation, Milford, USA). HPLC-MS/MS data was evaluated using Analyst software (Version 1.5.1, build 5218) and MultiQuant™ software (Version 3.0.2, build 8664.0), respectively (Sciex, Framingham, USA). HPLC-grade methanol and acetonitrile (ACN) were purchased from Carl Roth (Karlsruhe, Germany).

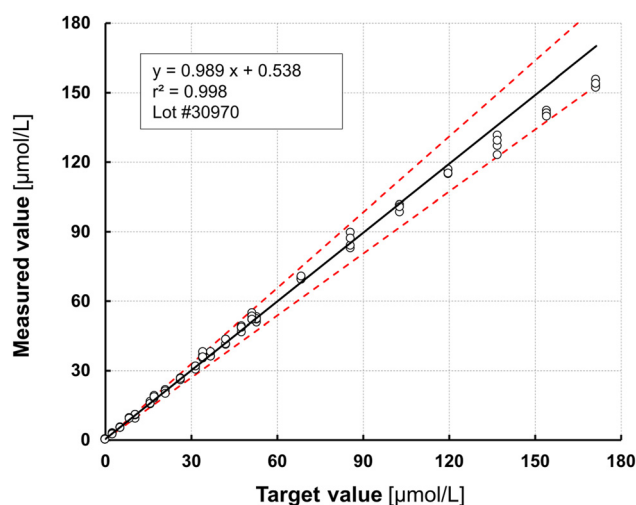
## Statistical analysis

Passing-Bablok regression and Spearman's rank correlation were used to compare methods and quantify the proportional differences between measurement results. As the Passing-Bablok method is only valid for linear relationships, linearity was assessed using the Cusum test ( $p > 0.05$ ) prior to the regression analysis. Statistical analyses were performed using MedCalc® (Version 18.10.2, MedCalc Software Ltd, Belgium) and Microsoft Excel 2021.

## Results

### Linearity

The Total bile acids 21 FS assay exhibited linear behavior between 3.5 and 130 µmol/L and met the CLSI acceptance criteria in this concentration range for all three reagent lots tested. The results for Lot 1 (#30970) on respons® 910 are



**Figure 2:** Linearity assessment. The linear range (3.5–130 µmol/L) was estimated according to CLSI acceptance criteria (CLSI EP05) by serial dilution of two different stool extracts for lower linearity (0–50 µmol/L) and upper linearity (0–170 µmol/L).

shown in Figure 2. See Supplementary Figure S1 for further details. The linear behavior up to 130 µmol/L could be confirmed on the respons® 940 analyzer (DiaSys Diagnostic Systems, Holzheim, Germany; see Supplementary Figure S2).

### Precision and detection capability

The results are summarized in Table 1 and Table 2. The within-run precision (repeatability) was between 1.09 and 2.06 % (CV near cut-off: ≤1.66 %), and the within-laboratory precision (total precision) was between 2.61 and 4.1 % (CV near cut-off: ≤3.51 %). The reproducibility (CV near cut-off) was found to be ≤3.01 % (Table 1). LoB, LoD and LoQ were ≤0.17, ≤0.3, and 3.5 µmol/L, respectively (Table 2). The good within-run precision for stool samples <20 µmol/L and ≥140 µmol/L was confirmed on respons® 940 (CV: 0.58–1.41 %) and BioMajesty® JCA-BM6010/C (CV: 0.60–1.04 %) (Supplementary Table S4). In addition, the TBA 21 FS reagent showed excellent inter-instrument agreement when run on different analyzers, with  $r = 0.998$  (respons® 940 vs. respons® 910) and  $r = 0.999$  (respons® 940 vs. BioMajesty® JCA-BM6010/C; Supplementary Figure S3).

### Interference

The results of the interference testing are summarized in Table 3. No significant interference from ascorbic acid, conjugated and unconjugated bilirubin, hemoglobin, triglycerides or immunoglobulin A (IgA) could be observed in

**Table 1:** Assay imprecision (CLSI EP05, 3rd Edition).

		<20 $\mu\text{mol/L}$		60–80 $\mu\text{mol/L}$		>100 $\mu\text{mol/L}$	
		Mean ( $\pm$ SD), $\mu\text{mol/L}$	CV, %	Mean ( $\pm$ SD), $\mu\text{mol/L}$	CV, %	Mean ( $\pm$ SD), $\mu\text{mol/L}$	CV, %
Repeatability (n=20)	Lot 1	14.7 (0.22)	1.50	70.8 (0.77)	1.09	115.0 (2.37)	<b>2.06</b>
	Lot 2	14.3 (0.24)	<b>1.68</b>	69.0 (1.06)	1.53	115.5 (1.55)	1.34
	Lot 3	14.5 (0.23)	1.59	71.0 (1.18)	<b>1.66</b>	118.8 (1.52)	1.28
Total precision (n=80)	Lot 1	15.1 (0.52)	3.46	73.3 (2.22)	3.02	122.2 (3.55)	2.91
	Lot 2	14.8 (0.61)	<b>4.09</b>	72.5 (2.54)	<b>3.51</b>	120.0 (4.13)	<b>3.44</b>
	Lot 3	14.9 (0.56)	3.74	72.9 (1.90)	2.61	121.6 (3.26)	2.63
Reproducibility (n=75)		15.0 (0.6)	<b>4.08</b>	74.2 (2.2)	<b>3.01</b>	124.7 (4.2)	<b>3.35</b>

Highest CV value is highlighted in bold.

**Table 2:** Detection capability (CLSI EP17, 2nd Edition).

	Limit of blank (LoB), $\mu\text{mol/L}$	Limit of detection (LoD), $\mu\text{mol/L}$	Limit of quantitation (LoQ), $\mu\text{mol/L}$
Lot 1	0.16	0.30	3.50
Lot 2	0.17	0.28	3.50
Lot 3	0.14	0.29	3.50

**Table 3:** Interference from endogenous substances (CLSI EP07, 3rd Edition).

Substance	Interference in stool extracts (dilution 1:100) $\leq 10\%$ up to	Analyte concentration [ $\mu\text{mol/L}$ ]
Ascorbic acid	1.2 mg/dL	30.5/86.6
Bilirubin (conjugated)	0.74 mg/dL	31.8/91.8
Bilirubin (unconjugated)	0.68 mg/dL	31.1/90.3
Hemoglobin	12 mg/dL	30.8/88.9
Immunoglobulin A	3 mg/dL	30.7/87.6
Triglycerides	24 mg/dL	30.1/84.7

stool samples with low (<40  $\mu\text{mol/L}$ ) and high (>80  $\mu\text{mol/L}$ ) analyte concentration (Table 3).

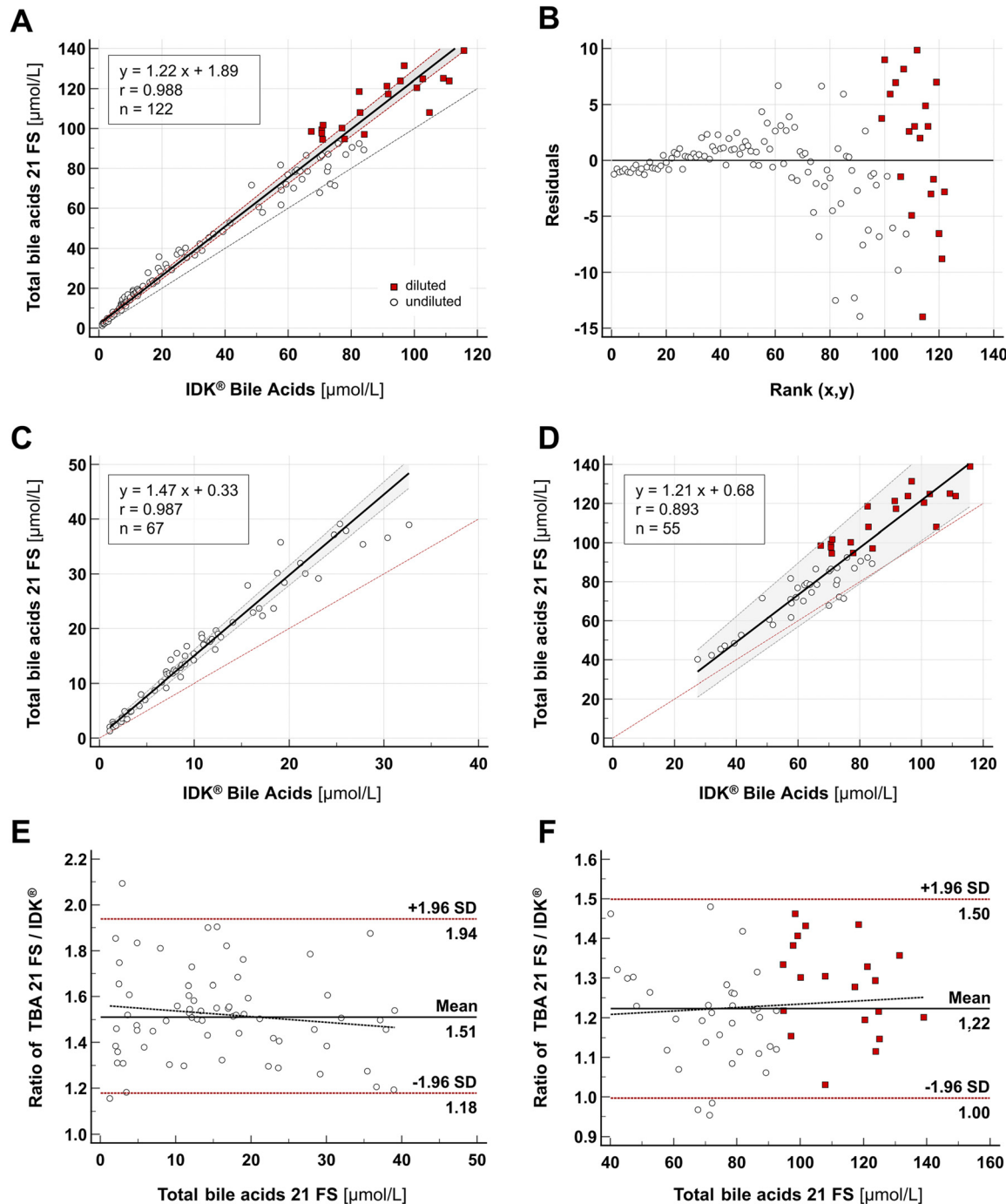
## Method comparison

To assess the agreement with an established method, patient stool extracts (n=122) were analyzed in parallel using both the TBA 21 FS reagent performed on a fully-automated responders<sup>®</sup> 910 analyzer and the microtiter-based IDK<sup>®</sup> Bile Acids kit. Samples exceeding the highest IDK standard (>90  $\mu\text{mol/L}$ ) were diluted 1:1 with IDK Extract<sup>®</sup> buffer as recommended by the manufacturer. Both methods showed good overall correlation (Spearman

r: 0.988; 95 % CI: 0.983–0.992;  $p < 0.0001$ ) with a proportional bias of approx. 22 % (slope: 1.22; 95 % CI: 1.19–1.27) (Figure 3A and B). Since the Cusum test indicated a significant deviation from linearity over the entire concentration range ( $p < 0.01$ ), the proportional differences were reevaluated by Passing-Bablok and Bland-Altman analysis for samples with TBA <40  $\mu\text{mol/L}$  (n=67) and >40  $\mu\text{mol/L}$  (n=55), respectively (Figure 3C–F). The correlation was lower for stool extracts in the upper measuring range (Spearman r: 0.893; 95 % CI: 0.823–0.937) compared to samples with low analyte concentration (Spearman r: 0.987; 95 % CI: 0.978–0.992), despite the recommended 1:1 dilution of samples >90  $\mu\text{mol/L}$ . The results are summarized in Table 4.

## Quantification of individual bile acids

Previous studies have shown that enzymatic TBA assays display variable recovery of individual bile acids [7, 9, 16, 17]. Therefore, method agreement will be affected by the individual BA composition of the sample, especially for samples with a fecal BA spectrum that differs significantly from that of the calibration standards. To compare the quantification of individual bile acids, both reagents were adapted for use on a BioMajesty<sup>®</sup> JCA-BM6010/C analyzer. Eight aqueous bile acid solutions were measured in single determination. The reference concentration was determined by HPLC-MS/MS. The recovery of deconjugated and T/G-conjugated bile acids is summarized in Figure 4. While CA was measured with the highest accuracy and lowest mean deviation from reference (3 %), TCA and GCA were consistently over- or undermeasured by both enzymatic assays, with mean recoveries of 123 % (TCA) and 82 % (GCA), respectively (Figure 4). The Total bile acids 21 FS assay recovered UDCA and CDCDA with a deviation of +1 % and –4 %, respectively, whereas the IDK assay deviated by –33 % (UDCA) and +42 % (CDCDA). The largest discrepancy



**Figure 3:** Method comparison. (A–B) Passing-Bablok regression analysis for the measurement of TBAs in stool samples (n=122). Stool extracts (dilution: 1:100) with fecal TBA concentration >90 μmol/L were further diluted according to the IDK® assay's instructions for use (red squares). (C–D) Passing-Bablok regression and (E–F) Bland-Altman analysis for stool extracts <40 μmol/L (n=67) and >40 μmol/L (n=55).

was observed for DCA, one of the most highly abundant fecal BAs, with a recovery of 77 % (DiaSys) vs. 144 % (IDK). Across all non-conjugated BAs tested, namely CA, CDCA,

UDCA, and DCA, the assays showed a mean deviation from HPLC-MS/MS-reference value of 7 % (DiaSys) and 31 % (IDK), respectively (Figure 4).

**Table 4:** Method comparison.

Analyte concentration range (Dilution 1:100)	Spearman correlation	Passing-Bablok regression			Bland-Altman Mean ratio: TBA FS 21/IDK ( $\pm 1.96$ SD)
	r (95 % CI) p<0.0001	Slope (95 % CI)	Intercept (95 % CI)	Cusum p	
1–140 $\mu\text{mol/L}$ (n=122)	0.988 (0.983–0.992)	1.22 (1.19–1.27)	1.89 (0.86–2.51)	<0.01 <sup>a</sup>	1.37 (1.01–1.87)
<40 $\mu\text{mol/L}$ (n=67)	0.987 (0.978–0.992)	1.47 (1.40–1.53)	0.33 (–0.18–0.84)	0.09	1.51 (1.18–1.94)
>40 $\mu\text{mol/L}$ (n=55)	0.893 (0.823–0.937)	1.21 (1.10–1.37)	0.68 (–9.30–7.41)	0.49	1.22 (1.00–1.50)

<sup>a</sup>Significant deviation from linearity (p<0.05).

		DiaSys TBA 21 FS			IDK® Bile Acids			HPLC-MS/MS	
		Measured [ $\mu\text{mol/L}$ ]	Recovery	Deviation	Measured [ $\mu\text{mol/L}$ ]	Recovery	Deviation	Difference [% points]	Mean Deviation
unconjugated	CA	39.4	101%	1%	40.9	105%	5%	4	3%
	UDCA	55.4	101%	1%	36.9	67%	33%	34	17%
	CDCA	37.3	96%	4%	55.2	142%	42%	46	23%
	DCA	35.0	77%	23%	65.3	144%	44%	67	34%
	Mean		94%	7%		115%	31%		
conjugated	TCA	41.8	123%	23%	41.9	123%	23%	0	23%
	GCA	42.4	80%	20%	43.9	83%	17%	3	19%
	TCDCA	41.5	88%	12%	56.7	121%	21%	33	17%
	GCDCA	38.1	78%	22%	56.7	116%	16%	38	19%
	Mean		92%	19%		111%	19%		

**Figure 4:** Recovery of individual BAs from HPLC-MS/MS reference value. Unconjugated and T/G-conjugated BAs are sorted by assay disagreement (arithmetic difference between recovery rates in percent points) from minimum (white) to maximum (yellow). Recovery: <100 % (blue), 100 % (white), >100 % (red). Deviation: minimum (white), maximum (green).

## Discussion

Although enzymatic TBA cycling assays are well established in clinical routine for the rapid and cost-effective quantification of serum TBA, their full potential has not been extended to the routine analysis of fecal bile acids. To our knowledge, this is the first study to report the evaluation and validation of a commercial 5th-generation TBA routine assay for the quantification of total bile acids in patient stool samples – collected in routine-compatible stool preparation tubes – on a fully-automated clinical chemistry platform.

Various hepatobiliary and gastrointestinal diseases are accompanied by pathological changes in bile acid synthesis,

metabolism, secretion, and absorption, which leads to detectable changes in the circulating BA pool [1, 18–21]. Especially the diagnostic value of serum TBA is well established since an elevation above the normal range (>10  $\mu\text{mol/L}$ ) is frequently observed in patients with liver diseases [18, 22, 23]. Furthermore, serum TBA is an important biomarker to assess hepatobiliary function in veterinary medicine [24, 25]. Enzymatic assays are the preferred detection method in clinical routine, although the recombinant 3 $\alpha$ -HSD cannot differentiate between individual BAs and is restricted to steroids with a free 3 $\alpha$ -OH group [7]. In 2022, Žižalová et al. reported that enzymatic 3rd-generation assays may systematically underestimate the serum TBA

concentration compared to the more specific LC-MS/MS method, mainly because 3 $\alpha$ -HSD exhibits different activity for different individual bile acids [17]. Grimm et al. (2024) recently compared the analytical performance of five 5th-generation TBA cycling assays for the measurement in patient serum samples on the same clinical chemistry platform [10]. All commercial routine assays showed excellent correlation for the TBA measurement in serum (Spearman  $r > 0.99$ ), despite substantial differences in the quantification of individual BAs [10]. Others reported similar results for patient plasma, with good correlation among the three commercial TBA assays tested (Spearman's  $r > 0.82$ ), and excellent correlation with the LC-MS reference method (Spearman  $r > 0.92$ ) [9]. The proportional bias between the commercial assays has been mainly attributed to the heterogeneous composition of proprietary calibrators, which typically contain different individual bile acids such as GCA, CDCA, or TDCA [9, 17]. In line with these results, we observed good correlation between the microtiter-based assay from IDK and the analyzer-based assay from DiaSys, especially for stool extracts with fecal TBA levels  $< 40 \mu\text{mol/L}$  (dilution 1:100), which corresponds to  $4 \mu\text{mol/g}$  stool (with  $1 \text{ g stool} \cong 1 \text{ mL}$ ). The correlation was lower for stool extracts with higher TBA concentration, despite the additional 1:1 dilution step recommended for the IDK assay for samples above the highest assays-internal IDK standard ( $> 96 \mu\text{mol/L}$ ). Of note, the IDK microtiter version (K7878W) demonstrated linearity between 1.9 and  $54.8 \mu\text{mol/L}$  (Supplementary Table S2), and the cuvette version (K7878CV) between 10.98 and  $36.83 \mu\text{mol/L}$ , according to the manufacturer [26]. Hence, a further dilution of stool extracts may be sufficient to improve the agreement with the DiaSys test in the upper TBA range.

Based on internal studies, both manufacturers estimated the upper reference range limit for healthy individuals to be  $6.5 \mu\text{mol/g}$  (as measured by IDK) or  $7.0 \mu\text{mol/g}$  (for women) and  $8.4 \mu\text{mol/g}$  (for men), respectively (as measured by DiaSys). This discrepancy is consistent with the  $\sim 20\%$  bias between the two assays reported in this study for stool extracts  $> 40 \mu\text{mol/L}$  (Table 4). The assays were calibrated according to the respective instructions for use, against a different assay-specific bile acid standard. Although the exact composition is a trade secret, it is reasonable to assume that a varying BA content may contribute to the observed proportional bias; in addition to other methodological differences, such as the recombinant enzyme, reaction conditions, and detection method. The calibration against a single bile acid may also explain, why most enzymatic methods seem to underestimate the TBA concentration compared to LC-MS-based techniques – especially in samples with pathological TBA levels and substantial overrepresentation of “atypical” individual BAs

[9, 17]. In serum from patients with liver disease, e.g., the conjugated primary BAs were found to be overproportionally elevated, between 13.8-fold for GCDCA and 97.2-fold for TCA, compared to the 3.1- and 2.4-fold increase observed for CA and CDCA, respectively [27]. A similar, non-uniform elevation under pathological conditions has been demonstrated for fecal bile acids. For example, Duboc et al. (2010) reported significantly higher levels of conjugated and 3-OH-sulfated BAs in the stool of patients with active inflammatory bowel disease (IBD), due to the IBD-related BA dysbiosis and dysmetabolism [16]. In contrast, the proportion of secondary BAs was significantly reduced compared to healthy individuals [16].

BAD is generally regarded as an underdiagnosed condition [28]. In a survey of UK patients, 44 % reported a delay of more than 5 years before the correct diagnosis [29]. When the 7-day SeHCAT retention test is not available, at least testing for fecal BAs should be considered [30]. Since fecal TBAs were found to be elevated in every second patient with chronic unexplained diarrhea (51 %), it has been proposed that a widespread biochemical analysis in clinical routine could help to reduce healthcare costs from unnecessary diagnostic imaging [31]. However, fecal analysis is generally more challenging than blood analysis [32]. This is primarily due to the higher complexity associated with sample collection, processing, and analyte normalization, as well as the higher variability and heterogeneity in sample composition [32, 33]. The individual BA content of each stool sample depends on numerous variables, including sex, with men having a higher mean TBA concentration than women, the previous diet, and the patient's gut microbiome [32, 34]. This is why the 48-h fecal bile acid test requires a standardized 4-day high-fat diet before the 2-day stool collection. Both an increase in the fecal TBA excretion ( $\geq 2,337 \mu\text{mol}/48 \text{ h}$ ) and the percentage of primary BAs ( $> 10\%$ ) are considered diagnostic for BA malabsorption and -diarrhea [3, 20, 28]. However, the BA concentration is usually determined by HPLC, which is time-consuming and not suitable for the routine analysis of large sample numbers [4, 8, 28]. In a prospective, observational study, Kumar et al. recently analyzed the TBA concentration in single random stool samples obtained from patients with chronic diarrhea ( $> 6$  weeks), using the microtiter plate-based IDK<sup>®</sup> Bile Acids test [11]. The authors reported a significantly higher fecal TBA mean concentration for subjects with mild and severe BAD, i. e. with  $^{75}\text{SeHCAT}$  retention  $< 15\%$  ( $4.95 \mu\text{mol/g}$ ; IQR: 2.6–10.5) and  $< 5\%$  ( $9.9 \mu\text{mol/g}$ ; IQR: 4.8–15.4), respectively, compared to the control group ( $2.6 \mu\text{mol/g}$ ; IQR: 1.6–4.2) [11]. At a fecal TBA cut-off of  $4.3 \mu\text{mol/g}$ , sensitivity and specificity were 57 and 77 %, respectively, relative to  $^{75}\text{SeHCAT}$  retention of  $\leq 15\%$  [11]. Furthermore, a fecal TBA concentration  $> 10.1 \mu\text{mol/g}$

(as determined enzymatically in a single, random stool sample) demonstrated 91 % specificity for mild, moderate, or severe BAD – providing a feasible measure to stratify patients with unexplained chronic diarrhea who should undergo further evaluation to confirm the diagnosis of BAD [11].

In conclusion, these results indicate that the performance of enzymatic TBA routine assays is appropriate for use in clinical routine to identify patients with elevated fecal bile acid levels; considering an upper reference limit between 6 and 8  $\mu\text{mol/g}$  ( $\approx 60\text{--}80\ \mu\text{mol/L}$  for the 1:100 diluted extract). The Total bile acids 21 FS assay (DiaSys) showed linearity up to 130  $\mu\text{mol/L}$  (Figure 1), as well as good repeatability, total precision, and reproducibility, especially near the presumable 60–80  $\mu\text{mol/L}$  cut-off (Table 1).

Further studies will be required to confirm the normal fecal TBA range in healthy individuals, when “total bile acids” are determined enzymatically. Furthermore, high-throughput analysis requires feasible, standardized, and routine-compatible methods for stool sampling and bile acid extraction. In this regard, commercial stool preparation systems, such as those used in this study and by Kumar et al., offer several advantages [11]. The dipstick-based collection tube enables simple and hygienic sampling at home – once or over several days, with or without a previous standardized diet – and a rapid and standardized extraction of fecal bile acids. The resulting stool extracts (dilution 1:100) can be directly measured on a clinical chemistry analyzer without further dilution, given the upper linear range limit of 130  $\mu\text{mol/L}$ . In this study, only 2/122 patient stool extracts (1.6 %) slightly exceeded this concentration (Figure 3A and B).

To further improve harmonization, commercial TBA cycling assays should be calibrated against a well-defined reference standard, comprising appropriate bile acids most relevant to the intended use and sample matrix. In human serum, the glycin-conjugated GCDCA, GDCA, and GCA are among the most abundant BAs. In contrast, the fecal BA pool of healthy individuals mainly comprises the unconjugated secondary DCA and LCA (approx. 32 % each), with only small amounts of CA, CDCA, and UDCA ( $\leq 2\%$  each) [34]. In general, the agreement between the two enzymatic methods was best for CA, TCA, and GCA. However, only CA was quantified with high accuracy by both methods, with a deviation of  $\leq 5\%$  from HPLC-MS/MS reference. For DiaSys, a similar accuracy could be observed for the quantification of CDCA ( $-4\%$ ) and UDCA ( $-1\%$ ), whereas the IDK stool test overestimated CDCA ( $+44\%$ ) and underestimated UDCA ( $-33\%$ ). Notably, the quantification of DCA, one of the most highly abundant fecal BAs, resulted in the highest disagreement and deviation from reference, ranging from  $-23\%$  (DiaSys) to  $+44\%$  (IDK).

While both enzymatic methods correlated well over the entire fecal TBA range, the proportional bias (between 20 and 50 %) may indicate that the development of TBA stool assays should focus on the most abundant fecal BAs, to further improve accuracy and inter-assay agreement.

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**Conflict of interest:** A. Masetto, T. Leber, and Kai Prager are employed by DiaSys Diagnostic Systems GmbH (Holzheim, Germany). M. Grimmmler is employed by DiaSys Diagnostic Systems GmbH (Holzheim, Germany) and DiaServe Laboratories GmbH (Iffeldorf, Germany). The other authors state no conflict of interest.

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