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A Review of Analytical Platforms for Accurate Bile Acid Measurement

Mainak Dutta^{#,1}, Jingwei Cai^{#,2}, Wei Gui², and Andrew D. Patterson^{2,*}

¹Department of Biotechnology, Birla Institute of Technology and Science, Pilani, Dubai Campus, Dubai International Academic City, Dubai, UAE

²Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, 322 Life Sciences Building, University Park, PA 16802, USA

Abstract

Bile acids are acidic steroids which help in lipid absorption, act as signaling molecules, and are key intermediate molecules between host and gut microbial metabolism. Perturbations in the circulating bile acid pool can lead to dysregulated metabolic and immunological function which often cause liver and intestinal diseases. Bile acids have chemically diverse structures and are present in broad range of biological concentrations in a wide variety of samples with complex biological matrices. Advanced analytical methods are therefore required to identify and accurately quantify individual bile acids. Though enzymatic determination of total bile acid is most popular in clinical laboratories, these methods provide limited information about individual bile acids. Advanced analytical methods such as gas chromatography- and liquid chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy have now emerged as highly informative techniques which help in identification and quantification of individual bile acids in complex biological matrices. Here, we review the detection technologies currently used for bile acid identification and quantification. We further discuss the advantages and disadvantages of these analytical techniques with respect to sensitivity, specificity, robustness, and ease of use.

Graphical Abstract

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*Corresponding author, adp117@psu.edu.

[#]((Footnote))Mainak Dutta and Jingwei Cai contributed equally to this work.

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Conflict of Interest

The authors have no conflict of interest to declare.

Keywords

bile acid; mass spectrometry; nuclear magnetic resonance spectroscopy; gas chromatography; liquid chromatography; enzymatic

Introduction

Bile acids (BAs) are 24 carbon amphipathic molecules with a hydroxylated steroid nucleus and a hydrocarbon chain that terminates in a carboxyl group. They are synthesized from cholesterol in the liver and play important roles in several physiological processes. Due to their amphipathic nature, BAs are known as powerful emulsifiers of dietary triacylglycerol and other complex lipids in the intestine where they help prepare these lipids for degradation by pancreatic digestive enzymes. BAs also act as signaling molecules which induce certain genes in turn regulating bile acid synthesis, transportation, uptake, and metabolism [1]. The pool of BAs consists of primary, secondary, and tertiary BAs. The chemical structures of some common and most abundant BAs found in humans are illustrated in Figure 1. Primary BAs including cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized in the liver from cholesterol. There, they bind with glycine or taurine to form conjugated BAs. Secondary BAs are formed when the structure of primary BAs undergoes biotransformation (including dehydroxylation and deconjugation) during enterohepatic cycling. This process is modulated by bacterial enzymes in the intestine. Secondary BAs may further undergo structural modifications such as glucuronidation, sulfation, glucosidation, and N-acetylglucosaminidation in the liver and gut to form tertiary BAs. BA synthesis and biotransformation thus yields a wide range of structural variants with varying range of concentration [2]. The pool size and composition of BAs is intimately related to dysregulated metabolic and immunological function [3]. Since the gut microbiome facilitates BA biotransformation, perturbations of the gut microbiota may significantly influence the circulating BA signature thereby contributing towards development of intestinal and liver diseases [4]. BAs, therefore, help in the crosstalk between host endogenous metabolism and gut microbial metabolism [5].

Given the biological and clinical significance of BAs, a reliable and efficient platform and method for robust detection and quantitation is important for understanding their physiologic roles. However, the development of sensitive and accurate analytical methods remains

challenging due to the chemical diversity of BAs, the broad spectrum of biological concentration (10^6 magnitude), as well as the molecular complexity of the biological matrix like plasma, urine, bile, and stool [6]. The present review focuses on recent studies on the main detection technologies of BAs. We further discuss the advantages and disadvantages of these analytical techniques with respect to sensitivity, specificity, robustness and ease of use.

1. Approaches for bile acid quantitation

Over the last decade, several methods using different platforms have been reported for BA separation, detection, and quantitation. These methods include simple, yet robust techniques such as enzymatic assays, enzyme-linked immunosorbent assay (ELISA), thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), gas-chromatography (GC) and supercritical fluid chromatography (SFC). More recently, several sensitive methods using high throughput platforms including GC coupled with mass spectrometry (GC-MS), liquid-chromatography mass spectrometry (LC-MS), SFC mass spectrometry (SFC-MS) and nuclear magnetic resonance (NMR) spectroscopy have also been developed which help with molecular characterization and detection of BAs. Table 1 shows a comparison of the different platforms based on sensitivity, selectivity, robustness, and ease of use for BA analysis.

1.1 Enzymatic assays

Enzymatic assays offer a relatively simple and rapid measurement for total bile acid (TBA) content making it the most widely used method in clinical laboratories. The most simple enzymatic assay measures the fluorescence of NADH generated by 3 α -hydroxysteroid dehydrogenase (3 α -HSD) catalyzed oxidoreduction reaction of BAs in presence of NAD + [7]. Since this assay requires a fluorimeter, an expensive instrument, an additional step was added so that a simple spectrophotometer can be used. Hydrogen in the NADH generated from the previous method was transferred to nitrotriazolium blue by diaphorase enzyme to yield diformazan. Absorbance of diformazan was then measured spectrophotometrically at 540 nm [8]. More recently, an improved and sensitive method for serum TBA estimation was developed. The method uses NADH, thio-NAD⁺ and genetically engineered 3 α -HSD to yield the product thio-NAD⁺ whose absorbance changes can be measured at 405/660 nm per minute to estimate serum TBA. This method, also known as enzymatic cycling method, showed a detection limit of 0.22 μ M without any interference from bilirubin, ascorbates, hemoglobin, or lactate dehydrogenase [9]. To further improve the efficiency of enzymatic determination of TBA, an indirect electrochemical detection (IED) method was developed using screen printed carbon electrode [10]. It consumes less reagent but provides similar detection sensitivity (upto 5 μ M) to the enzymatic cycling method [10]. Though enzymatic methods for BA analysis are popular in clinical laboratories, identification of individual BAs is restricted using these methods because total BAs, instead of individual BAs, are quantified.

1.2 Enzyme-linked immunosorbent assay (ELISA)

Like enzymatic assays, ELISA is also one of the most popular methods for estimation of certain BAs in clinical laboratories. Kano and co-workers developed a simple ELISA

method for estimating CA and deoxycholic acid (DCA) in human feces [11]. More recently, a monoclonal ELISA method for human urinary glycolithocholic acid sulfate (GLCA-Sul) with limited cross-reactivity for 3-sulfates of CA, CDCA and DCA was developed [12]. The method exhibited 6 pM sensitivity and therefore allowed the measurement of GLCA-Sul in trace amount of urine specimen (less than 50 μ l). Liu and co-workers developed an indirect competitive enzyme linked immunosorbent assay (icELISA) method for determination of five major BAs including, CA, CDCA, DCA, ursodeoxycholic acid (UDCA) and hyodeoxycholic acid (HDCA), using a combination of four different monoclonal antibodies [13]. Serum TBA concentrations determined using this method were also found to be consistent with values obtained by enzymatic methods. Further, the icELISA method showed significant sensitivity for detection of BAs (as low as 0.29 μ M) in saliva where they are present typically in low concentrations [13]. Though ELISA is highly sensitive and shows limited variation across assays, antibody cross-reactivity limits its usage.

1.3 Chromatography-based techniques

Most of the routine methods used in clinical laboratories for BA measurements are non-chromatographic and provide information about TBA concentrations; however, these methods provide limited information about individual BAs. Individual BAs serve important physiological function and clinical significance, especially hydrophobic secondary BAs including lithocholic acid (LCA) and DCA. Elevated LCA and DCA levels have been implicated in the promotion of colon cancer, chronic inflammation, and hepatobiliary diseases like cholestasis and gallstone formation [14]. It is therefore imperative to use selective techniques for accurate and specific bile acid quantitation in clinical practice. Chromatographic techniques depend on the selective affinity of the migrating components in the stationary phase and mobile phase, thus providing a good separation to purify individual bile acid from the complex biological matrix [15]. Choice of chromatographic system is dependent on the type of BA to be analyzed and also the type of molecules present in the interfering biological matrix.

1.3.1 Thin-Layer Chromatography (TLC)—TLC methods have been used in routine qualitative analysis of primary and secondary bile acids and their glycine or taurine conjugates for its relatively simple and inexpensive procedure [15]. Though several methods exist for separation of CDCA and DCA and their conjugates using either silica (normal phase TLC) or alkyl-bonded silica (reverse phase TLC) TLC plates, resolution remains poor at higher sample concentration. Improved resolution was obtained using two-dimensional TLC separation where two different solvent systems with different selectivities were used to separate CDCA, DCA, CA, glycocholic acid (GCA), LCA, glycodeoxycholic acid (GDCA) and glycolithocholic acid (GLCA) [16]. Separation of isomeric dihydroxy conjugates including glycochenodeoxycholic acid (GCDCA), taurodeoxycholic acid (TCDCA) and taurocholic acid (TDCA) remains poor using TLC. Recently, Dolowy and co-workers have used TLC for quantitative estimation of BAs using densitometry in the UV region (360 nm). They were able to successfully separate and quantify five BAs (CA, DCA, CDCA, LCA and UDCA) with their limit of detection and quantitation ranging from 0.119-2.085 μ g/spot and 0.396-6.951 μ g/spot, respectively [17].

1.3.2 High Performance Liquid Chromatography (HPLC)—HPLC is the most widely used chromatographic technique for BA analysis. The technique offers much better sensitivity and separation of the analytes in a complex matrix compared to the techniques discussed in the previous sections. Modification of chromatography and choice of detectors and columns used in HPLC is dependent on sample type and analysis purpose. For samples with high bile acid concentrations (mM), HPLC coupled with RI (refractive index) or UV (ultraviolet) detectors yielded satisfactory measurement. For samples with low bile acid concentrations (nM) or more unconjugated bile acids with poor spectroscopic absorption, pre-column derivatization is often used. Esterification with derivatization reagents like 4-bromomethyl-7-methoxycoumarin and 1-bromoacetylpyrene, or the post-column immobilized 3 α -hydroxysteroid dehydrogenase (3 α -HSD) enzymatic reactions using octadecyl-silica (ODS) column offer significantly improved sensitivity and resolution of the bile acid detected by either UV or fluorescence detector [15]. More recently, use of nitrophenacyl bromide was used as a derivatization agent to detect BAs at a wavelength of 263 nm [18]. The method was found to be suitable for separation and detection of CA, CDCA, DCA and HDCA with limit of detection and quantitation ranging from 0.28-0.31 ng and 0.83-1.02 ng, respectively. Further, phenacyl bromide was used as a derivatizing agent for the detection of CA, CDCA, DCA, LCA, UDCA at a wavelength of 253 nm which significantly improved the dynamic range to 500 fold [19]. The method was also found suitable for detection of stereoisomers for secondary BAs including isoLCA and isoDCA [19]. For determination of conjugated BAs in serum, improved HPLC using ion pair chromatography or ion exchange chromatography on piperidino-hydroxy-propyl-Sephadex LH-20 were developed for better separation and selectivity [20, 21]. Irrespective of diversity of HPLC method developed, the main disadvantages including matrix effect and restricted specificity of the detectors, thus rendering these method unsuitable for measurement of the non-principal BA species, (including taurine-, glycine-, sulfated- and glucuronidated-conjugated BAs) in more complex biological matrices.

1.3.3 Gas Chromatography (GC)/ GC-Mass Spectrometry (GC-MS)—GC is a separation technique for volatile or semi-volatile constituents in gas phase. A temperature-controlled oven is used to heat the column above the boiling points of the constituents to convert them into gas phase. BAs however, contain –COOH, –OH, and oxo-functional groups which increase their ability to form hydrogen bonds between compounds, often leading to low volatility and thermal instability. In their native state, BAs are not sufficiently volatile for direct GC analysis. A derivatization step is thus required prior to GC injection to improve the volatility and thermal stability of the constituents. The earliest GC application for BA series analysis was described in 1960, with only 4 methyl-bile acid derivatives detected [22]. With the development of diverse detection techniques including electron capture detection (ECD) and flame ionization detection (FID), wider applications for BA analysis in bile, serum, urine and feces has been enabled [15, 23]. However, the time-consuming sample preparation procedure involving extraction, purification, hydrolysis, derivatization, and inaccurate identification of stereoisomeric forms of BA with a single GC column have limited the extensive application for BA quantitation.

GC coupled with MS detection is a robust yet simple and inexpensive method that has been extensively used for BA separation and quantitation [24]. Unlike GC coupled with ECD and FID detectors, GC-MS is quite sensitive and leads to definitive identification of BAs based on their characteristic MS fragmentation patterns, thus bypassing the need for reference standards. Several GC-MS methods have been developed for identification of BAs in biological samples. However, these methods require large sample volumes (5 mL or more) [25–27] with multiple prior chromatographic (solid phase, gel, and ion-exchange) sample clean up steps to remove interferences from biological matrices. The derivatization steps in these methods are also complex. Kumar and colleagues developed a relatively simple GC-MS method for detecting seven BAs including CA, LCA, DCA, CDCA, UDCA, α -muricholic acid (α -MCA) and β -MCA [28]. The sample preparation steps involved liquid-liquid extraction using methyl-t-butyl-ether followed by a derivatization step with MSTFA:NH₄I:DTE mixture. The electron impact (EI) ionization mode in GC-MS was used for identification of the BAs, and all of the ions were monitored in the selected ion monitoring (SIM) mode. They further reported that unlike other derivatization agents, MSTFA:NH₄I:DTE mixture showed improved stability and highest abundance of MS fragments [28].

1.3.4 Liquid Chromatography-Mass Spectrometry (LC-MS)—HPLC or ultra-high pressure liquid chromatography (UHPLC) coupled with MS (and MS/MS) has been the most sensitive and widely used analytical tool for accurate BA detection and quantitation in human and rodent urine [29–33], plasma/serum [30, 34–38], bile [30, 39, 40], intestinal contents [41, 42], liver [36, 42] and feces [42, 43]. Currently, most of the available HPLC or UHPLC methods involve reverse-phase chromatography which allows wider choices of flow rates and column dimensions [6, 44]. pH of the mobile phase is an important factor that influences chromatographic separation and ionization efficiency of BAs. Neutral analytes are more non-polar than ionized analytes and will show different degrees of retention on reverse phase chromatography. pK_a values of BAs ranges from 1.5 to 6 (taurine-conjugated BAs: ~1.5, glycine-conjugated BAs: ~4.5, and unconjugated BAs: ~6). Varying the mobile phase pH can therefore change the ionization state of different types of BAs and thus influencing the overall chromatographic separation and MS (or MS/MS) ionization [45, 46]. Several MS ionization techniques including electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and fast atom bombardment (FAB) along with different mass analyzers such as ion trap (IT), time-of-flight (TOF) and Fourier transform ion cyclotron resonance coupled with HPLC/UHPLC have been reported for the analysis of BAs. A linear ion trap Fourier transform mass spectrometer was used to identify 17 bile acids including their taurine, sulfate and glycine conjugates. The method showed a linear dynamic range of 100 fold and the limit of quantitation ranged between 10-50 pg [45]. Further, LC/ESI-MS instrument was used to detect as low as 10 fmol of bile acid 24-glucuronides from human urine [29]. Though LC-MS spectrum can be used for identification of BAs based on their mass, one must be careful while postulating the structure. A more definitive method for identification of BAs without the need for reference standards is by tandem mass spectrometry using their MS/MS fragmentation patterns.

Conjugated and unconjugated BAs provide distinctive fragmentation patterns in MS/MS which is used for their identification. Qiao and colleagues investigated the fragmentation pattern of 18 BAs including six free BAs and their glycine or taurine conjugates [47]. Four isomeric hydroxyl BAs (UDCA, HDCA, CDCA and DCA) showed different neutral loss patterns of CO, CO₂, CH₂O and CH₂O₂ in ESI MS/MS which allowed for their structural differentiation. Their chromatographic separation using RP C18 column was also found to be distinctive and their order of elution was UDCA, HDCA, CDCA, and DCA. For conjugated BAs, Qiao and coworkers reported that predominant [M+H]⁺ and [M-H]⁻ ions were observed in both positive and negative ion modes. [M+H-H₂O]⁺ and [M+NH₄]⁺ ions were commonly detected in positive ion mode. They reported that fragmentation of the [M-H]⁻ ions consistently produced [M-H-H₂O]⁻ and glycine (m/z 74) or taurine (m/z 124 and 80) daughter ion peaks [47]. BAs conjugated with carbohydrate moieties are usually found in urine and blood; but to a lesser extent in the bile. BAs conjugated with glucose, glucuronic acid or *N*-acetylglucosamine, show neutral losses of 162, 176 and 203 Da, respectively in ESI MS/MS [24]. Conjugation of BAs with carbohydrate occurs via the anomeric carbon of the sugar and carboxyl group (ester conjugate) or with a hydroxyl group of the bile acid. These two types of conjugation can be differentiated by the fact that neutral loss of the sugar group from ester conjugate is effortless and easily achieved [29].

Though the full scan mode in MS and MS/MS leads to identification and qualitative analysis of the BAs, selected ion monitoring (SIM), selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) modes help in optimal selectivity and accurate quantification of BAs. In SIM mode a very small mass range is scanned to detect compounds within the selected mass which provides increased specificity. SRM experiment is broadly similar to the SIM experiment except that tandem mass spectrometry is used to select a specific product ion from a specific precursor ion. In MRM, an application of the SRM approach, more than one fragment ions are selectively monitored from one or more than one precursor ions for detection and quantitative analysis. SIM, SRM and MRM approaches have been applied for BA profiling and quantitation of taurine-, glycine-, sulfated-glucuronidated-conjugated BAs [6, 48–50]. Ando and co-workers developed a SRM based method to quantify 8 BAs and their glycine and taurine conjugates with a dynamic range of 400 fold [51]. MRM approach was used to quantify 15 BAs, including free and conjugated bile acids, using LC-MS/MS in negative mode. The detection and quantitation limits ranged from 1– 6 nM and 3– 18 nM, respectively for different bile acids [52]. Another group reliably quantified 31 BAs with limit of quantification being 2.5–20 nM in different biological samples including human, mouse, and rat serum and liver extracts [53]. More recently, Sarafian and co-workers developed a method for identification of 145 BAs. They developed a 15 minutes UHPLC-MS/MS method for identification of 145 BA species. Of them, 36 non-conjugated, 12 conjugated with taurine, 9 conjugated with glycine BAs were accurately quantified by using either SIM or MRM modes. They also developed the MRM transitions for 44 BAs sulfated in position OH-C3, 15 sulfated in position OH-C6, 21 sulfated in position OH-C7 and 8 sulfated in position OH-C12(6).

1.3.5 Supercritical fluid chromatography (SFC)/SFC-MS—SFC is a complementary separation technique to both LC and GC. Unlike HPLC, SFC shows

significant reduction in analysis time and higher transparency of the eluent at low UV wavelengths [54]. In comparison to GC, SFC is not restricted by compound volatility and thermal lability [54]. SFC uses supercritical carbon dioxide (SCCO₂), a fluid state of CO₂ critical temperature and pressure, as the mobile phase. Low viscosity and high diffusivity of SCCO₂ have been shown to improve the separation of BAs. Further, SCCO₂ provides polarity flexibility which enables the use of wide range of polarity in the mobile phase leading to elution of not only hydrophobic compounds but also more hydrophilic compounds [55, 56]. Scalia and co-workers developed the first SFC method coupled with UV detector for simultaneous determination of the glycine and taurine conjugates of BAs [54]. They further showed that with the use of a thermospray mass spectrometer as a detector coupled with SFC, interfering peaks encountered in biological samples may be eliminated [57]. More recently, the first SFC/ESI-MS/MS method for the simultaneous profiling of 25 bile acids was reported [56]. They also reported unique selectivity of BAs with minimal run time of 13 mins. Moreover, the method required minimal sample preparation steps for precise quantitation of 24 BAs in rat serum [56]. These results indicate that SFC may be used as a complementary tool to GC and LC.

1.4 Nuclear Magnetic Resonance (NMR) Spectroscopy based Assays

High resolution NMR spectroscopy has emerged as a well-established analytical tool for characterizing the composition of biological samples. NMR is highly reproducible and requires minimal sample preparation avoiding the use of extraction or derivatization steps. As the most reliable and simplest sample preparation analytical method, high field NMR spectroscopy has been exploited for BA identification and quantification. Proton NMR spectroscopy has been used to study the molecular conformation of seven different bile salts in micelles [58]. A separate study demonstrated that C18 methyl proton signal (around 0.7 ppm) obtained from ¹H-NMR spectroscopy can be used for quantitative analysis of total and taurine-conjugated BAs [59]. However, accurate determination of total BAs using C18 methyl proton peak is questionable since the 0.7 ppm peak partially or completely overlaps with other bile metabolites [60]. Ijare and coworkers developed a simple method for quantification of total glycine or taurine conjugated BAs. They showed that by reducing the pH of the BA solution to 6.0 ± 0.5 the phenomenon of amide exchange can be suppressed. This led to the detection of amide proton signal in the region of 7.8–8.1 ppm. The integral of this signal was then be used for accurate quantification of total conjugated BAs [61]. They further showed that since C26 proton signal (3.08 ppm) usually do not overlap with other signals, the integral value of this peak can be used to estimate the total quantity of BAs conjugated with taurine. Total glycine conjugated BAs quantification was subsequently performed by subtracting the quantity of taurine conjugated BAs from the amide signal detected for total conjugated BAs [61]. Duarte and colleagues reported a method for hepatic whole bile analysis using 800 MHz ¹H NMR spectra. They were able to assign as many as 40 compounds including amino acids, organic acids, carbohydrates, polyols and also 3 major BAs (CA, DCA and CDCA) using two-dimensional ¹H-¹H TOCSY and ¹H-¹³C HSQC spectra [62]. Despite the fact that NMR is a more reliable method for quantitative analysis and virtually requires no sample preparation step the method has limited application in BA analysis due to relative low sensitivity compared to MS based approaches.

Conclusion

It is now well established that BAs contribute towards several pathological and physiological processes. Simple and rapid measurement for TBA content can be performed using enzymatic assays and are widely used in clinical laboratories. However, since the structural and physico-chemical diversity of BAs are key to their different physiologic roles, it is imperative to develop analytical methods for identification and quantification of individual BAs. ELISA is one of the most popular methods for estimation of certain BAs in clinical laboratories. However, antibody cross-reactivity with minor structurally different BAs limits its usage for individual BA detection and quantitation. Chromatographic techniques on the other hand offer simultaneous detection and quantitation of a wide range of structurally different BAs.

With the advancement of mass spectrometry over last decade, sensitivity of chromatographic techniques have increased many fold. Use of SIM, SRM and MRM methods in chromatographic techniques coupled with mass spectrometry have significantly improved the precision for quantitation of individual BAs. However, capturing all the BAs in a single chromatographic run still remains challenging as they are present in a wide dynamic range spanning numerous orders of magnitude. Further, complex biological matrices continue to interfere with chromatographic experiments. Pre-fractionation steps with minimal analyte loss during the extraction procedure therefore needs to be developed for optimal GC-MS and LC-MS/MS results. Despite some of the concerns of chromatography coupled with mass spectrometry techniques, their sheer power and accuracy is undeniable. However, these techniques require significant initial capital investment and also require highly trained operators which has forced clinical diagnostics laboratories to use traditional enzymatic assays for total BA analysis. A more automated clinical chemistry analyzer with MS as detector could help diminish barriers for clinical laboratories to adopt this technology.

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Figure 1:

Structures of the most abundant bile acids found in humans and the advantages (▲) and disadvantages (▼) of the various analytical platforms used to detect them

Table 1:

A comparison of currently available technologies used for BA detection, identification, and quantitation.

Technique	Sensitivity	Specificity and selectivity	Robustness	Matrix interference/sample processing steps/ease of use
Enzymatic assays	Detects BAs in micromolar range (9, 10, 63)	Detects total BA content	Both intra- and inter-day variation < 5% for enzymatic cycling method (9) and < 10% for IED method (10). Significantly underestimates total BA content because enzymatic methods using 3 α -hydroxysteroid dehydrogenase do not detect iso (3 β -hydroxy) bile acids (19)	Minimal matrix effect and minimal sample processing required. Few steps involved during the experiment and requires minimal technical expertise.
ELISA	Detects certain BAs as low as 6 pM (12) to 0.29 μ M (13)	Detects GLCA-Sul (12), CA, DCA, CDCA, UDCA, and HDCA based on the different types of antibodies used (13).	Intra- and inter-assay variation < 10% (12, 13).	Minimal matrix effect and minimal sample processing required. Requires minimal technical expertise.
TLC	Detects BAs as low as 0.7 μ g/spot (16) and 0.119-2.085 μ g/spot (17). Quantitative analysis possible for as low as 0.396-6.951 μ g/spot of BAs (17).	Separation possible for CDCA, DCA, CA, GCA, LCA, GDCA GLCA. Separation of isomeric dihydroxy conjugates including GCDCA, TCDCa and TDCA remains poor (16, 64, 65)	Visual evaluation of bands is subjective whereas densitometric method provides precise estimation of concentration (17).	Less matrix effect however sample processing steps required for BA extraction. Few steps involved during the experiment and requires minimal technical expertise.
HPLC	Limit of detection: 0.25-0.31 ng (18); 1.2-1.5 pmol (19). Limit of quantitation: 0.83-1.02 ng (18); 2.4-7.3 pmol (19). Linear dynamic range: 16 (18) to 500 fold (19)	Primary and secondary BAs including CA, CDCA, DCA, HDCA, LCA, UDCA (18, 19). Some stereoisomeric forms of secondary BAs including isoLCA and isoDCA can also be separated and detected (19)	Overestimation of total BAs in some samples (19). Both intra- and inter-day variation < 5% (18).	Matrix effect causes variation in chromatographic separation and therefore sample processing required for BA extraction. A derivatization step is also required for introduction of UV-absorbing for selective and sensitive determination of unconjugated Bas (18, 19). Requires moderate technical expertise.
GC and GC-MS	Limit of detection: 1-10 ng/ml. Limit of quantitation: 4-20 ng/ml. Linear dynamic range: 500-1000 fold (28, 66)	Primary and secondary BAs including CA, LCA, DCA, CDCA, UDCA, α -muri cholic acid (α -MCA) and β -MCA (28)	Robustness in quantitation may be obtained with the use of SIM method (28)	Matrix effect causes variation in chromatographic separation and therefore sample processing required for BA extraction. Derivatization steps are also required to improve the thermal stability and volatility of the BAs (15, 23, 66). Requires moderate to extensive technical expertise.
LC-MS and LC-MS/MS	Limit of detection: 10 fmol (29); 2.5-75 pg (36); 1- 6 nM (52); 0.6-10 nM (53); 0.05-7.5 nM (6, 67). Limit of quantitation: 5-7.5 pg (36); 10-50 pg (45); 3- 18 nM (52); 2.5-20 nM (53); 7.8-156.3 nM (37); 0.25 nM (6, 67). Linear dynamic range: 64 (37) and 100 (45) to 10000 fold (6, 51, 52, 67)	LC-MS: 17 bile acids including their taurine, sulfate and glycine conjugates (45) and 24-glucuronides (29) detected using LC-MS. However, reference standards and retention time must be used for accurate identification. LC-MS/MS: 24 BAs including 8 unconjugated and their glycine and taurine conjugates quantified using SRM approach (51). 15 BAs, including free and conjugated bile acids quantified (52)	Intra-day variation: < 3.8% (52); < 8.6% (53); < 15% (37); < 22.8% (36). Inter-day variation: < 6.1% (52); 16.0% (53); < 15% (37); < 29.5% (36). Though the technique is prone to intra- and inter-day variations due to change in column conditions, significant robustness can be obtained with following of good lab practices	Matrix effect causes variation in chromatographic separation as well as suppression of signals in MS and therefore extensive sample processing required for BA extraction. Requires extensive technical expertise.

Technique	Sensitivity	Specificity and selectivity	Robustness	Matrix interference/sample processing steps/ease of use
SFC-MS	Limit of detection: 0.37–1.46 pg/μl Limit of quantitation: 1.11–4.43 pg/μl Linear dynamic range: upto 5000 fold (56).	31 BAs accurately quantified using MRM approach (53). 36 non-conjugated, 12 conjugated with taurine, 9 conjugated with glycine BAs could be accurately quantified by using either SIM or MRM modes (6)	Variation ranged from 1.0–14.7% (56).	Minimal sample preparation for biological samples. Requires extensive technical expertise.
NMR	The technique is not very sensitive and requires large volume of sample	Accurate quantification possible for total taurine and glycine conjugated BAs. Unable to distinguish individual BAs (61)	Excellent for total bile and total taurine and glycine conjugated BAs quantitation with minimal variation (61, 68)	Minimal matrix effect and minimal sample preparation steps Requires extensive technical expertise