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Determination of bile acids in pig liver, pig kidney and bovine liver by gas chromatography-chemical ionization tandem mass spectrometry with total ion chromatograms and extraction ion chromatograms

Suh-Jen Jane Tsai*, Yao-Shen Zhong, Jen-Feng Weng, Hsiu-Hua Huang, Pei-Yin Hsieh

Department of Applied Chemistry, Providence University, 200 Chungchi Road, Sha-Lu, Taichung Hsien 43301, Taiwan

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ABSTRACT

An effective method has been developed for quantitative determination of six bile acids including lithocholic acid (LCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), hydodeoxycholic acid (HDCA), cholic acid (CA) and ursodeoxycholic acid (UDCA) in biological tissues including pig liver, pig kidney and bovine liver by gas chromatography-chemical ionization/tandem mass spectrometry (GC-CI/MS/MS). Camphor-10-sulphonic acid (CSA) was proposed as effective catalyst for bile acid derivatization. Reactions were accelerated ultrasonically. The effects of different catalysts and reaction times on derivatization efficiency were evaluated and optimized. Bile acids were determined as methyl ester-trimethylsilyl ether and methyl ester-acetate derivatives. The efficiency of trimethylsilylation and acetylation was evaluated. Trimethylsilylation was done with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) as the trimethylsilyl donating reagent in a ultrasonic bath for 20 min. Acetylation was done in pyridine with acetic anhydride at 40–45 °C for 4 h. The former reaction was faster than the latter. Thus, trimethylsilylation was employed for the quantitative analysis. Negligible interferences from sterols in biological matrices were observed when the biological samples were treated with solid phase extraction before GC-CI/MS/MS. The linearity, reproducibility, detection limit and recovery were evaluated under the optimized conditions. Satisfactory results were obtained when bile acid derivatives of LCA, CDCA, HDCA, and UDCA were determined with total ion chromatograms (TIC) while DCA and CA were determined with extracted ion chromatograms (EIC), respectively. The detection limits ($S/N = 3$) for six bile acids in biological tissues were ranging from 0.40 to 1.6 ng/mL and the recoveries indicated that the proposed method was feasible for the determination of trace bile acids in the biological samples studied. The experimental results for the animal tissues purchased from five different markets were compared. Interestingly, all of the six bile acids were present in pig liver while only the dihydroxy bile acids, DCA, CDCA and HDCA were found in pig kidney. In addition to DCA and CDCA, trihydroxy bile acid, CA, are the major bile acids in bovine liver.

1. Introduction

Due to the significance of bile acids, the development of reliable methodology for the analysis of such metabolism products has gained a lot of attention for quite many years [1,2]. Different analytical methodologies have been reported for the determination of conjugated and unconjugated bile acids in biological material. For examples, liquid chromatography/electrospray ionization tandem MS has been used for high sensitive analysis of bile acids in rat serum [3,4]. Tandem mass spectrometry (MS/MS) reduced the background due to complex matrix by excluding all ions except the parent ion which further dissociated to produce a unique product ion mass spectrum. MS/MS has been used to determine biologically and clinically important compounds. LC–FT–MS [5] and LC–MS/MS [6] have proposed for the quantitative profiling of bile acids and their conjugates in biological samples, respectively. The improvement in analytical methods increases the understanding of the physiological and pathophysiological roles of bile acids [7,8].

Bile acids are important end products of cholesterol metabolism in the livers by multiple complex path ways. Bile acids undergo enterohepatic circulation involving the small intestine, liver and kidney. Daily average amount of 400–800 mg bile salts is released from this enterohepatic circulation and is effectively involved in microbial bio-transforming reactions in the large bowel [9]. Bile acids are not only the major metabolic products of cholesterol, but also amphipathic molecules with multiple physiological functions. More than 95% of the bile acids passing through the ileum are reabsorbed by an active re-absorptive mechanism in the terminal ileum,

* Corresponding author. Tel.: +886 4 2632 3692; fax: +886 4 2632 7554.
E-mail address: sjtsai@pu.edu.tw (S.-J.J. Tsai).

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leaving less than 5% of bile salt pool to enter the colon [10]. The total amount of bile acids is maintained relatively constant by the daily synthesis of newly formed bile acids from cholesterol in response to losses through urine and feces [11]. This represents a major route for elimination of excess cholesterol. However, excessive bile acids in the body appear to be toxic and the relevant mechanisms are still under study [12]. The determination of bile acids in tissues is important in the diagnosis of hepatobiliary diseases. Bile acids also have been applied to therapeutic treatments. Gallstone disease remains a significant health problem worldwide, particularly in the female adults. There are a relatively high percentage, about 10–20%, of adults who develop gallstones, mostly cholesterol-rich stones in the United States and European countries [13]. Bile acids are widely used for the dissolution of cholesterol-rich gallstones. For example, chenodeoxycholic acid and ursodeoxycholic acid have been used as an alternative treatment for patients with gallstone [14,15]. However, a review article indicates that bile acids cause DNA damage and the mechanism seems to be indirect [16]. Abnormalities in cholesterol biosynthesis or metabolism are often reflected in the concentration and the proportion of different bile acids in various tissues. Targeting bile-acid signaling is also important for studying metabolic diseases [17].

Bile acids are saturated C24 steroid carboxylic acids with mono or polyhydroxy groups. They are different from cholesterol which is C27 unsaturated, monohydroxylated, non-polar compounds. The primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized in the liver from cholesterol [18] and are conjugated at the carboxylic acid carbon with either glycine or taurine. Thus, their polarity and solubility in water are increased. A portion of the primary bile acids is subsequently converted by microbial flora into secondary bile acids. The secondary bile acids thus produced during the enterohepatic cycling including deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) through the action of intestinal organisms [19]. Hyodeoxycholic acid (HDCA) is also a secondary bile acid and one of the metabolic byproducts of intestinal bacterial. However, HDCA is not marketed for any medical condition, unlike CDCA and UDCA, which are approved drugs for the treatment of gallstones [20]. UDCA can also be employed to reduce hepatic damage and cholestasis induced by high concentrations of other bile acids [21]. LCA was the most potent inhibitor in colon cancer [22]. An interesting mechanism for the reduction of blood cholesterol levels which involved the binding of bile acids to dietary fibers in the small intestine was proposed [23].

Gas chromatographic separation with mass spectrometric detection (GC–MS) is an alternative with high efficiency, sensitivity and specificity for bile acids determination when LC–MS is not available. GC–MS was employed to study the inborn errors of bile acid metabolism [24]. Besides, different types of MS, such as FAB–MS [25], CI–MS [26], EI–MS, and electrospray–MS [27] have been employed routinely for the determination of bile acids. Bile acids were often analyzed by GC–MS as methyl ester-trimethylsilyl ether derivatives because of their good resolution and stability on different capillary columns. Besides, their mass spectra are often easier to interpret. Our goal was to develop a highly sensitive method for the detection and quantification of six bile acids including LCA, DCA, UDCA, CA, CDCA and HDCA in pig liver, pig kidney, and bovine liver using a gas chromatography-chemical ionization tandem mass spectrometry (GC–MS/MS). GC–MS/MS separation and identification of bile acids as methyl ester-trimethylsilyl ether derivatives were compared with methyl ester-acetate derivatives. Bile acids were determined as the methyl ester-trimethylsilyl ether derivatives with camphor-10-sulphonic acid as the esterification catalyst. The effects of catalysts, reaction times on derivatization efficiency were evaluated and optimized. Interferences from sterols in biological samples were eliminated by CI–MS/MS with solid-phase extraction. Analytical characteristics such as the dynamic linear range of the calibration curves, reproducibility, and recovery were evaluated under the optimized experimental condition. The contents of bile acid in pork liver, pork kidney and bovine liver were compared.

2. Experimental

2.1. Chemicals

Chemicals of cholic acid (CA, CAS#81-25-4), 3α,7α,12α-hydroxy-5β-cholan-24-oic acid; deoxycholic acid (DCA, CAS#83-44-3), ursodeoxycholic acid (UDCA, CAS#128-13-2), 3α,7β-hydroxy-5β-cholan-24-oic acid; lithocholic acid (LCA, CAS#434-13-9), 3α-hydroxy-5β-cholan-24-oic acid and hyodeoxycholic acid (HDCA, CAS#83-49-8), 3α,6α-hydroxy-5β-cholan-24-oic acid, and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, CAS#25561-30-2) were purchased from Sigma (St. Louis, MO, USA). Hydrochloric acid (CAS#7647-01-0), n-hexane (CAS#110-54-3), dichloromethane (CAS#75-09-02), methanol (CAS#67-56-1), acetic anhydride (CAS#108-24-7) and pyridine (CAS#110-86-1) were from Merck (Darmstadt, Germany). Chenodeoxycholic acid (CDCA, CAS#474-25-9), 3α,7α-hydroxy-5β-cholan-24-oic acid was obtained from Aldrich (Milwaukee, WI, USA). Camphor-10-sulphonic acid (CSA, CAS#5872-08-2) was from Fluka (Buchs, Switzerland). Ultra pure water was obtained from Barnstead Nano Pure II (Boston, USA). Bakerbond SPE™ Octadecyl (C18) cartridges (1000 mg/6 mL columns) were obtained from J.T. Baker (Philipsburg, NJ, USA).

2.2. Extraction of bile acids from tissues

The tissue samples were purchased from the local markets. They were kept at −20 °C for 24 h to remove the moisture. The freeze-dried sample was ground to fine powder and accurately weighed (0.4–0.5 g) and then digested with 20 mL of 0.2 M NaOH at 80 °C for 20 min. The digested sample was extracted twice with 6.0 mL of n-hexane, centrifuged (1600 rpm for 10 min) to remove the lipid components. The collected aqueous layer was adjusted to pH 7.0 using concentrated hydrochloric acid to keep bile acids in molecular state for solid-phase extraction. After removing the impurities by high speed centrifuge (1.0×10^5 rpm for 10 min) the sample was loaded to C18 Bakerbond cartridge which had been preconditioned with 12 mL of methanol and 12 mL of pure water. Then, the cartridge was washed with 6.0 mL pure water to remove the water soluble components. Afterwards, bile acids and their conjugates were recovered from the cartridge by elution with 6.0 mL of anhydrous methanol.

2.3. Derivatization

The methyl ester-trimethylsilyl ether of bile acid was prepared as the followings. The dried extract obtained from tissue sample was esterified with 3.0 mL of anhydrous methanol and 60 mg of CSA (catalyst). The content was sonicated using a Ney Ultrasonik (Burlington, NJ, USA) for 30 min at room temperature to complete the esterification reaction and to be dried on a rotary evaporator. The resultant residue was resuspended in 20 mL of dichloromethane, 5.0 mL of water added and extracted for 10 min to remove excess CSA. After phase separation, the organic layer was evaporated to dryness. The esterified bile acids were converted into trimethylsilyl ethers using a mixture of pyridine (0.3 mL) and N,O-bis(trimethylsilyl)trifluoroacetamide, BSTFA (0.3 mL) containing 1% trimethylchlorosilane (TMCS) in an ultrasonic bath for 20 min.

In order to prepare methyl ester-acetyl ether, the ester bile acid was treated with 0.3 mL of acetic anhydride in 0.3 mL of pyridine at 110 °C for 30 min. Stock solutions were prepared by dissolving them in methanol and followed by the derivatization. Working solutions were prepared by series dilutions of stock solutions in n-hexane.

2.4. Instrumentation and component identification

GC–MS/MS analysis of all samples were performed on a Varian CP 3800 gas chromatograph (Walnut creek, California, USA) equipped with a split/splitless injector, Varian 8410 autosampler, and a Varian Saturn 2000 mass spectrometric detector equipped with an electron impact (EI)/chemical ionization (CI) source. Chromatographic separation was carried out using a non-polar fused silica capillary column, Equity – 1 (30 m × 0.25 mm I.D., × 0.25 μm film thickness) from Supelco (Bellefonte, PA, USA).

For optimal separation, different column temperature programs were selected for trimethylsilyl ether and acetate derivatives. For trimethylsilyl ether derivatives, the temperature was initially held at 100 °C for 5 min, ramped to 290 °C at 35 °C/min and held for 15 min. For acetate derivatives, the temperature was held at150 °C for 1 min. ramped to 290 °C at 45 °C/min and held for 16 min. Helium (purity 99.999%) was used as carrier gas at a constant flow rate of 1 mL/min. Electron impact (EI) and chemical ionization (CI) were performed at 70 eV and 15 eV, respectively with an ion source temperature at 180 °C. The sample (1 μL) was injected in split mode (1:20). The injector temperature was adjusted to 260 °C, while the transfer line temperature was set at 300 °C. In CI mode, acetonitrile was used as a reagent gas when bile acids were analyzed as methyl ester-trimethylsilyl ether derivatives whereas methanol was used when they were injected as methyl acetate derivatives. Instrument control and data acquisition were carried out using Saturn GC–MS Workstation Varian version 6.3. Component identification was based on fragmentation and comparison of the retention times with those of standards.

2.5. Quantification

Six bile acids in various biological tissues were determined by GC-CI/MS/MS with total ion and extracted ion chromatograms (TIC and EIC). TIC for the standard solutions of six bile acids were obtained first. Those with some degrees of overlapping were further analyzed with EIC. In EIC, a single analyte was monitored throughout the entire run. The calibration curves were established for those obtained with TIC and EIC, separately for quantitative analysis.

3. Results and discussion

Derivatization was required for the analysis of bile acids with GC–MS due to the relative low volatility of bile acids. In this work, the derivatization for CA, DCA, UDCA, LCA, HDCA, and CDCA which involved the methylation of carboxyl group and the trimethylsilyl ether derivatization of the hydroxyl group were performed with improved derivatization process. The derivatives were then analyzed with gas chromatography-chemical ionization tandem mass spectrometry. Table 1 and Fig. 1 give the structures for the six bile

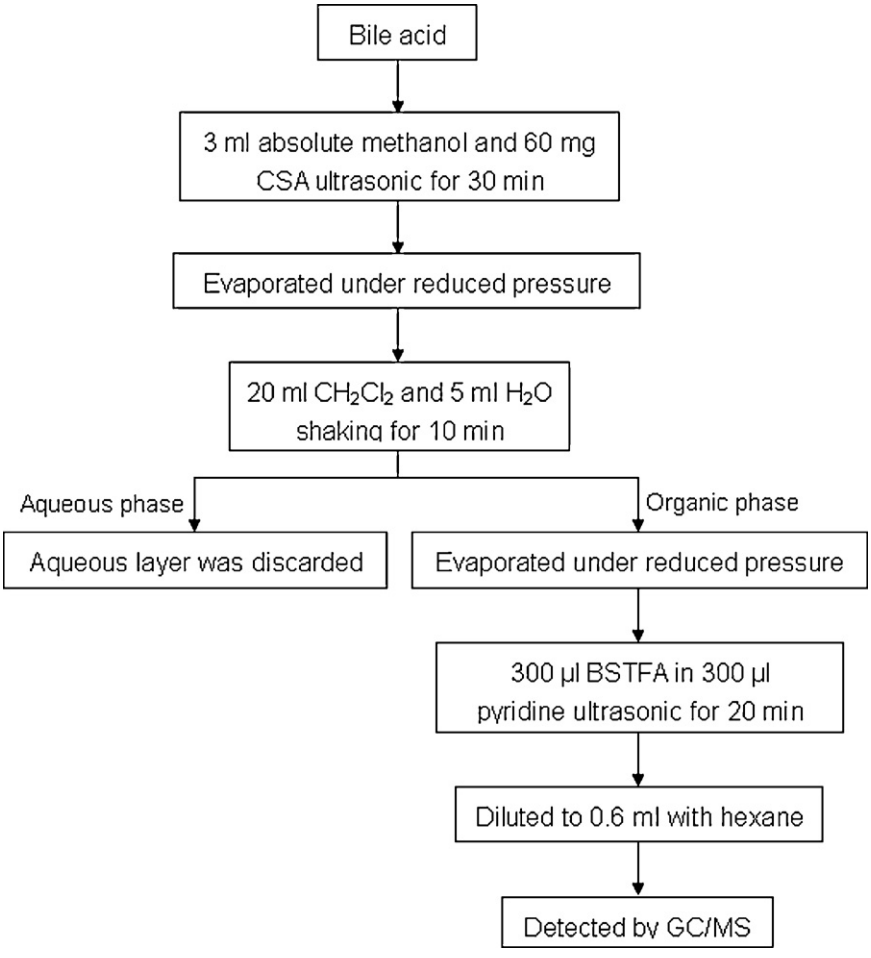


Fig. 1. The flow-chart for the analysis of biological samples.

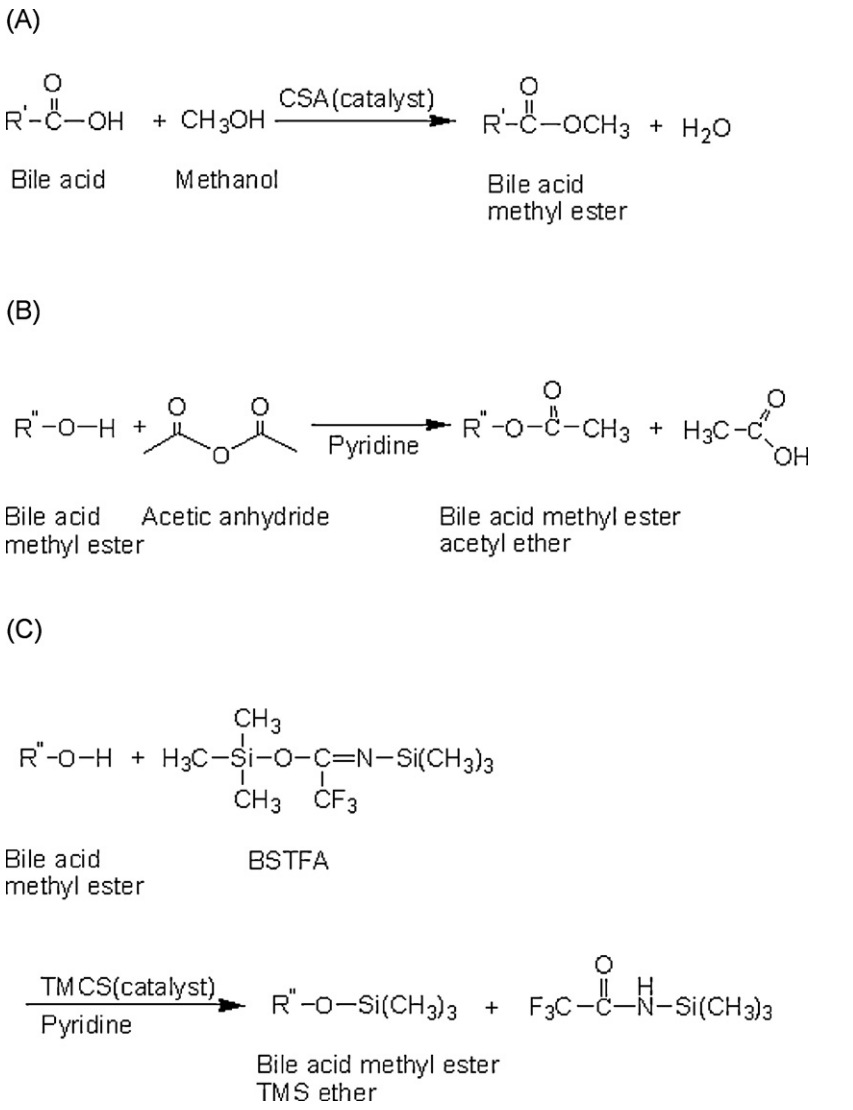
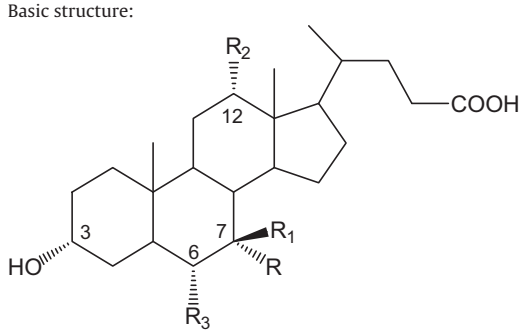


Fig. 2. The derivative reactions involved for bile acids: (A) the esterification of carboxyl group; (B) the acetyl ether derivatization of hydroxyl groups; (C) the trimethylsilyl (TMS) ether derivatization of hydroxyl groups. R′ and R″ are the moieties of bile acids.

Table 1
Name and structure of six bile acids.

Bile acid	Abbreviation	–OH position
Lithocholic acid	LCA	None
Deoxycholic acid	DCA	R_2
Chenodeoxycholic acid	CDCA	R
Hydodeoxycholic acid	HDCA	R_3
Cholic acid	CA	R, R_2
Ursodeoxycholic acid	UDCA	R_1
Basic structure:		



acids and the flow chart for the biological sample analysis, respectively. Fig. 2 gives the reactions involved.

3.1. Optimization of the derivatization condition for bile acids

Bile acids were often analyzed as methyl ester-trimethylsilyl (TMS) ether with GC–MS, due to their relatively low volatility. The derivatization of bile acids often involved two major reactions, the esterification of the carboxyl group and ether formation of the hydroxyl group as mentioned previously. Methyl, ethyl, n-propyl, n-butyl and isobutyl esters all had been used in the derivatization of carboxylic group for the GC analysis of bile acids [28,29]. Marschall et al. [30] compared three different methods for derivatization: (i) diazomethane in diethyl ether, (ii) trimethylsilyldiazomethane in toluene and (iii) concentrated hydrochloric acid in dimethoxypropane for methylation of bile acids in GC–MS analysis. They found that concentrated hydrochloric acid was suitable for the derivatization of unconjugated bile acids. Diazomethane in ether reacted with bile acids instantly and methyl esters were formed, however, its application was limited by its carcinogenic effect. Besides, it was hazardous when preparing large amounts of bile acid methyl esters. Iida et al. [31] analyzed acyl glycosides of bile acids as trifluoroacetyl (TFA) derivatives with N-methyl-bis-

trifluoroacetamide by GC–MS. They concluded that TFA derivatives were unsuitable for using conventional middle-bore capillary column at the column temperature above 280 °C.

In order to optimize derivatization condition, several parameters which affected the derivatization of analytes were studied. Camphor-10-sulphonic acid (CSA) had been used as an effective catalyst for preparing crystalline methyl ester [32] and polyaniline-camphorsulphonic acid nanotube film [33]. In this work, the acid-catalyzed esterification was performed as shown in Fig. 2(A). CSA was used as a new catalyst for bile acids derivatization at room temperature in an ultrasonic bath [34]. The mixture of bile acids was treated with 3.0 mL of anhydrous methanol and three different catalysts either concentrated hydrochloric acid (1.0 mL), 3N methanolic hydrochloric acid (1.0 mL) or CSA (70 mg). All six bile acids studied in this work were converted to methyl esters of respective bile acids and were denoted as, LCA-M, DCA-M, CDCA-M, HDCA-M, CA-M, and UDCA-M, respectively. The effects of CSA, methanolic hydrochloric acid, and conc. HCl were compared as shown in Fig. 3. CSA and 3N methanolic hydrochloric acid gave comparable results while lowest yield of methyl ester of bile acids was found when conc. HCl was used as the catalyst. Water in concentrated HCl caused reversed esterification. One of the disadvantages for using methanolic hydrochloric acid was its hygroscopicity; in other words, it readily absorbed water from atmosphere. Therefore, dehydration process was required before use. Based on the above observation, CSA was chosen as the catalyst for the followed studies as it was more effective and more easily to handle. Both the reaction time and the amount of CSA required to complete the esterification reaction were optimized. There were no further increasing in the peak area after 30 min and 60 mg of CAS catalyst was enough for the esterification of the carboxyl group. However,

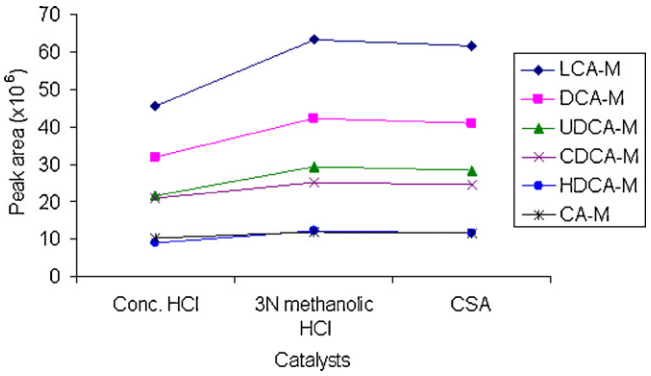


Fig. 3. Comparison of three different catalysts on esterification efficiency for bile acids. The amount for concentrated hydrochloric acid, 3N methanolic hydrochloric acid, and camphor-10-sulphonic acid (CSA) were 1.0 mL, 1.0 mL and 70 mg, respectively.

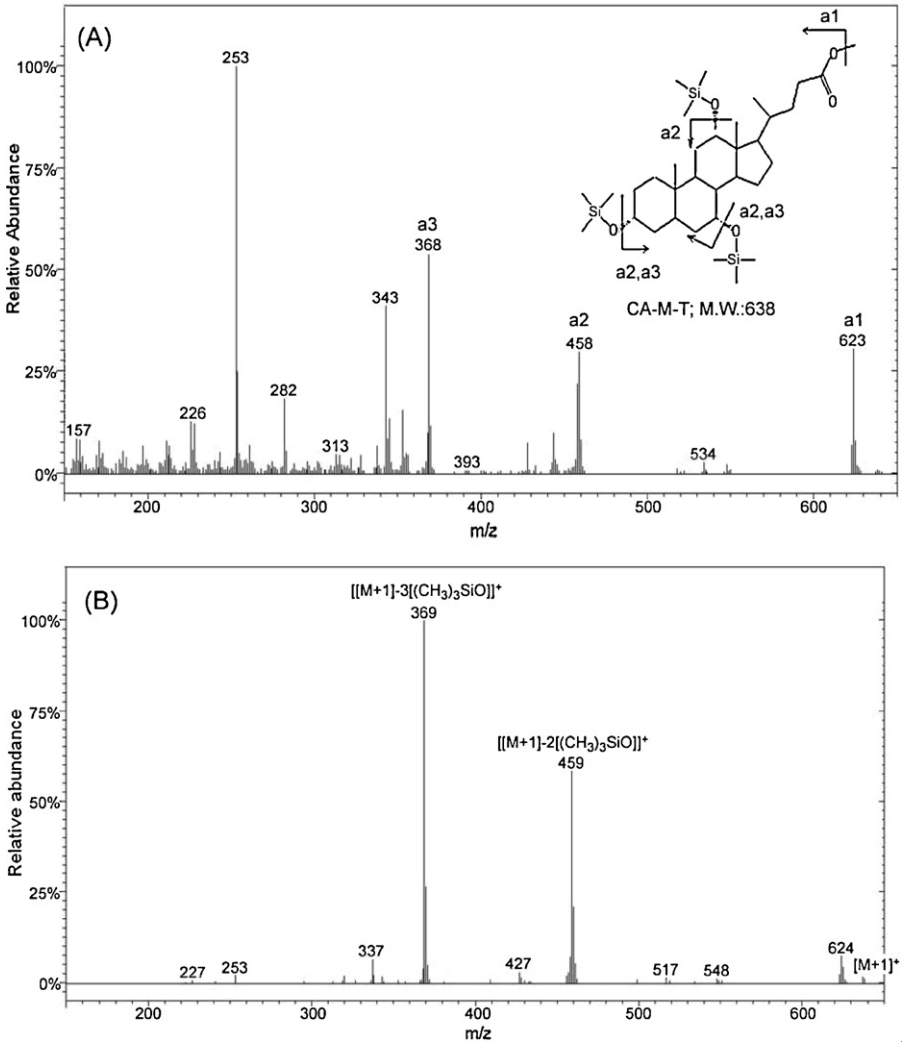


Fig. 4. Mass spectra of methyl ester trimethylsilyl ether derivative of cholic acid in electron impact mode (upper spectrum) and in chemical ionization mode (lower spectrum).

Table 2
GC-CI/MS data for methyl ester trimethylsilyl ether derivatives of bile acids.

Bile acid	Peak no.	Retention time (min)	Base peak[a] (*m*/*z*) [[M+1]–*n*[$(CH_3)_3SiOH$]]$^+$	Fragment ions (*m*/*z*) (relative intensity, %)
LCA	1	15.43	373	463(2)
DCA	2	16.09	371	536(5), 551(1)
CDCA	3	16.35	371	551(1)
HDCA	4	16.45	371	461(5), 551(2)
CA	5	16.55	369	459(59), 624(5), 639(2)
UDCA	6	16.74	371	461(6), 536(4), 551(1)

[a] *N* was different for different bile acid. *N* was 1, 3, and 2 for LCA, CA and others, respectively.

GC EI/MS chromatograms of methyl esters of those bile acids studied showed that UDCA-M and CDCA-M were seriously overlapped. Besides, there was serious tailing effect for CA-M. Therefore, the hydroxyl groups of bile acids were converted into ethers to lower the polarity and to increase the volatility. Consequently, better resolution was obtained.

The efficiency for ether derivatization was compared in this work. The ether derivatives of bile acid methyl esters were often obtained by reacting with acetic anhydride [34,35] or through trimethylsilylation (TMS) [36] as shown in Fig. 2(B) and (C), respectively. The derivatization for methyl ester acetyl ether was done in pyridine with acetic anhydride at 40–45 °C for 4 h. Trimethylsilylation was done with N,O-bis(trimethylsilyl)trifluoroacetamide, BSTFA as the trimethylsilyl donating reagent in a supersonic bath for 20 min. The latter reaction was much faster than the former. Besides, TMS derivative was less polar and more thermally stable. The conversion of hydroxyl groups into alkyl silyl ether derivatives enhanced peak shape, resolution and sensitivity. Based on those observations, bile acid esters studied in this work were further treated with BSTFA to converted to methyl ester-trimethylsilyl ethers and the corresponding derivatives were denoted as LCA-M-T, DCA-M-T, CDCA-M-T, HDCA-M-T, CA-M-T and UDCA-M-T, respectively.

3.2. Identification of bile acid derivatives in GC–MS: CI vs. EI

The optimum experimental condition established in this work allowed the six bile acids been studied by GC–MS as methyl ester TMS ethers without interfering products. Fig. 4 shows the typical mass spectra of CA-M-T derivative of the standard CA solution with EI and CI modes. An amount of 10 μg/mL of bile acid standard solution prepared as methyl ester- TMS ether derivatives was analyzed with GC–MS from *m*/*z* 150 to 650 in both EI and CI modes to confirm the structures of bile acids. The base peaks at *m*/*z* 253 and 369 were observed in EI and CI modes, respectively. The GC-CI/MS data for bile acid derivatives are summarized in Table 2, showing retention times and characteristic fragment ions accompanied by their relative abundance. The retention of bile acid derivative in the capillary tube was highly dependent on its molecular weight and structure, the higher the molecular weight, the longer the retention time. Besides, symmetric molecules retained in the column longer compared to those with less symmetric structures. The retention times of all bile acid derivatives were highly reproducible under identical chromatographic conditions. Mass spectra of the derivatives showed the characteristic ions arising from the loss of trimethylsilanol ion, $[(CH_3)_3SiOH]^+$, and forming base peaks at *m*/*z* 372, 370, 370, 370, 368 and 370 for LCA-M-T, DCA-M-T, CDCA-M-T, HDCA-M-T, CA-M-T and UDCA-M-T, respectively. The fragment

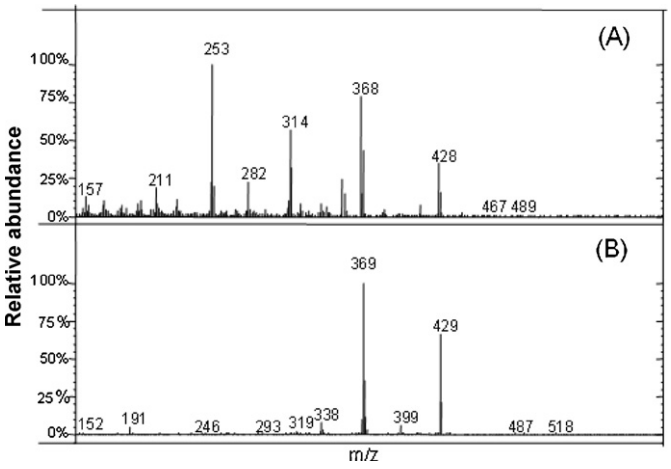


Fig. 5. Mass spectra of methyl ester acetate derivative of cholic acid in electron impact mode (upper spectrum) and in chemical ionization mode (lower spectrum).

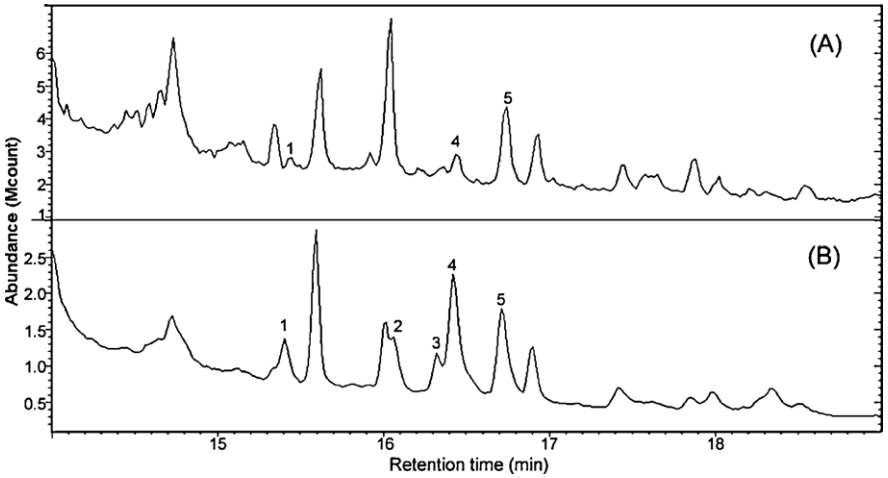


Fig. 6. Chromatogram obtained by GC-EI–MS for pig liver. The sample solution was injected as methyl ester trimethylsilyl ether derivatives: (A) without solid-phase extraction and (B) with solid-phase extraction. Peaks were identified as: (1) LCA-M-T; (2) DCA-M-T; (3) CDCA-M-T; (4) HDCA-M-T; (5) UDCA-M-T.

ion found in EI mass spectra was the dominate fragment ion (base peak) with 1 Da positive shift in CI mode. For example, the base peak for CA-M-T was present at m/z 369 and m/z 368 for EI and CI modes, respectively. The characteristic fragment ions at m/z 623 (EI) and m/z 624 (CI) indicated the elimination of methyl group from the original CA-M-T molecule. Further loss of trimethylsilanol units resulted in the fragment ions at m/z 458 $[M-2[(CH_3)_3SiOH]]^+$, and m/z 459 $[(M+H)-2[(CH_3)_3SiOH]]^+$ for EI and CI, respectively. The peak at m/z 253 was an ABCD-ring fragment, formed by the loss of the side chain and three hydroxyl groups along with one methyl group. The fragmentation pattern was further confirmed by means of spectrum matching in NIST MS library.

The mass spectra for methyl ester TMS ether and methyl ester acetate cholic acid (CA) derivatives were compared and shown in Fig. 5. The base peaks at m/z 253 and 369 were observed in EI and CI modes, respectively. The fragmentation ions at m/z 428 and 429 were from $[M-2[CH_3COOH]]^+$ and $[(M+H)-2[CH_3COOH]]^+$, respectively. Further elimination of acetate group which confirmed the presence of three hydroxyl groups, produced ions at m/z 368 and 369 due to $[M-3[CH_3COOH]]^+$ and $[(M+H)-3[CH_3COOH]]^+$, respectively. There was no molecular ion peak at m/z 253 (carbon–carbon skeleton fragmentation) in CI mode because CI was a relative mild ionization process compared to EI. Other bile acid derivatives gave similar MS fragmentation pattern (data not shown).

3.3. Determination of bile acids in biological samples

Solid-phase extraction (SPE) had often been employed in the sample preparation for increasing sensitivity and removing potential interfering matrix components, such as lipid content in biological samples [37,38]. Fig. 6 gives a comparison of the chromatograms of methyl ester-TMS ether of bile acids in pig liver without and with solid-phase extraction. More peaks from bile acids, such as peaks 2 and 3 for DCA-M-T and CDCA-M-T, respectively, were found in the chromatograms obtained with solid-phase extraction. Based on this observation, SPE was employed for the analysis of biological tissues including pig liver, pig kidney, and bovine liver from local market. Each bile acid was identified by comparing the GC retention time and the corresponding NIST mass spectrum to the known compounds. The analytical characteristics obtained by GC-EI/MS, GC-CI/MS, and GC-CI/MS/MS were studied and the analysis of bile acids in pig liver was discussed in the followings as a representative example. Fig. 7 shows the gas chromatograms for methyl ester TMS ether derivatives of bile acids in pig liver obtained with (A) GC-EI/MS, (B) GC-CI/MS and (C) GC-CI/MS/MS, respectively. As shown in Fig. 7(A), relatively poor base line with severe fluctuation was found in EI mode. The base line was improved when CI mode, Fig. 7(B), was used for the same pig liver sample solution. MS/MS offered best selectivity, besides, it also gave lower background and better detection limit for those bile acids studied. GC-CI/MS/MS spectra were shown in Fig. 7(C). The methyl ester TMS ethers derivatives of all of the six bile acids studied including LCA-M-T, DCA-M-T, CDCA-M-T, HDCA-M-T, CA-M-T and UDCA-M-T were identified and denoted as 1–6, respectively. The base peaks for LCA-M-T, DCA-M-T, CDCA-M-T, HDCA-M-T, CA-M-T, and UDCA-M-T obtained by GC-CI/MS were m/z 373,371, 371, 371, 369, and 371, respectively. In addition, cholesterol which was the major sterol component was still present in the pig liver samples even with solid phase extraction and some derivatives of steroid compounds were found.

3.4. Quantification with total ion and extracted ion chromatograms

Bile acid concentrations in the biological tissues were determined by GC-CI/MS/MS with total ion and extracted ion

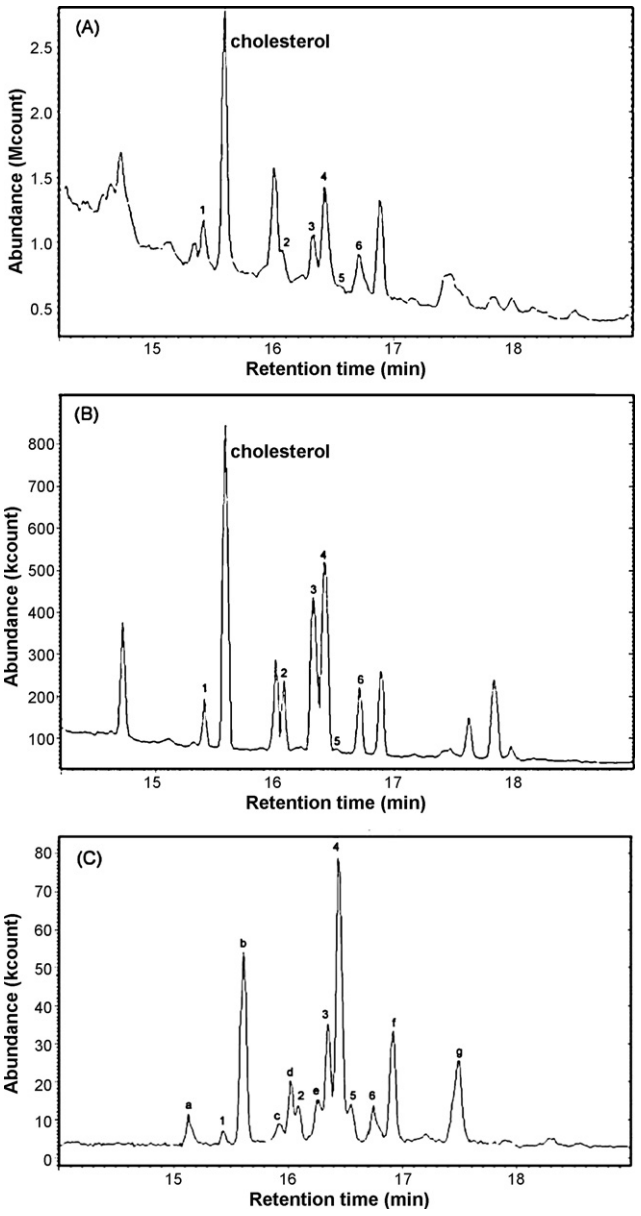


Fig. 7. Chromatograms of pig liver injected as methyl ester-trimethylsilyl ether derivatives: (A) GC-EI/MS; (B) GC-CI/MS; (C) GC-CI/MS/MS. Peaks identified: (1) LCA-M-T; (2) DCA-M-T; (3) CDCA-M-T; (4) HDCA-M-T; (5) CA-M-T; (6) UDCA-M-T. Peaks (a)–(g) may be the derivatives of steroid compounds.

chromatograms (TIC and EIC). TIC and EIC obtained at m/z 371 and m/z 369 for the standard solution of six bile acids studied were shown in Fig. 8. LCA-M-T(1), DCA-M-T(2), and UDCA-M-T(6) were well resolved from others. There were some degrees of overlapping for bile acids, CDCA-M-T(3), HDCA-M-T(4), and CA-M-T(5) as shown in Fig. 8(A). LCA-M-T(1) was the only one bile acid which gave EIC at m/z 373 as shown in Fig. 8(B). CDCA-M-T(3) and HDCA-M-T(4) could not be resolved because they both had m/z 371 as the dominate peak (base peak) as shown in Fig. 8(C). CA-M-T(5) was well resolved from CDCA-M-T(3) and HDCA-M-T(4) in EIC obtained with m/z 369 as shown in Fig. 8(D). LCA-M-T(1), DCA-M-T(2), CDCA-M-T(3), HDCA-M-T(4), CA-M-T(5) and UDCA-M-T(6) in pig liver were identified in TIC, however, with some degree of overlapping as shown in Fig. 9(A), DCA-M-T(2), CDCA-M-T(3), HDCA-M-T(4), and UDCA-M-T(6) were shown in EIC at m/z 371 while CA-M-T(5) was identified at m/z 369 as shown in Fig. 9(B) and (C), respectively.

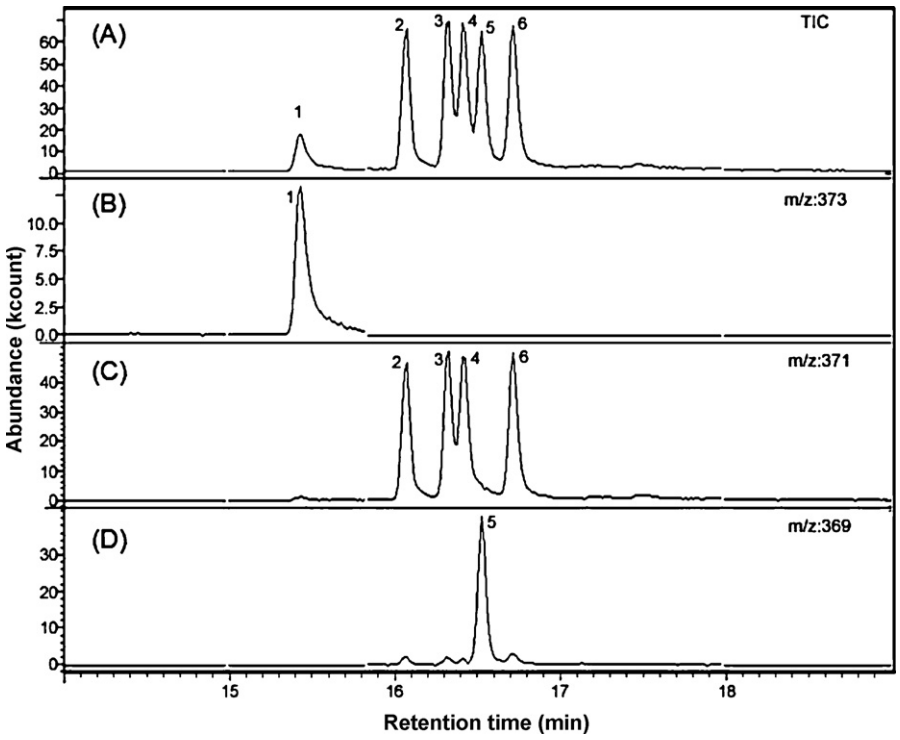


Fig. 8. Total ion and extracted ion chromatograms of bile acid solution injected as methyl ester-trimethylsilyl ether derivatives: (A) TIC; (B) EIC at *m*/*z* 373; (C) EIC at *m*/*z* 371; (D) EIC at 369. Peaks were identified as in Fig. 7.

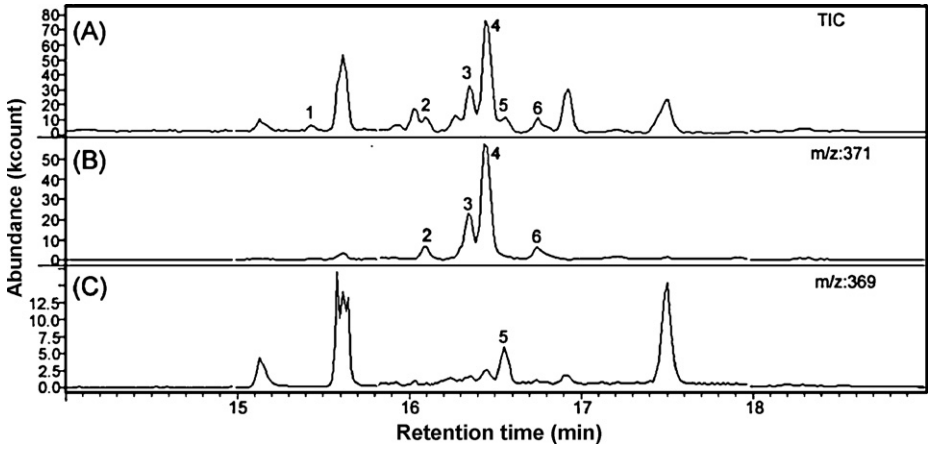


Fig. 9. Total ion and extracted ion chromatograms of bile acids in pig liver injected as methyl ester-trimethylsilyl ether derivatives: (A) TIC; (B) EIC *m*/*z* 371; (C) EIC *m*/*z* 369. Peaks identified – as in Fig. 7.

Table 3
Calibration curves ($y = ax + b$) and detection limits for bile acids determined by GC-CI–MS/MS ($n = 5$).

Bile acid derivatives	Linear range (μg/mL)	$y = ax + b$ ($\times 10^4$)	r^2	Detection limit (ng/mL)
LCA-M-T(TIC)	0.008–10.0	*a*: 17.0566 *b*: +0.0818	0.9999	0.40
DCA-M-T(TIC)	0.008–8.0	*a*: 31.9028 *b*: +0.6831	0.9993	0.40
DCA-M-T(EIC)	0.008–8.0	*a*: 19.0093 *b*: +0.5449	0.9998	0.40
CDCA-M-T(TIC)	0.04–8.0	*a*: 29.8412 *b*: +0.1768	0.9984	0.60
HDCA-M-T(TIC)	0.04–8.0	*a*: 24.3935 *b*: +0.1527	0.9989	1.60
CA-M-T(TIC)	0.04–8.0	*a*: 27.0774 *b*: +0.8925	0.9979	0.80
CA-M-T(EIC)	0.04–8.0	*a*: 13.5666 *b*: +0.8599	0.9962	0.80
UDCA-M-T(TIC)	0.04–6.0	*a*: 26.1137 *b*: +0.3199	0.9987	0.80

Table 4
Bile acid concentration for biological samples purchased from different markets ($n = 5$).

Biological sample	Content (μg/g)					
	LCA-M-T	DCA-M-T	CDCA-M-T	HDCA-M-T	CA-M-T	UDCA-M-T
Pig liver 1	0.154 ± 0.011	0.156 ± 0.012	0.459 ± 0.015	1.23 ± 0.08	0.223 ± 0.022	0.269 ± 0.014
Pig liver 2	0.755 ± 0.022	1.35 ± 0.05	1.39 ± 0.03	0.886 ± 0.013	1.01 ± 0.02	1.03 ± 0.04
Pig liver 3	N.D.	0.259 ± 0.012	0.421 ± 0.027	1.07 ± 0.06	0.026 ± 0.003	0.125 ± 0.012
Pig liver 4	0.028 ± 0.001	0.023 ± 0.004	0.166 ± 0.012	0.977 ± 0.068	N.D.	0.025 ± 0.001
Pig liver 5	0.147 ± 0.012	0.112 ± 0.008	0.419 ± 0.017	0.485 ± 0.013	0.097 ± 0.003	0.100 ± 0.007
Range	0.028–0.755	0.023–0.349	0.166–1.388	0.485–1.232	0.026–1.006	0.025–1.025
Pig kidney 1	N.D.	0.011 ± 0.001	0.023 ± 0.001	0.062 ± 0.001	N.D.	N.D.
Pig kidney 2	N.D.	0.018 ± 0.001	0.021 ± 0.002	0.060 ± 0.002	N.D.	N.D.
Pig kidney 3	N.D.	0.018 ± 0.002	0.019 ± 0.002	0.057 ± 0.003	N.D.	N.D.
Pig kidney 4	N.D.	0.010 ± 0.001	0.018 ± 0.002	0.062 ± 0.005	N.D.	N.D.
Pig kidney 5	N.D.	0.015 ± 0.001	0.024 ± 0.002	0.058 ± 0.005	N.D.	N.D.
Range	N.D.	0.010–0.018	0.018–0.024	0.057–0.062	N.D.	N.D.
Bovine liver 1	N.D.	0.012 ± 0.001	0.012 ± 0.002	N.D.	0.895 ± 0.053	N.D.
Bovine liver 2	N.D.	0.082 ± 0.003	0.044 ± 0.002	N.D.	1.428 ± 0.020	N.D.
Bovine liver 3	N.D.	0.045 ± 0.002	0.029 ± 0.001	N.D.	1.003 ± 0.055	N.D.
Bovine liver 4	N.D.	0.035 ± 0.003	0.023 ± 0.002	N.D.	1.029 ± 0.033	N.D.
Bovine liver 5	N.D.	0.031 ± 0.002	0.017 ± 0.002	N.D.	0.777 ± 0.015	N.D.
Range	N.D.	0.012–0.082	0.012–0.044	N.D.	0.777–1.428	N.D.

N.D.: not detectable.

The calibration curves established with TIC and EIC are summarized in Table 3. The calibration curves were constructed by plotting the peak area obtained with a mixture of standard bile acids solution. Satisfactory results were obtained when bile acid derivatives of LCA-M-T(1), CDCA-M-T(3), HDCA-M-T(4), and UDCA-M-T(6) were determined with TIC while DCA-M-T(2) and CA-M-T(5) were determined with EIC. Therefore, the real samples were spiked with known quantities of bile acids before sample treatment and recoveries were calculated to verify the reliability of the proposed method. The recovery for all the bile acids in the animal tissues studied here ranged from 93.3 ± 2.5 to 96.7 ± 3.4% ($n = 5$). The detection limit was determined as the concentration of bile acids at which the signal-to-noise ratio (S/N) of 3 and found to be in the range of 0.4–1.6 ng/mL. Repeated analysis of the sample was carried out in order to determine reproducibility of the analysis. Correlation coefficients were grater than 0.99 for all the bile acids which indicated a well defined linear relationship between bile components concentration and the peak area as shown in Table 3.

The proposed method was employed to determine bile acid contents in pig liver, pig kidney and bovine liver purchased from various markets. The experimental results are listed in Table 4. LCA, DCA, CDCA, HDCA, CA, and UDCA were found in almost all of the pig liver samples except in pig liver 3 in which the content of LCA was not detectable. Besides, CA was not detectable in pig liver 4, either. Only DCA, CDCA, and HDCA were found in pig kidney while DCA, CDCA and CA were found in bovine liver. LCA, HDCA, and UDCA were below the detection limit in bovine liver. Bile acids are important end products of cholesterol metabolism in the livers by multiple complex path ways. Bile acids undergo enterohepatic circulation involving the small intestine, liver and kidney. Dihydroxy bile acids (DCA, CDCA, HDCA and UDCA) and trihydroxy bile acid (CA) were the major bile acids in pig liver. Guinea-pigs also had higher concentration of CDCA than CA in their biles as reported in the literature [39]. Cholic acid (CA) which was the first metabolite of cholesterol was the dominant bile acid in both bovine liver and ox bile as shown in our experimental results and in the literature [40], respectively.

4. Conclusions

1. An improved derivation method is proposed that combines the formation of the methyl ester from the carboxylic groups and the trimethylsilyl ether from hydroxyl groups in the bile acids. Camphor-10-sulphonic acid (CSA) was proved effective in acid-catalyzed methyl esterification which was performed at room temperature ultrasonically.
2. Solid-phase extraction was required for the determination of bile acids in biological samples.
3. Methyl ester-trimethylsilyl ether derivatives of bile acids can be quantified by GC-CI/MS/MS with shorter retention times with the proper interpretation of total ion chromatograms (TIC) and extracted ion chromatograms (EIC).
4. Satisfactory recovery, precision and accuracy was found for the proposed method.
5. The proposed method enables the identification of the main components of bile acid in the biological samples without matrix interference.
6. Successful application for the analysis of pig liver, pig kidney and bovine liver has been demonstrated. The presence of major bile acids in those biological samples was consistent with those reported in the literature.

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