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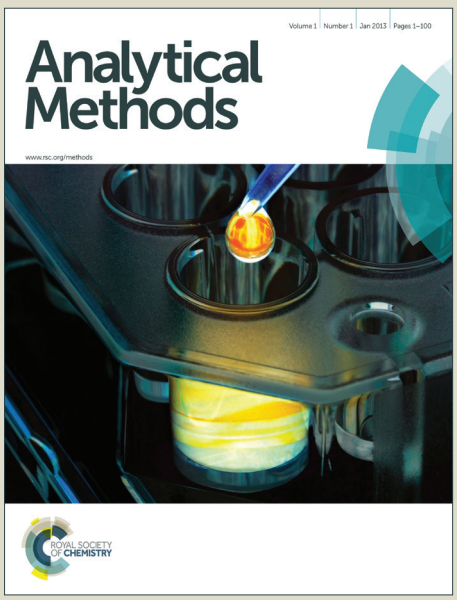

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Development of a Rapid and Simple LC-MS/MS Method for Identification and Quality Control of Natural *Calculus Bovis* and *Calculus Bovis Sativus*

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Calculus Bovis Sativus (*C. Bovis Sativus*, CBS), with the same pharmacodynamics activities as those of natural *C. Bovis* (CB), has been approved as an ideal substitute in China. However, in the latest edition of the Chinese Pharmacopoeia, only cholic acid and bilirubin are used as the markers for quality control. To overcome current limitations and to further improve its quality control method, a rapid and reliable method was herein developed for simultaneous qualitative and quantitative analyses of taurine and twelve bile acids (BAs) in natural CB and CBS by liquid chromatography coupled with tandem mass spectrometry. Taurine and BAs were separated using a Diamonsil C_{18} column (150 mm × 2.1 mm i.d., 5 μm) with acetonitrile: water containing 10 mM ammonium acetate (pH 3) as mobile phase in a

gradient elution program. Mass spectra were obtained in the negative ion mode through multiple reaction monitoring of respective mass transitions. This method had satisfactory separation efficiency, high sensitivity, little potential interference, and short running time. The average recoveries ranged from 93.0% to 107.3%, with relative standard deviations of less than 9.0% for all thirteen analytes of interest. Taurine and characteristic BAs, especially conjugated BAs, may partially reflect the internal quality of CBS and natural CB. Combining the ratios of cholic acid/deoxycholic acid and unconjugated BAs/conjugated BAs with quantitative data of single BA and chromatographic profiles may be able to control the quality of CBS. In conclusion, this method can be used for explicit identification and quality control of CBS and natural CB.

Key words: Traditional Chinese Medicine; *Calculus Bovis Sativus*; natural *Calculus Bovis*; bile acids; quality control; LC-MS/MS

Abbreviations: CBS, *Calculus Bovis*; CBS, *Calculus Bovis Sativus*; QC, Quality Control; LOQ, lower limit of quantification; MRM, multiple reaction monitoring; CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; HDCA, hyodeoxycholic acid; UDCA, ursodeoxycholic acid; TCA, taurocholic acid; TDCA, taurodeoxycholic acid; GCA, glycocholic acid; GDCA, glycodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; TCDCA, taurochenodeoxycholic acid.

Introduction

Calculus Bovis (*C. Bovis,* CB), commonly known as 'Niuhuang', is one of the incomparable and therapeutic traditional Chinese medicines (TCM).[1, 2] It is widely used in Oriental countries, and has been included in both the Japanese Pharmacopoeia 16th edition [3] and the Chinese Pharmacopoeia 10th edition.[2] Naturally derived from the dried pigment gallstones of *Bos Taurus domesticus* Gmelin and/or *Bubalus*

bubalis L, CB was first documented in "Shennong Bencao Jing" and has been classified as top grade for millennia. Niuhuang has traditionally been used to treat coma and delirium, epileptic convulsions, retropharyngeal abscesses, carbuncles, and furuncles,[4] with its versatile pharmacological effects also confirmed by a large number of modern findings.[5-11] Now it has been included in 650 out of 4500 TCM.[12] Due to the scarce resource and high price of natural Niuhuang, its substitutes, artificially synthesized CB and *C. Bovis Sativus* (CBS, also called *In-vitro Cultured C. Bovis*), have already been developed and widely used in medicine preparation. The pharmacological activities of artificial CB are considered inferior to natural CB, while those of CBS are generally proved equal and have been considered as an ideal substitute.[13] From the analysis of chemical composition,[14-16] the bioactive components of CB mainly include bile acid analogs (BAs) (structures are shown in Figure 1) such as cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), hyodeoxycholic acid (HDCA), ursodeoxycholic acid (UDCA), taurocholic acid (TCA), and glycocholic acid (GCA), bilirubin as well as some inorganic salts. CA and bilirubin, the most abundant constituents, have been generally considered as the main active components of natural CB and its substitutes.[17] Being in accordance with the herbal quality guidelines of the European Medicines Evaluation Agency (EMEA),[18] CA and bilirubin have already been used as the '*active markers'* for quality control in the China Pharmacopoeia (2010 edition). However, CA and bilirubin cannot fully explain the variations of therapeutic effects of CB, as many other active ingredients such as taurine and conjugated BAs also contribute to its pharmacological effects.[19-21] Besides, CA and DCA cause injuries of cardiomyocytes and/or fibroblasts, while additional treatments with taurine can attenuate these harmful actions.[19] Furthermore, although conjugated BAs have low contents and poor absorption, their effects are

comparable to those of unconjugated BAs.[22] Therefore, whether the structural (compositions and contents) differences of conjugated BAs affect their therapeutic values remains elusive, and it is of great significance to propose a quality control method that simultaneously determines more components such as taurine and conjugated BAs in CBS. This strategy helps partially explain why CBS is an ideal substitute for natural CB by comparing their material compositions.

A number of methods have been developed to determine BAs in TCM. Up to now, spectrophotometry,[23] thin-layer chromatography,[24] capillary electrophoresis (CE),[25, 26] high performance liquid chromatography[27-29] and gas chromatography [30, 31] have commonly been employed to determine BAs in raw materials, TCM preparations and other bio-samples derived from CB. Given the absence of chromophore in these BAs, they cannot be detected with a UV detector, which can be circumvented by applying evaporative light scattering detection (ELSD). However, only a small number of BAs can be monitored by ELSD-based methods, and it fails to detect conjugated BAs due to insufficient sensitivity. Currently, mass spectrometry (MS) detector has become more popular in quantitative analysis of drugs owing to high selectivity and sensitivity. Qiao et al. [16] presented a liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) method for simultaneous determination of eighteen BAs in bile-based crude drugs and quality control of these drugs. Unfortunately, this method uniquely focused on clarifying the differences between BAs in raw materials and formulated products, without taking CBS into consideration. Then, Peng et al. [17] established another method for simultaneous determination of unconjugated, taurine-conjugated and glycine-conjugated BAs in artificial CB. However, they did not systematically clarify the chemical constituents, especially conjugated BAs, in natural CB or CBS.

The aim of this study was to develop a sensitive and reliable LC–MS/MS method that simultaneously determined taurine and BAs in CBS for quality control. In addition, the chemical constituents of natural CB and CBS were compared in order to partially elucidate the mechanisms underlying their similar pharmacological activities.

Experimental

Chemicals and Reagents

Taurine, UDCA, CDCA, HDCA, tauroursodeoxycholic acid (TUDCA) and taurochenodeoxycholic acid (TCDCA) were purchased from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). CA, DCA, TCA, taurodeoxycholic acid (TDCA), GCA, glycodeoxycholic acid (GDCA) and glycochenodeoxycholic acid (GCDCA) were purchased from Sigma-Aldrich (St Louis, MO, USA). Fifteen batches of CB samples were all gifted from Wuhan Jianmin Dapeng Pharmaceutical Co., Ltd. (Wuhan, P. R. China), among which samples marked 1-5 were natural CB and samples marked 6-15 were CBS. Detailed information including production date and lot number is listed in Table 1. Voucher specimens were deposited at Herbarium of Department of Pharmacy, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, P. R. China.

Liquid Chromatography

All analyses were conducted on a Shimadzu liquid chromatography system equipped with two LC-20AD pumps, an SIL-20ACHT autosampler, an SCL-10Avp control system, a DGU-20A3 on-line degasser, and a CTO-20AC column oven (Chiyoda-Ku, Kyoto, Japan). Separation was performed on a Diamonsil C_{18} column (150 mm × 2.1 mm i.d., 5 μm, Dikma, Beijing, China) equipped with a phenomenex guard

column (5.0 mm × 2.0 mm i.d., Phenomenon, Guangzhou, China). The mobile phase, which consisted of acetonitrile (A) and 10 mM ammonium acetate in water, was adjusted to pH 3.0 using formic acid (B). Gradient elution was utilized, and the gradient was as follows: 70% B for 7.0 min, then decreased to 60% B over 0.2 min, held for 17.8 min, then decreased to 40% B over 0.2 min, held for 4.6 min, then returned to 70% B over 0.2 min and equilibrated for 5 min before the next injection. The chromatographic run time of each sample was 35 min. The temperature was maintained at 35°C for the column and at 15°C for the autosampler. The flow rate was 0.3 ml/min, and the injection volume was 10 μL.

Mass Spectrometry

MS analyses were conducted on an API 3200 triple quadruple mass spectrometer system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization source (ESI). The curtain gas (CUR) and collisionally activated dissociation (CAD) were 25 psi and 4 psi, respectively. The other working parameters were set as follows: spray voltage -4500 V, source temperature 370°C, GAS1 50 psi, GAS2 40 psi. The dwell time was kept at 200 ms for all compounds. LC-ESI-MS/MS was performed in the negative ionization mode with multiple reaction monitoring (MRM) of the transitions. The MRM ion pair transitions and main working energy parameters of each component are listed in Table 2. Data acquisition and analysis were controlled using the Analyst 1.5 software (Applied Biosystems, Foster City, CA, USA).

Calibration Standards and Quality Control Samples

Thirteen primary stock solutions including CA (148.0 μg/ml), DCA (96.0 μg/ml), UDCA (103.2 μg/ml), HDCA (100.4 μg/ml), CDCA (111.6 μg/ml), TCA (164 μg/ml),

TDCA (100.0 μg/ml), TUDCA (86.4 μg/ml), TCDCA (105.6 μg/ml), GCA (108.0 μg/ml), GDCA (124.8 μg/ml), GCDCA (124.8 μg/ml), and taurine (101.2 μg/ml) were prepared in methanol, respectively. Methanol (60%) was used to dissolve taurine. Then the mixed stock solution of standards was prepared by mixing the thirteen standard solutions in different ratios, containing CA, DCA, UDCA, HDCA, CDCA, TCA, TDCA, TUDCA, TCDCA, GCA, GDCA, GCDCA and taurine. The obtained solution was then serially diluted with 60% aqueous methanol to achieve standard working solutions. Lansoprazole, chosen as internal standard (IS), was dissolved with 60% methanol and diluted to 645.0 ng/ml. To a 100 μL aliquot of mixed standard solutions, an equal volume of IS was added, and then the mixture was vortex-mixed for 1 min and centrifuged at 12,000 rpm for 10 min. Quality control (QC) samples were prepared with the same procedure at low, median and high concentration levels. All solutions were sealed and stored at 4°C until use, and were kept at 15°C during analysis.

Sample Preparation

All batches of CB were ground to fine powders and filtrated with 20 mesh sieves. Appropriate amount of each sample was accurately weighed and dissolved in a 10 ml volumetric flask with mixed solvent (CH_3OH:H_2O, 3:2, v/v). The samples were vortexed for 2 min, ultrasonicated for 30 min and then centrifuged (3,500 rpm) for 5 min. The supernatants were sealed and stored at 4°C, filtered through 0.22 μm Millipore membranes, and diluted by 10-fold with extraction solvent before LC–MS/MS analysis. The fine powders of samples were stored at -20°C and away from light.

Results and Discussion

Optimization of Extraction Conditions

According to a previous study of Can et al., the primary active components in CB, i.e. taurine and BAs, were multicomponent with large differences in polarity and molecular structure.[32] A suitable extraction solvent system was required to ensure successful establishment of methodology. Taurine and some BAs such as CA and DCA can be completely dissolved in water due to strong polarity, while other BAs with weaker polarity can be easily dissolved in methanol or acetonitrile. To completely extract taurine and BAs simultaneously, certain proportions of methanol and water were used to prepare the calibration solution of taurine, CA and DCA as well as sample solutions of CB.

In order to get satisfactory extraction efficiencies of taurine and twelve BAs from the tested samples, the sample labeled 8 in Table 1 was randomly selected to optimize extraction conditions. Different proportions of methanol – water, extraction repetitions (1, 2, 3 and 4 times) and time (15, 30, 60 and 90 min) were tested for this ultrasonic procedure. Ultrasonication of samples with methanol and water at proportion of 3:2 (V/V) showed the best extraction efficiency among these methods, and extraction once for 30 min was sufficient with respect to extraction yield. Notably, chemical structures and properties of many components like conjugated BAs might be unstable at high ambient temperature or under light,[33] thus keeping the sample preparation procedure at 25°C and away from light was also required.

Optimization of Chromatographic Separation Conditions

Since the active components in CBS (taurine and BAs) are multicomponent with large differences in polarity and molecular structure, gradient elution was recommended. Firstly, methanol-water and acetonitrile-water solvent systems were compared, and the latter was chosen because of lower and more stable column pressure during

elution, which may be attributed to the low viscosity of acetonitrile. Then, whether the addition of formic acid, acetic acetate or ammonium formate could enhance column performance was tested. Adding 10 mM ammonium acetate resulted in desirable separation and allowed more flexible adjustment. Moreover, the peak narrowed when pH of the solvent system decreased. Considering the acid range that the column could withstand was pH 1-12, a mixture of acetonitrile and water containing 10 mM ammonium acetate (adjusted pH to 3 with formic acid) was chosen as mobile phase, which gave good peak shapes as well as high sensitivity for BAs, taurine and IS. Meanwhile, several commercially available columns, i.e. Dikma Diamonsil ar (150 mm × 2.1 mm, 5 μm, Beijing China), Waters silica (50 mm × 2.1 mm, 1.7 μm, Massachusetts, USA), Ultimate XB-CN (50 mm × 2.1 mm, 5 μm, MD, USA) and Ultimate C_{18} (150 mm × 2.1 mm, 5 μm, MD, USA), were tested. Eventually, Diamonsil C_{18} (150 mm × 2.1 mm, 5 μm) was selected, which could generate narrow, relatively symmetric peaks and short analytical time with an optimal resolution during gradient elution.

Optimization of MS Conditions

To optimize MS conditions, the standard solutions of every component (500-1000 ng/ml) were injected into the mass spectrometer via a syringe pump with a flow rate of 10 μl/min, respectively. MS scans were carried out in both positive and negative ionization modes. The negative ionization mode produced higher sensitivity, less baseline interference and fewer fragments for taurine and BAs than the positive one did, so the former was used to analyze all BAs. Then declustering potential (DP) was optimized for each component gradually, showing that the best DPs for taurine and conjugated BAs were high, i.e. from -43 to -120 V. Probably, all components were weak acids which hindered the dissociations of hydrogen ions. Interestingly, for

unconjugated BAs, either no prominent product was generated at lower collision energy (CE) or too many fragile, unstable product ions appeared when CE was raised, probably because the steroid backbone of BAs is very resistant to ESI, being consistent with previous reports.[34-36] Taking CDCA as example, no prominent product ion was found when CE was decreased to lower than -10 V, while so many product ions were produced when CE was raised to -30 V that they could not be stable enough for quantitative determination. When CE was further increased to -50 V, all precursor ions were completely dissociated into unstable fragment ions. Based on this finding, molecular ions were chosen as both precursor and product ions for taurine and most of the unconjugated BAs, as previously reported also[17]. For conjugated BAs, taurine (*m/z* 80) and glycine (*m/z* 73.2) were the typical fragment ions of taurine-/glycine-conjugated BAs. Since the intensities of these two fragment ions were sufficiently abundant, they were selected as product ions. All the MS parameters are listed in Table 2.

Validation of LC-MS/MS Method

Specificity, Linearity and Limit of Quantification. Specificity of this method was identified by comparing the retention time of each peak with those presented in the chromatogram of the mixed standard solution. The typical MRM chromatograms are demonstrated in Figure 2, and those of samples are illustrated in Figure 3. There were no potential inferences near retention time of each analyte and IS. All calibration curves were obtained by analyzing six-point calibration standards of every analyte. Linear regression calibration curves of the thirteen marker constituents (analyte peak area / IS peak area versus analyte concentration) were obtained based upon least square linear regression fitting ($y = ax + b$) with a weighting factor of $1/x^2$. Limit of quantification (LOQ, defined as S/N beyond 10) was detected. Detailed information

about all calibration curves and LOQ is listed in Table 2. All thirteen compounds showed good linearity ($r > 0.9951$) and high sensitivity in a wide dynamic range under the chromatographic condition.

Precision, Accuracy and Stability. The intra-day, inter-day accuracies and precision were validated by analyzing QC samples (n=6) at three concentration levels on a single day and three consecutive days, respectively. Precision was calculated as relative standard deviation (RSD), and accuracy was assessed as percent bias from the nominal concentration. For acceptance, the intra- and inter-day precisions were required not to exceed 15%, and accuracy should be within ±15%. As shown in Table 3, the overall inter-day and intra-day RSDs are less than 10.2%, and accuracies are within the range of 1.2 ~ 10.2%, indicating this method was precise, accurate and reproducible. Just because all four analytes achieved satisfactory baseline separation and stable retention time, and the precisions and accuracies of the validated method met determination needs also, it made sense for taking the same MRM transition with same precursor ion and daughter ion to monitor these four analytes. The stability was tested under different storage conditions by analyzing QC samples (n=3). The autosampler stability was evaluated by analyzing prepared QC samples kept in the autosampler (15°C) for 12 h. Ambient temperature and long-term stabilities were assessed using untreated QC samples kept at ambient temperature for 12 h and stored at -20°C for 30 days, respectively. RSDs of all analytes ranged from 0.98% to 3.5%, indicating the 13 marker components were stable under our experimental conditions.

Recovery. Recovery was evaluated by adding three accurately known quantities of the corresponding marker components (approximately equivalent to 0.7 times ~ twice the concentrations of the matrix) to one batch sample of CB. However, low, median, high concentration levels of HDCA were added into the matrix to investigate the

corresponding recovery. In this study, pre-analyzing sample solutions of batch 8 (Table 1) were further diluted three times and used as the matrix. The average recoveries were calculated by formula: recovery (%) = (amount found−original spiked)/amount spiked × 100%. The recoveries ranged from 93.0 to 107.3%, with RSD of less than 9.0% for all the thirteen marker analytes, indicating that the developed method was reliable and accurate enough (Table 4).

Sample Analysis

The validated LC-MS/MS method was successfully employed for the simultaneous analysis of taurine and twelve BAs in five batches of natural CB and ten batches of CBS. Under optimized conditions, the thirteen target analytes were baseline-separated within 33 min. By comparing the retention time and typical MRM chromatograms of standard solutions with those of the counterparts of samples in Figure 2-4, twelve and eleven components were identified in CBS and natural CB, respectively. All batches of CBS contained noticeable taurine, CA, DCA, CDCA as well as their glycine-conjugated and taurine-conjugated derivatives, including TCA, TDCA, TCDCA, GCA, GDCA and GCDCA (Table 5). Compared with CBS, BAs in natural CB were structurally simpler, and the contents, except for that of DCA, were lower. Interestingly, HDCA was almost absent in either natural CB or CBS, being consistent with some previous studies.[37] Since HDCA, a characteristic constituent of pig bile, has been used to manufacture artificial CB,[14] it may be a chemical marker of pig bile-derived products to differentiate artificial CB from natural CB and CBS.

The quality control method of components in TCM is required to reflect its internal quality. In current quality control methods of natural CB or CBS, only CA and DCA are analyzed. Nevertheless, CA and bilirubin fail to fully explain the variations of therapeutic effects of CB, so it is necessary to monitor other ingredients to estimate

the quality of natural CB and CBS. The contents of individual BAs in CBS overall exceeded those in natural CB. Meanwhile, CBS showed smaller coefficients of batch-to-batch variation than those of natural CB, with RSD of 3.62-10.86% versus 12.56-223.61%, respectively (Table 5). The larger inter-batch differences of natural CB may result from the complicated environment of formation. In contrast, CBS is produced *in vitro* under conditions mimicking the gallstone formation process *in vivo*, so the internal quality is maintained by the rigid and stable environments. Moreover, the proportions of unconjugated, taurine-conjugated and glycine-conjugated BAs were subjected to similar variations (Figure 6). However, the contents of individual BAs in natural CB showed more evident batch-to-batch differences. Hence, CBS, as an eligible substitute for natural CB, is more suitable for large-scale production and clinical applications. Regardless, the merits of natural CB and CBS cannot be evaluated only based on respective contents of unconjugated or conjugated BAs.

The quantitative data were further analyzed by making statistic comparison of the multicomponent structures. As shown in Figure 5 and Table 4, the ratio of CA/DCA in CBS (2.67±0.38) is nearly twice that of natural CB (1.27±0.23), and the ratios of (CA +GCA+ TCA)/(DCA +GDCA+ TDCA) (2.83±0.32 versus 1.52±0.28) remain unchanged for both varieties. The ratio of unconjugated BAs/conjugated BAs in natural CB was much higher than that of CBS (28.33±16.65 versus 3.27±0.25). Thus, combining the ratios of CA/DCA and unconjugated BAs/conjugated BAs with quantitative data for single BA and chromatographic profiles may be able to differentiate CBS from natural CB or to identify them. It is now well-established that BAs, which mainly exist as taurine conjugates in both CBS and natural CB, are better absorbed than glycine-conjugated BAs. As a consequence, recent studies presumed that taurine and its conjugated BAs were probably the major components contributing

to their wide pharmacological activities. Based on the above results, the ratios of taurine/ (TCA+TDCA+TCDCA) in CBS and natural CB were compared, showing no significant difference (3.71±0.36 versus 4.16±2.06, $P > 0.05$). This may partially explain the reasons behind CBS being an ideal substitute for natural CB.

Conclusion

In China, CBS is currently regarded as an ideal substitute for natural CB with definite chemical components. In this study, a fully validated LC-MS/MS method was established and applied to simultaneously determine taurine and twelve BAs in CBS and natural CB. This method could be used to comprehensively reveal the differences between structures of natural CB and CBS as well as contents of ingredients in them. Detection of taurine and characteristic BAs, especially that of conjugated BAs, at least partially reflected the similarities and differences between internal quality of CBS and natural CB. Combining the ratios of CA/DCA and unconjugated BAs/conjugated BAs with quantitative data of single BA and chromatographic profiles may be a feasible strategy for quality control of CBS.

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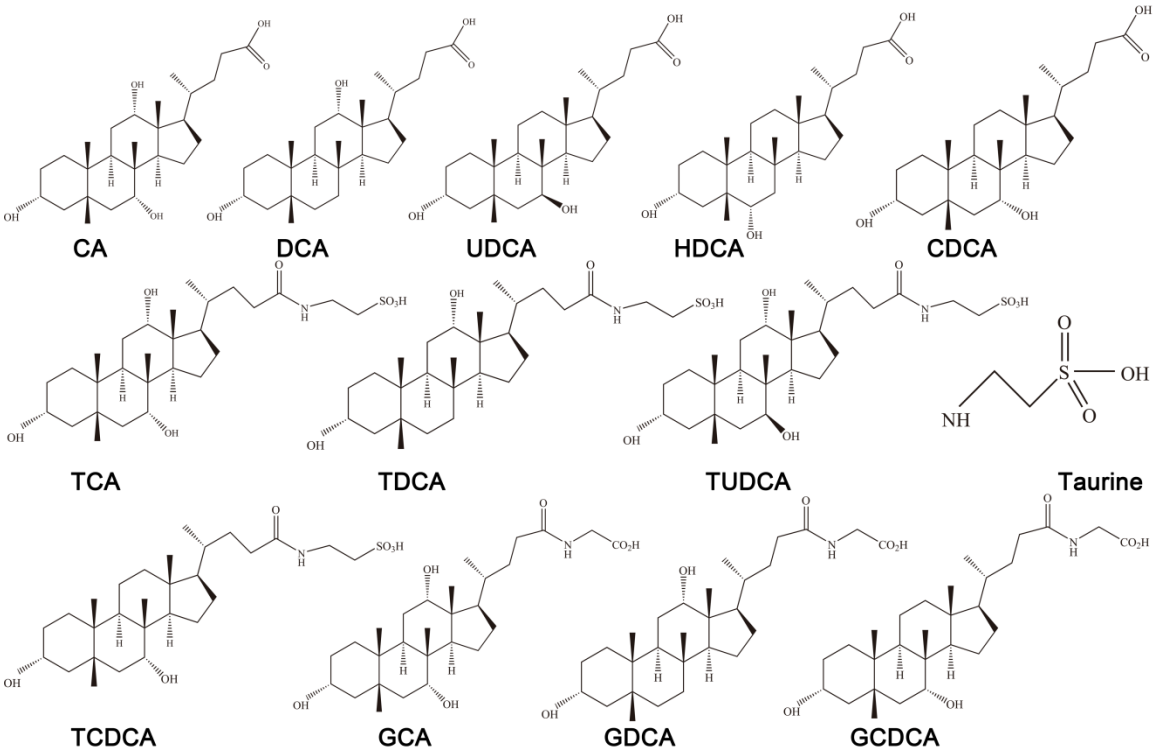

Fig. 1. Structures of representative bile acids.

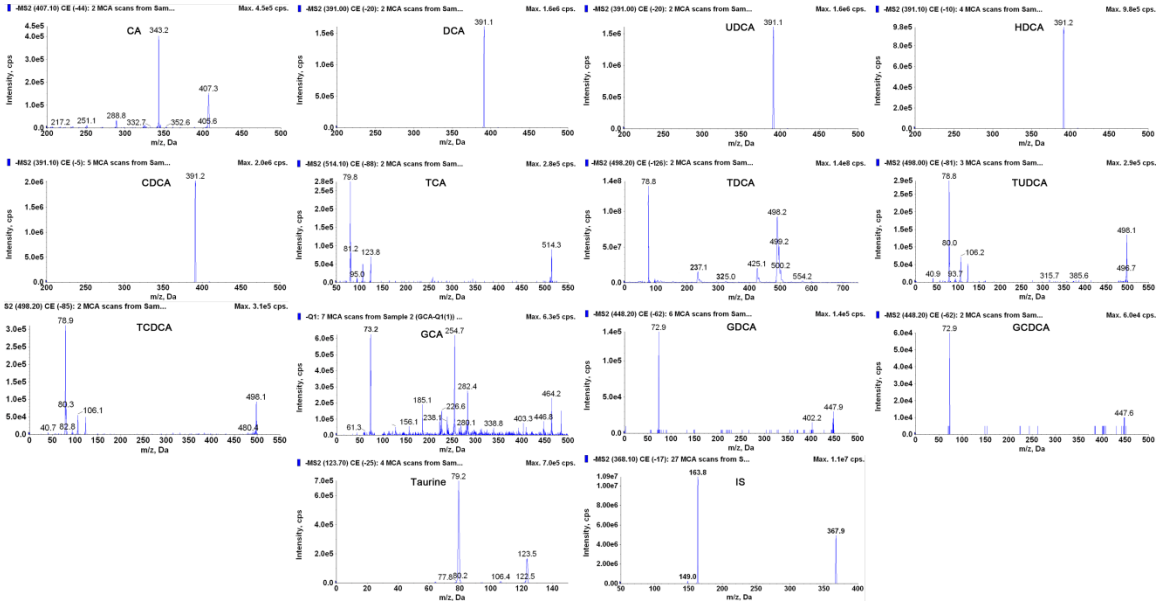

Fig. 2. Electrospray ionization-tandem mass spectra of taurine and twelve bile acids in the negative ion mode.

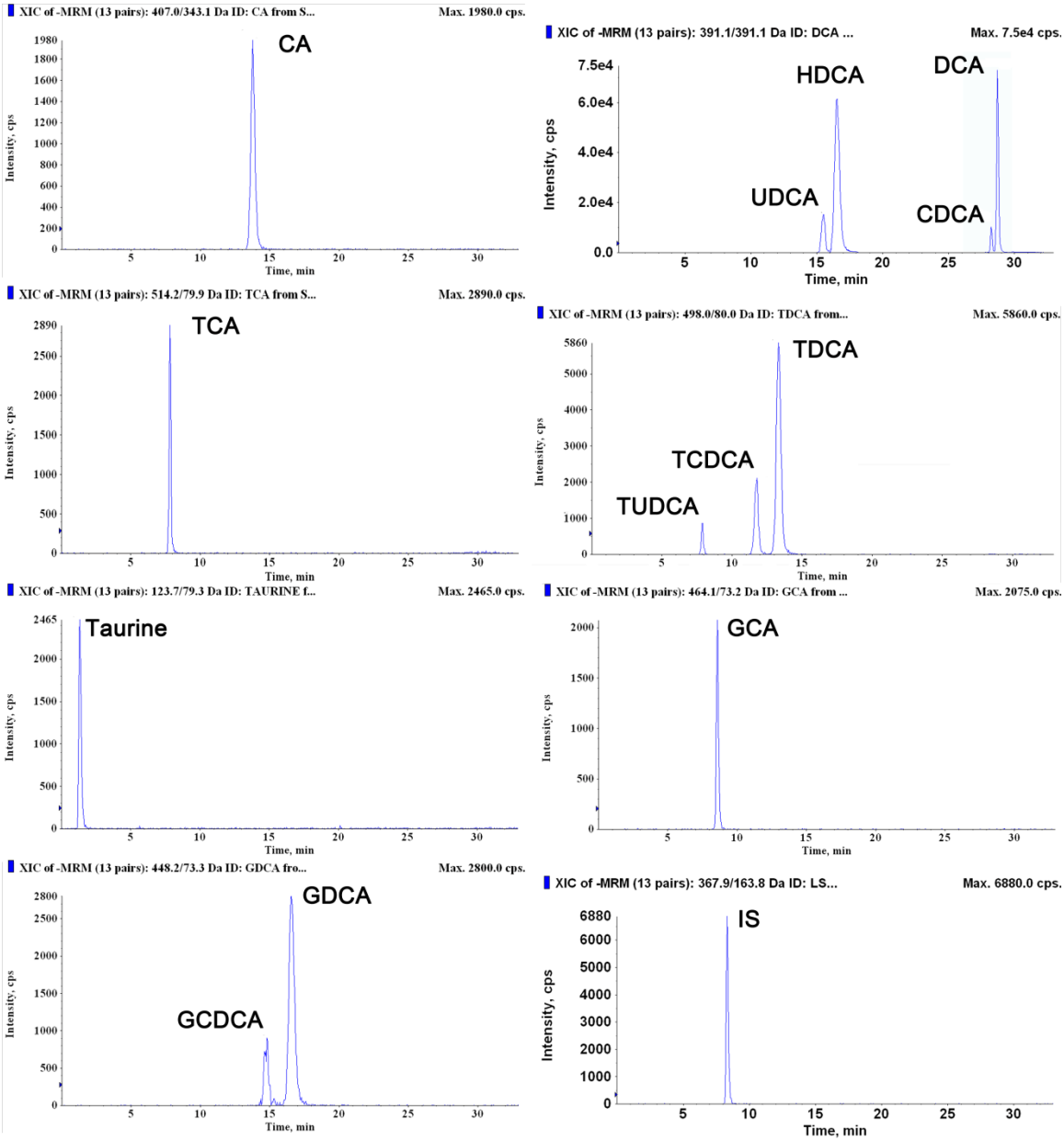

Fig. 3. Typical chromatograms of mixed standards.

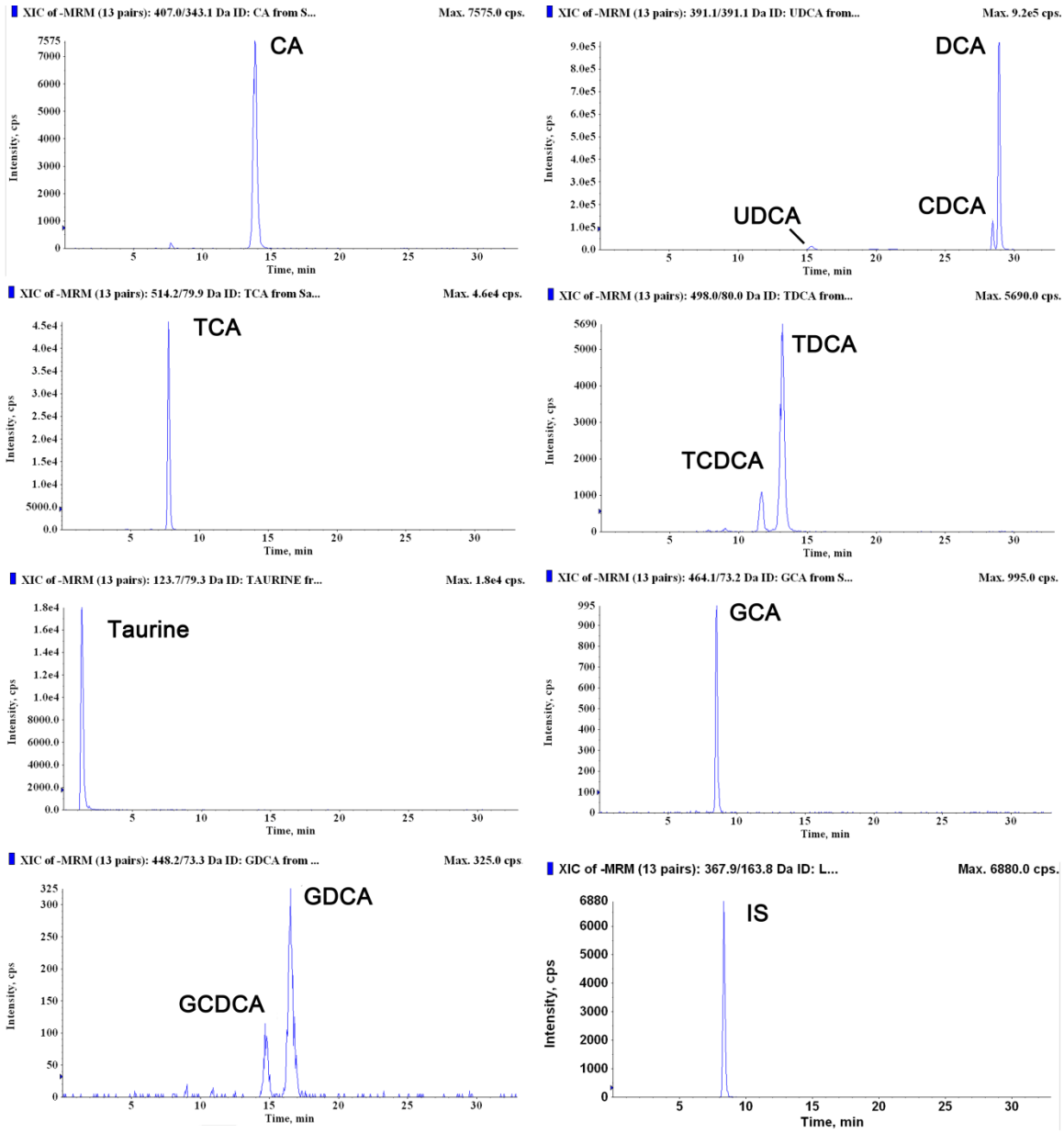

Fig. 4. Typical chromatograms of various samples.

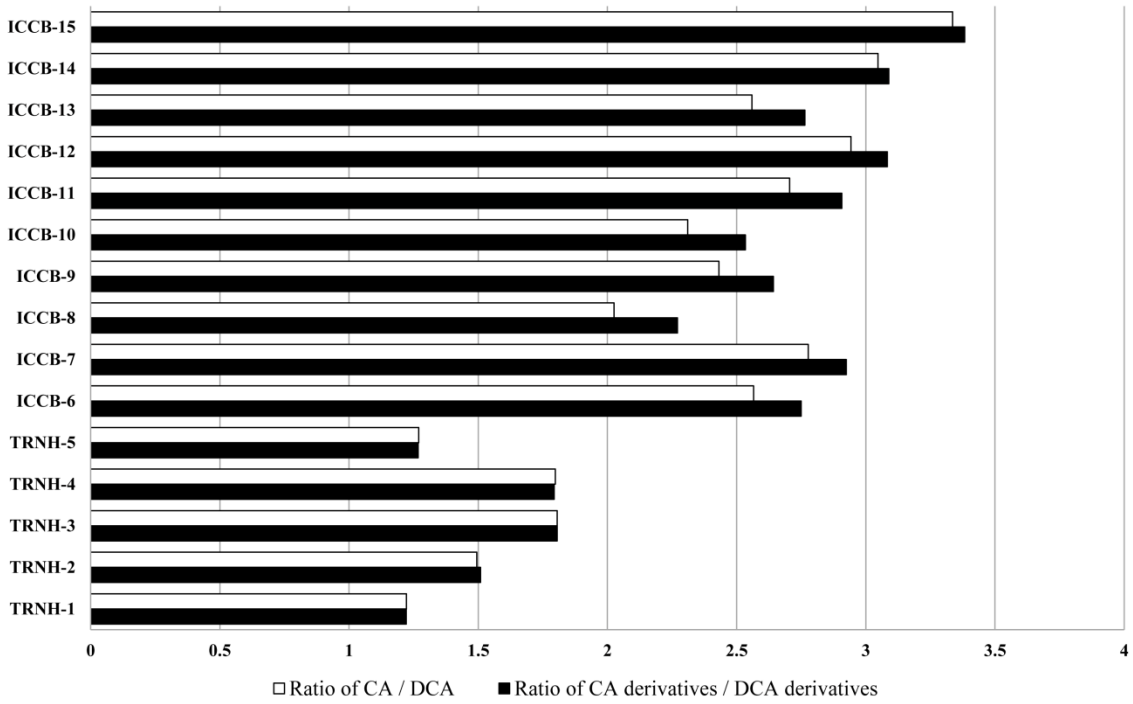

Fig. 5. Distribution of CA and DCA derivatives in natural *Calculus Bovis* and *Calculus Bovis Sativus*.

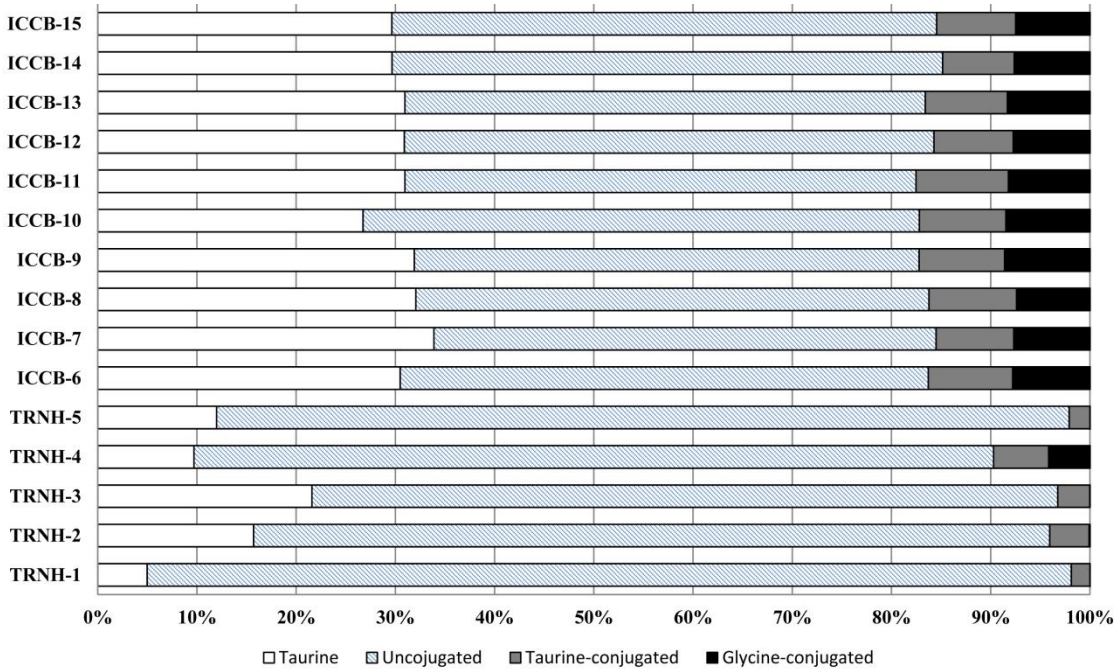

Fig. 6. Distribution of unconjugated, glycine-conjugated and taurine-conjugated BAs in natural *Calculus Bovis* and *Calculus Bovis Sativus*.

Table 1 Summary of investigated samples.

Number	Collection sites	Manufacture date	Lot No.
1	Wuhan	2013/10/14	130803
2	Wuhan	2013/11/22	131015
3	Wuhan	2014/01/16	140101
4	Wuhan	2014/02/12	140201
5	Wuhan	2014/04/17	140302
3	Wuhan	2014/01/05	140101
4	Wuhan	2014/02/11	140201
5	Wuhan	2014/03/02	140301
6	Wuhan	2014/04/02	140401
7	Wuhan	2014/04/05	140402
8	Wuhan	2014/04/10	140403
9	Wuhan	2014/05/09	140501
10	Wuhan	2014/05/12	140502
11	Wuhan	2014/06/09	140602
12	Wuhan	2014/06/15	140603

Note: Batches 1-5 are natural *Calculus Bovis* while batches 6-15 represent *Calculus Bovis Sativus*.

Table 2 Transitions, main working energy parameters and calibration Curves of all analytes.

Analyte	Molecular weight (Da)	transitions (*m/z*)	DP (V)	EP (V)	CE (V)	CXP (V)	Calibration curve	*r*	Linear range (ng/mL)	LOQ (ng/mL)
Taurine	125.15	123.5→79.2	-43	-6	-26	-1	Y=0.000879*X+0.00181	0.9949	12.7-4048	2.5
TCA	514.7	514.3→79.8	-120	-9	-115	-1	Y=0.00896*X+0.0142	0.9958	32.2-1030	8.0
GCA	465.62	464.2→73.2	-45	-10	-50	-4	Y=0.000193*X+0.00222	0.9998	13.5-1350	3.4
TUDCA	499.7	498.1→78.8	-110	-8.5	-123	-50	Y=0.00108*X++0.001	0.9984	27-864	27
TCDCA	499.7	498.1→78.9	-109	-10	-120	-54	Y=0.00126*X+0.0071	0.9960	20.60-660	20.6
TDCA	499.7	498.0→78.8	-109	-7.8	-126	-2	Y=0.00721*X+0.00335	0.9984	12.5-1250	10.0
CA	408.57	407.0→343.2	-92	-9	-44	-3	Y=0.000346*X+0.00512	0.9949	185-5960	185
GCDCA	448.62	447.6→72.9	-45	-10	-50	-4	Y=0.00069*X+0.00112	0.9993	15.6-1560	15.6
GDCA	448.62	447.9→72.9	-45	-10	-50	-4	Y=0.00071*X+0.000852	0.9896	15.6-1560	15.6
UDCA	392.57	391.1→391.1	-96	-10	-15	-6	Y=0.00637*X+0.0886	0.9988	8.0-258	8.0
HDCA	392.57	391.1→391.1	-94	-6.5	-15	-5.5	Y=0.00555*X+0.0343	0.9997	19.6-627.5	9.8
CDCA	392.57	391.1→391.1	-110	-5.5	-14	-6	Y=0.00924*X+0.128	0.9973	55.8-2790	55.8
DCA	392.57	391.1→391.1	-100	-7	-15	-6	Y=0.0177*X+0.155	0.9995	37.5-1200	18.8
Lansoprazole	369.36	367.9→163.8	-20	-5	-21	-4	-	-	-	-

Table 3 Precision, accuracy and repeatability of all analytes.

		Accuracy and precision					
		inter-day (n=6)			intra-day (n=6)		
	Con. (ng/ml)	Con. (ng/ml)	Accuracy (%)	RSD (%)	Con. (ng/ml)	Accuracy (%)	RSD (%)
Taurine	127	115	90.6	5.9	119	93.7	4.9
	759	768	101.2	4	761	100.2	5.2
	4048	3922	96.9	4.4	3854	95.2	4.8
CA	185	166	89.7	3.2	158	85.4	5.6
	1110	955.1	86.0	2.9	962	86.7	3.3
	5920	5496	92.8	5.5	5308	89.7	3.6
DCA	37.5	32.58	86.9	7.2	33.4	89.1	6.5
	225	211	93.8	2.1	216.5	96.2	3.8
	1200	1070.5	89.2	5.4	1088.5	90.7	4.6
UDCA	8.05	6.9	85.7	3.4	7.2	89.4	6.7
	48.38	42.05	86.9	2.4	41.99	86.8	5.1
	258	272.4	105.6	2.3	272.7	105.7	3.4
CDCA	55.8	60.3	108.0	8.4	57.7	103.3	7.9
	418.5	398.3	94.9	5.2	405.1	96.6	4.6
	2790	2699.5	96.8	3.7	2731	97.9	4.1
HDCA	19.61	17.34	88.4	6.8	17.2	87.7	4.2
	117.68	105.8	89.9	4.6	108.7	92.4	4.7
	627.5	631.1	100.6	3.2	629.9	100.4	3.8
TCA	16.1	14.65	91.0	6.6	17.48	108.6	7.3
	193.15	183.4	95.0	3.4	187.7	97.2	2.4
	1030	884.5	85.9	5.6	1031.4	100.1	9.2
TDCA	12.5	13.5	108.0	10.2	13.3	106.4	9.9
	150	149	99.3	4.9	140.1	93.4	5.2
	1250	1102	88.2	3.3	1112.8	89.0	3.7
TUDCA	13.5	13.5	100.0	1.2	12.7	94.1	8.6
	162	153.1	94.5	4.3	151.4	93.5	3.7
	864	909.4	105.3	5.4	891.6	103.2	4.3
TCDCA	20.6	19.5	94.7	8.1	18.7	90.8	8.3
	123.75	117.3	94.8	4.1	117.8	95.2	5.5
	660	647.2	98.1	6.4	670.2	101.5	6.8
GCA	13.5	13.6	100.7	8.4	13.9	103.0	8.7
	162	140.9	87.0	5.6	142.8	88.1	4.7
	1350	1295.3	95.9	6.4	1303.6	96.6	6.5
GDCA	15.6	13.6	87.2	9.8	14	89.7	9.2
	187.2	172.4	92.1	3.7	175.5	93.8	3.8
	1560	1379.3	88.4	7.6	1406.7	90.2	8.7
GCDCA	15.6	13.7	87.8	5.1	15.8	101.3	7.9
	187.2	171.2	91.5	4.8	174.6	93.3	6

	1560	1470	94.2	7.4	1534	98.3	6.9

Table 4 Recovery data for the assay of thirteen analytes.

	Original (ng/ml)	Spiked (ng/ml)	Found (ng/ml)	Recoveried (%)	Mean (%)	RSD (%)
Taurine	1340	1012	2292.2	94.1	95.9	3.4
	1340	1214.4	2479.4	93.8		
	1340	1619.2	2954	99.7		
CA	1870	1480	3327.5	98.5	95.7	6.2
	1870	1776	3448.1	88.9		
	1870	2368	4233	99.8		
DCA	430	300	697	89.0	94.3	4.9
	430	360	782	97.8		
	430	480	890.8	96.0		
UDCA	51	64.5	107.9	88.2	90.4	2.2
	51	77.4	122.3	92.1		
	51	103.2	144.7	90.8		
CDCA	116	89.3	208.6	103.7	104.1	3.6
	116	111.6	236.5	108.0		
	116	167.4	284.4	100.6		
HDCA	0	78.5	70.3	89.6	93.0	4.0
	0	117.7	114.1	96.9		
	0	156.9	145.2	92.5		
TCA	242	257.5	508.3	103.4	95.8	7.1
	242	309	521	90.3		
	242	412	627.7	93.6		
TDCA	60.3	50	113.4	106.2	107.3	5.8
	60.3	50	117.3	114.0		
	60.3	75	136.6	101.7		
TUDCA	0	108	109	100.9	99.9	2.8
	0	108	104.5	96.8		
	0	108	110.2	102.0		
TCDCA	28.2	20.2	49.3	104.5	99.0	9.0
	28.2	24.8	50.2	88.7		
	28.2	33	62.5	103.9		
GCA	172.3	162	315.8	88.6	93.6	4.8
	172.3	162	329.5	97.0		
	172.3	217.6	379.5	95.2		
GDCA	52.3	49.9	94.1	83.8	87.7	4.2
	52.3	62.4	107.4	88.3		
	52.3	93.6	137.5	91.0		
GCDCA	48.7	49.9	97.2	97.2	104.2	6.1
	48.7	62.4	114.8	105.9		
	48.7	93.6	151.3	109.6		

Table 5 Contents (mg/g) of investigated analytes in natural *Calculus Bovis* and *Calculus Bovis Sativus*.

batch	Taurine (mg/g)	CA (mg/g)	DCA (mg/g)	UDCA (mg/g)	CDCA (mg/g)	HDCA (mg/g)	TCA (mg/g)	TDCA (mg/g)	TUDCA (mg/g)	TCDCA (mg/g)	GCA (mg/g)	GDCA (mg/g)	GCDCA (mg/g)
1	3.57	34.84	28.51	0.2	3.28	ND	0.67	0.56	BL	0.12	ND	0	0.01
2	19.76	56.68	37.94	0.44	5.87	ND	3.09	1.61	BL	0.3	ND	0.07	0.04
3	27.48	57.67	31.94	0.36	5.73	ND	2.49	1.34	BL	0.27	ND	0.03	0
4	11.74	58.51	32.53	0.35	6.18	ND	3.84	2.24	0.33	0.32	3.09	1.7	0.26
5	9.34	35.52	27.97	0.21	3.34	BL	0.79	0.68	BL	0.17	ND	0	0
6	59.33	69.74	27.18	0.29	6.37	BL	12.74	3.35	BL	0.44	9.13	2.78	3.27
7	68.93	71.03	25.57	0.27	6.07	BL	11.85	3.66	BL	0.43	10.15	2.58	2.90
8	62.44	63.03	31.12	0.29	6.28	ND	13.44	3.37	BL	0.38	8.74	3.03	2.64
9	60.50	63.88	26.27	ND	6.33	ND	12.04	3.80	BL	0.48	10.59	2.67	3.06
10	50.74	61.84	26.76	0.30	6.48	ND	12.61	3.57	BL	0.38	10.19	3.07	2.81
11	61.13	69.04	25.52	0.30	6.88	ND	14.36	3.58	BL	0.46	10.23	3.11	2.89
12	62.04	75.82	25.77	0.28	5.31	ND	11.99	3.57	BL	0.47	10.29	2.48	2.78
13	60.04	68.69	26.84	0.28	5.88	BL	11.97	3.62	ND	0.46	10.58	2.54	3.02
14	62.82	83.60	27.44	0.27	6.22	ND	11.29	3.58	BL	0.39	10.26	3.02	2.88
15	63.00	85.40	25.60	0.27	5.44	ND	12.18	3.82	0.40	0.50	10.20	2.44	3.30

Note: Batches 1～5 were natural *Calculus Bovis*, while batches 6～15 represent *Calculus Bovis Sativus*; The samples were coded in Table 1;

ND, not detected;

BL, below the limit of quantification.