A new method for identification of natural, artificial and *in vitro* cultured Calculus bovis using high-performance liquid chromatography-mass spectrometry

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ABSTRACT

Objective: Calculus bovis have been widely used in Chinese herbology for the treatment of hyperpyrexia, convulsions, and epilepsy. Nowadays, due to the limited source and high market price, the substitutes, artificial and *in vitro* cultured Calculus bovis, are getting more and more commonly used. The adulteration phenomenon is serious. Therefore, it is crucial to establish a fast and simple method in discriminating the natural, artificial and *in vitro* cultured Calculus bovis. Bile acids, one of the main active constituents, are taken as an important indicator for evaluating the quality of Calculus bovis and the substitutes. Several techniques have been built to analyze bile acids in Calculus bovis. Whereas, as bile acids are with poor ultraviolet absorbance and high structural similarity, effective technology for identification and quality control is still lacking. **Methods:** In this study, high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (LC/MS/MS) was applied in the analysis of bile acids, which effectively identified natural, artificial and *in vitro* cultured Calculus bovis and provide a new method for their quality control. **Results:** Natural, artificial and *in vitro* cultured Calculus bovis were differentiated by bile acids analysis. A new compound with protonated molecule at m/z 405 was found, which we called 3α, 12α-dihydroxy-7-oxo-5α-cholanic acid. This compound was discovered in *in vitro* cultured Calculus bovis, but almost not detected in natural and artificial Calculus bovis. A total of 13 constituents was identified. Among them, three bio-markers, including glycocholic acid, glycodeoxycholic acid and taurocholic acid (TCA) were detected in both natural and artificial Calculus bovis, but the density of TCA was different in two kinds of Calculus bovis. In addition, the characteristics of bile acids were illustrated. **Conclusions:** The HPLC coupled with tandem MS (LC/MS/MS) method was feasible, easy, rapid and accurate in identifying natural, artificial and *in vitro* cultured Calculus bovis.

Key words: Artificial Calculus bovis, high-performance liquid chromatography-mass spectrometry, *in vitro* cultured Calculus bovis, natural Calculus bovis

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INTRODUCTION

Calculus bovis, the dry gallstone of Bostaurus domesticus Gmelin, have been recognized for centuries in traditional Chinese medicine (TCM) for its multiple pharmacological actions, including sedation, relieving fever, diminishing inflammation, normalizing function of the gallbladder and anti-hyperspasmia.[1] As the source of natural Calculus bovis is limited, alternatives for natural Calculus bovis were used in the medicinal preparations. However, the constituents of Calculus bovis and their substitutes are different, which may reflect the various inherent qualities. There are many bioactive components in Calculus bovis, including bilirubin, bile acids, amino acid, fatty acid, and mineral.[2] Therefore, these components could be chosen as the bioactive marker compounds for the quality control of Calculus bovis. In China Pharmacopoeia, bile acids, as one of the main active constituents, are important indicator for evaluating the quality of Calculus bovis and the substitutes, which have the effects of diminishing inflammation, antianaphylaxis and antidote. Bile acids are a mixture of steroids, mainly including cholic acid (CA), deoxycholic acid (DCA), hyodeoxycholic acid (HDCA), chenodeoxycholic acid (CDCA) and others.[3,4] Several techniques are available to analyze bile acids in Calculus bovis, such as thin layer chromatography,[5]

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capillary electrophoresis,[6,7] and high-performance liquid chromatography (HPLC).[8] Among them, HPLC with various detectors is most widely used. However, ultraviolet detector is inadequate in detecting bile acids due to the absence of a chromophore. Evaporative light scattering detector as a universal detector with high sensitivity has been successfully applied for the simultaneous analysis of nonchromophoric compounds in TCM.[9-13]

High-performance liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) could be the most sensitive and reliable technology for the analysis of bile acids. HPLC could provide effective chromatographic separation and MS could supply abundant information for structural elucidation of these compounds, especially when high-resolution tandem MS is applied. Cao *et al.*[14] identified four bile acids from Liu Shen Wan using multi-dimension HPLC tandem MS system. Qiao *et al.*[15] established a method of simultaneously determination of 18 bile acids in bile-based crude drugs both qualitatively and quantitatively using HPLC/MS/MS. But there was very few reports about identifying natural, artificial and *in vitro* cultured Calculus bovis by distinguishing bile acids.

In this work, a new method for identification natural, artificial and *in vitro* cultured Calculus bovis was established using HPLC-MS. A total of 13 constituents were identified. The Characterizations of these compounds are showed in Table 1. Among them, a novel compound was found in *in vitro* cultured Calculus bovis. According to the result of Gaussian calculation, this compound was indentified as 3α, 12α-dihydroxy-7-oxo-5β-cholanic acid. Three bio-markers, including glycocholic acid (GCA), glycodeoxycholic acid (GDCA) and taurocholic acid (TCA) were detected in both natural and artificial Calculus bovis, in which TCA density could be used to distinguish natural Calculus bovis from the artificial substitutes.

EXPERIMENTAL

Chemicals and materials

Cholic acid and CDCA were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile (E. Merck, Darmstadt, Germany) were HPLC grade. The water used for HPLC was purified by Milli-Q system (Millipore, Milford, MA, USA). Phosphoric acid (AR grade), glacialacetic acid (AR grade), dichloromethane (AR grade) and methanol (AR grade) was obtained from Beihua Fine Chemicals Co., Ltd. (Beijing, China).

Preparation of samples

The samples were weighed accurately (0.1 g) and placed into a 25 mL flask containing 25 mL of water saturation methylene chloride/methanol/water (100:50:2, v/v/v), then the mixture was extracted in ultrasonic bath (Eima Ultrasonics Corp., Germany) at room temperature for 0.5 h. The solution was filtered through a 0.45 μm membrane before injection to the HPLC-MS system for analysis.

Apparatus and operation conditions

Liquid chromatography

Samples were separated on an Agilent SB C_{18} column (250 mm × 4.6 mm I.D., 5 μm). The mobile phase consisted of acetonitrile (A) and water containing 0.5% (v/v) phosphoric acid (B) a gradient program was used as follows: 0–2 min, 90% A; 2–30 min, 90% A ~78% A; 30–40 min, 78% A; 40–60 min, 78% A ~66% A; 60–70 min, 66% A ~50% A; 70–80 min, 50% A ~10% A; 80–85 min, 10% A ~5% A; 85–110 min, 5% A. Flow rate, 0.8 mL/min. The column temperature was 30°C. The samples were detected at 200 nm.

Mass spectrometry

High-resolution MS and MS/MS spectral analysis were performed on an LTQ-orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) connected to the HPLC instrument via an electrospray ionization (ESI) interface in a postcolumn splitting ratio of 1:3. The mass spectrometer was monitored in the negative ESI mode. High purity nitrogen was used as sheath (30 arb) and aux gas (5 arb). Parameters were as follows: Spray voltage of 3.0 kV (negative), capillary temperature of 300°C, capillary voltage of 25 V, tube lens voltage of 110 V. The injection time was 50 ms and the number of microscans was 2. The collision energy for collision induced dissociation (CID)

Table 1: The bile acids of natural, artificial and *in vitro* cultured calculus bovis

	TCA	CA	GCA	GDCA	3α, 12α-dihydroxy-7-oxo-5β-CA	LCA
Artificial (120,209)	+	+	+	+	−	−
Artificial (120,301)	+	+	+	+	−	−
Natural (Beijing)	+	+	−	+	−	−
Natural (Hebei)	+	+	−	+	−	−
In vitro cultured (111,001)	−	+	+	−	+	+
In vitro cultured (111,002)	−	+	+	−	+	+

+: Containing the compound; −: Not containing the compound; TCA: Taurocholic acid; CA: Cholic acid; GCA: Glycocholic acid; GDCA: Glycodeoxycholic acid; LCA: Lithocholic acid

was adjusted to 35% of maximum, and the isolation width of precursor ions was m/z 2.0 Da.

RESULTS AND DISCUSSION

Optimization of extraction and analytical conditions

The selection of HPLC conditions was guided by the requirement for obtaining chromatograms with better resolution of adjacent peaks, including type of column, column temperature, mobile phase system, and flow rate. With the optimized conditions, most peaks could be well separated within 110 min [Figure 1]. Furthermore, all factors related to MS performance including ionization mode, nebulizer gas pressure, electrospray voltage of the ion source and collision energy have been experimented. The results showed that ESI in negative ion mode was necessary for the analysis. Most of the investigated compounds exhibited quasi-molecular ions $[M-H]^-$ and product-ions with rich structural information in the CID-MS/MS experiment.

Identification of 3α, 12α-dihydroxy-7-oxo-5β-cholanic acid

A compound, with its pseudo-molecular ion at m/z 405, was found in Calculus bovis [Figure 2]. The prominent $[M-H]^-$ ion of cholic acid was at m/z 407.2798, while this new compound was at m/z 405.2636. Its MS^2 was similar to

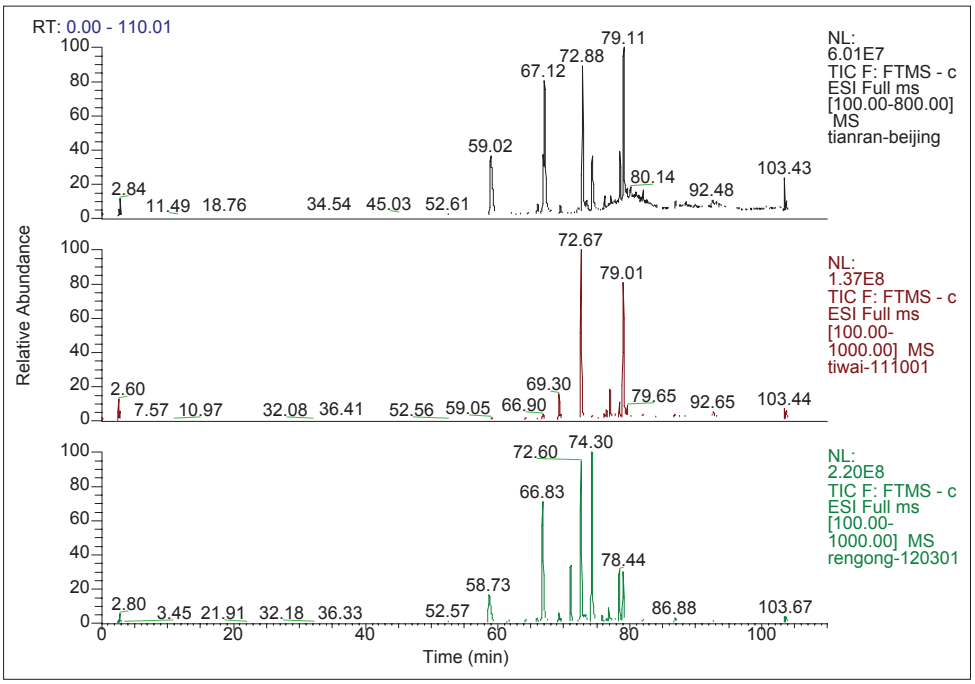

Figure 1: The total ion chromatogram of natural, *in vitro* cultured and artificial Calculus bovis

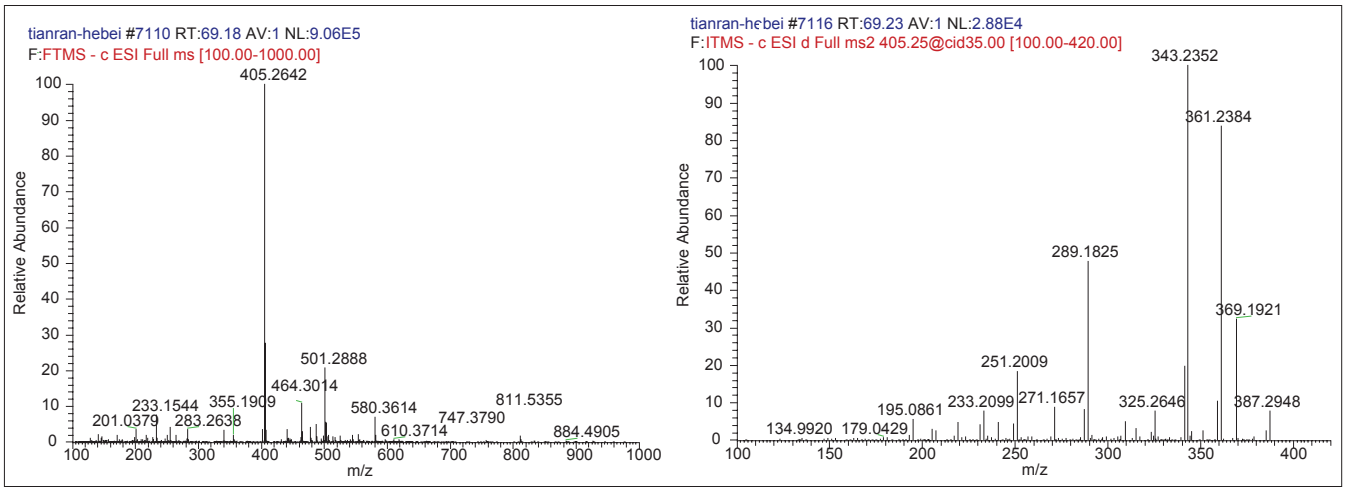

Figure 2: The mass spectrometry1 (MS) and MS2 of 3α, 12α-dihydroxy-7-oxo-5β-cholanic acid

cholic acid, and its MS^2 was at m/z 343 and m/z 289. According to above data, we deduced the compound has similar core structure with cholic acid and has lost H_2 from cholic acid. However, according to the structure of cholic acid, there are three hydroxyls in C_3, C_7 and C_{12}, respectively, which could generate carbonyl by losing hydrogen on O and C. There are three kinds of possible structure in this compound. In this paper, we solved this problem using quantum chemistry. The theoretical calculation of Gaussian calculation was done using Gaussian 03w pack. Quantum chemical calculations were also performed for a better analysis of the results, which would help to have a deep insight into the compound.[16-18] Density functional theory and 6–31 g basis set were used in the calculation. Geometry optimization analysis was completed at the B3LYP/6-31 g level. Three optimal structure and energy were obtained by Gaussian calculation. As shown in Figure 3, the structure of this compound was the most stable, with the lowest energy. The HF value was −1313.1769. The same method was used to optimize the structure of cholic acid, which was with the HF value at −1313.1769, and the structure is shown in Figure 4. Based on the result of Gaussian calculation, the structure of the new compound was for C_7 carbonyl structure, which could be called 3α, 12α-dihydroxy-7-oxo-5β-cholanic acid. It was discovered in *in vitro* cultured and almost not detected in natural and artificial Calculus bovis.

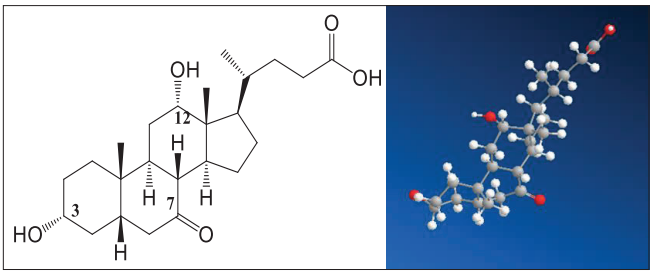

Figure 3: The structure of 3α, 12α-dihydroxy-7-oxo-5β-cholanic acid

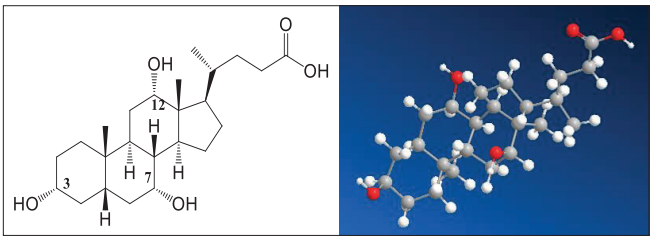

Figure 4: The structure of cholic acid

Identification of bio-makers of natural, artificial and *in vitro* cultured Calculus bovis

Glycocholic acid, GDCA and TCA, were detected in Calculus bovis. Their chemical structures were shown in Figure 5. Compound 1 showed the $[M-H]^-$ ion at m/z 514.2831 ($C_{26}H_{45}NO_7S$), and its MS^2 product ion at m/z 353.3042 was a loss of taurine and $2H_2O$ [Figure 6]. It also produced the $[M-H_2O-H]^-$ ion at m/z 496.4588. The third fragment ion of MS^2 spectrum was at m/z 371.3727 with losing taurine and H_2O. Thus, it was characterized as TCA. As shown in Table 1, TCA was not found in *in vitro* cultured Calculus bovis, and the density of TCA in natural Calculus bovis was much higher than in artificial Calculus bovis [Figure 1]. Compound 3 was identified as GCA according to the prominent $[M-H]^-$ ion at m/z 464.3018, and its MS^2 product ion at m/z 402.2941 represented a loss of C_2H_4NO [Figure 7]. The other MS^2 fragment ions were at m/z 420.2153 and 376.0374, and 420.2153 was a loss of CO_2. GCA was not detected in natural Calculus bovis, and the density of it in artificial Calculus bovis was much higher than in *in vitro* Cultured Calculus bovis. Compound 8 and 7 showed the $[M-H]^-$ ion respectively at m/z 448.3068 and m/z 448.3065 [Figure 8]. They also generated MS^2 product ion at m/z 386.2315 and m/z 386.3240, which was a loss of C_2H_4NO. In addition to the MS^2 fragment ions at m/z 404.4071 and m/z 404.4156 were a loss of

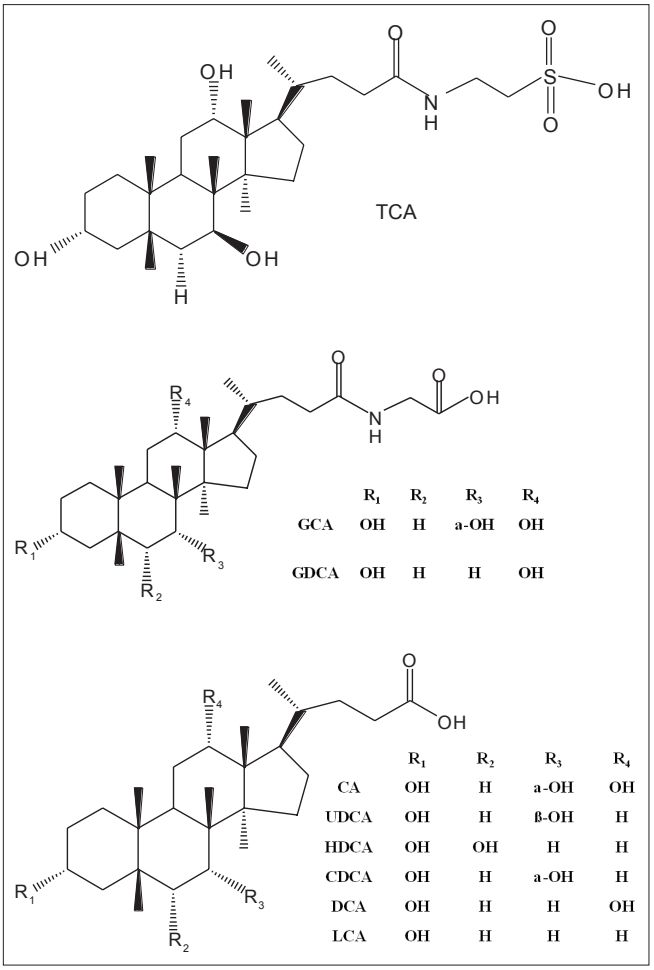

Figure 5: The structure of bile acids

CO_2, similarly. But the polarity of GDCA was stronger than iso-GDCA according to the polarity of DCA and its isomers. Therefore, compound 8 and 7 were identified as

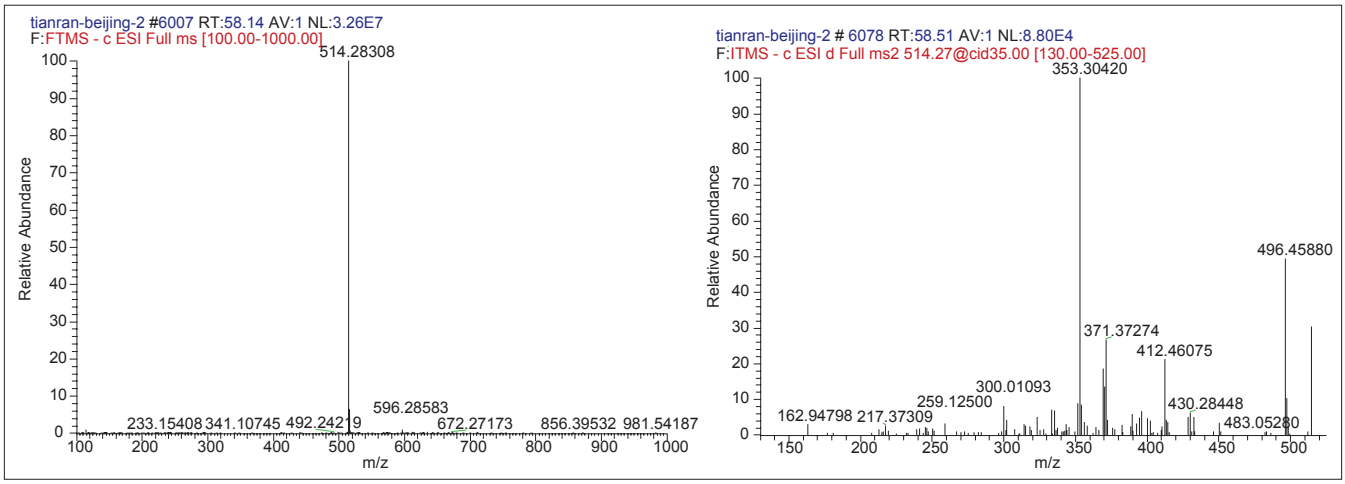

Figure 6: The mass spectrometry[1] MS and MS[2] of taurocholic acid

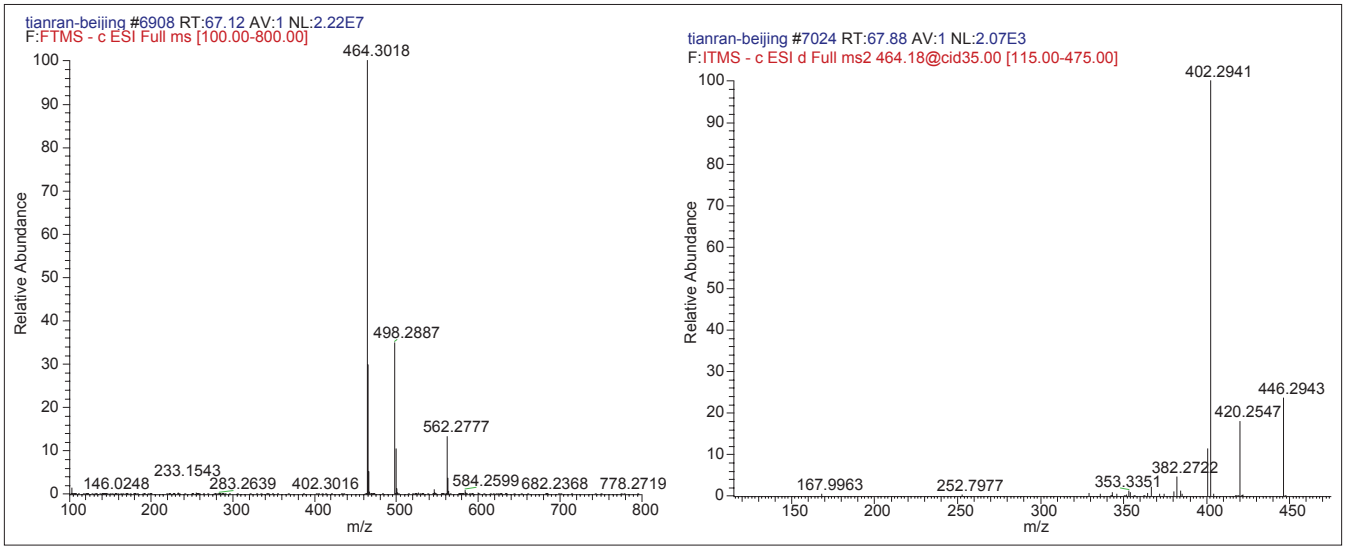

Figure 7: The mass spectrometry[1] (MS) and MS[2] of glycocholic acid

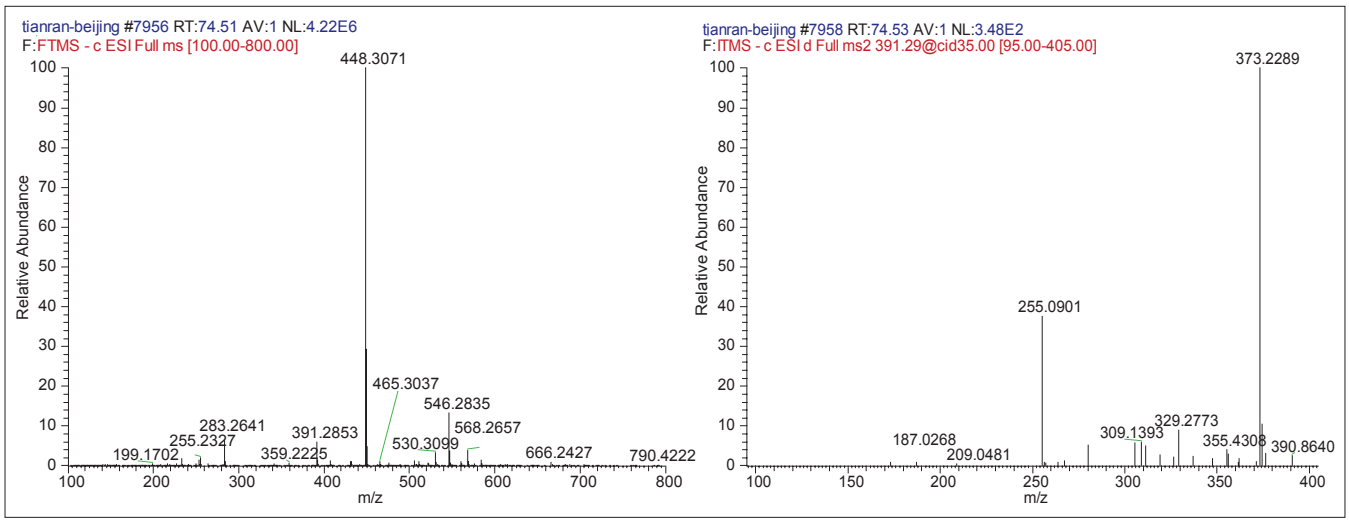

Figure 8: The mass spectrometry[1] (MS) and MS[2] of glycodeoxycholic acid

Table 2: Identification of constituents of calculus bovis by LC-DAD/ESI-MS^n

t_R (min)	Formula	Observed mass	Calculated mass	Error (ppm)	MS^2	Identification
58.14	$C_{26}H_{45}NO_7S$	514.2831	514.2833-0.427	−0.427	353.3042, 496.4588, 371.3727	TCA
64.25	$C_{24}H_{40}O_5$	407.2798	407.2792	0.281	389.1498, 345.3354, 325.2583	iso-CA
66.88	$C_{26}H_{42}NO_6$	464.3016	464.3007	0.547	420.2153, 402.3163, 376.0374	GCA
69.18	$C_{24}H_{38}O_5$	405.2642	405.2636	1.503	343.2352, 361.2384, 289.1825	3α,12α-dihydroxy-7-oxo-5β-CA
71.52	$C_{24}H_{40}O_5$	407.2798	407.2792	0.041	361.2221, 341.3354, 371.2583	HDCA
72.61	$C_{24}H_{40}O_5$	407.2798	407.2792	0.001	343.2910, 289.1625, 353.1927	CA
73.31	$C_{26}H_{42}NO_5$	448.3065	448.3057	0.162	386.3240, 404.4156, 340.1675	iso-GDCA
74.19	$C_{26}H_{42}NO_5$	448.3068	448.3057	0.372	404.4071, 430.2695, 386.2315	GDCA
74.30	$C_{24}H_{40}O_4$	391.2850	391.2843	0.135	373.2746, 347.3000, 293.0165	UDCA
76.02	$C_{24}H_{40}O_4$	391.2850	391.2843	0.165	345.2654, 355.2810, 371.2582	HDCA
78.31	$C_{24}H_{40}O_4$	391.2844	391.2843	−0.445	373.2087	CDCA
79.00	$C_{24}H_{40}O_4$	391.2847	391.2843	−0.165	345.2941, 327.2352, 355.2016	DCA
84.62	$C_{24}H_{40}O_3$	375.2901	375.2894	0.210	357.2033, 329.3588, 171.3024	LCA

LC-DAD/ESI-MS^n: Liquid chromatography-diode array detector-electrospray ionization/mass spectrometric; TCA: Taurocholic acid; GCA: Glycocholic acid; HDCA: Hyodeoxycholic acid; GDCA: Glycodeoxycholic acid; UDCA: Ursodeoxycholic acid; DCA: Deoxycholic acid; LCA: Lithocholic acid; CDCA: Chenodeoxycholic acid

GDCA and iso-GDCA. GDCA was discovered in natural, and artificial Calculus bovis, but not in the *in vitro* Cultured ones, and the density of GDCA in artificial Calculus bovis was much higher than in natural Calculus bovis. Based on the above discussion, these compounds could be considered as bio-markers of different Calculus bovis.

Lithocholic acid was detected in *in vitro* Cultured Calculus bovis, but not in natural and artificial Calculus bovis. Three kinds of Calculus bovis could be distinguished according to the above discussion, which provides a new method for the quality control of Calculus bovis and their substitutes.

Characterization of cholic acids

As shown in Table 2, a total of 13 bile acids was identified from natural, artificial and *in vitro* cultured Calculus bovis, which chemical structures are shown in Figure 5. Bile acids were identified according to their pseudo-molecular ions and their pseudo-molecular fragmentations in the negative ion mode. The pseudo-molecular $[M-H]^-$ of cholic acids were at 407. Compound 5 could be identified as hyocholic acid in accordance with the MS^2 fragment ion at m/z 371.2583, which represented a loss of $2H_2O$. Compound 6 could be considered as CA on the basis of the MS^2 fragment ion at m/z 353.1927, a loss of $3H_2O$.[14] Compound 2 was iso-CA due to its $[M-H]^-$ ion at m/z 407, but it could not be deduced what kind of compound it is. Compound 9-12 all generated $[M-H]^-$ at m/z 391. According to previous reports,[14,15] they were identified as ursodeoxycholic acid, HDCA, CDCA, and DCA respectively.

CONCLUSIONS

This study introduces a new method to differentiate the Calculus bovis and their substitutes based on the analysis of bile acids efficiently using HPLC coupled with tandem MS, which provides a novel way to control the quality of Calculus bovis.

The chemical compositions could be identified with high-resolution MS when the structure of compounds is given. What's more, MS coupled with quantum chemical technology has an advantage in speculating new compound that has to be validated further using nuclear magnetic resonance spectrum. However, samples were analyzed in small batches in this work. It is much better to increase batch number in further study.

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