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Research Article

Determination of multicomponent contents in *Calculus bovis* by ultra-performance liquid chromatography–evaporative light scattering detection and its application for quality control

A fast ultra-performance liquid chromatography–evaporative light scattering detection (UPLC-ELSD) method was established for simultaneous quantification of seven components in natural *Calculus bovis* (*C. bovis*) and its substitutes or spurious breeds. On a Waters Acquity UPLC® BEH C_{18} column, seven analytes were efficiently separated using 0.2% aqueous formic acid–acetonitrile as the mobile phase in a gradient program. The evaporator tube temperature of ELSD was set at 100°C with the nebulizing gas flow-rate of 1.9 L/min. The results showed that this established UPLC-ELSD method was validated to be sensitive, precise and accurate with the LODs of seven analytes at 2–11 ng, and the overall intra-day and inter-day variations less than 3.0%. The recovery of the method was in the range of 97.8–101.6%, with RSD less than 3.0%. Further results of PCA on the contents of seven investigated analytes suggested that compounds of cholic acid, deoxycholic acid and chenodeoxycholic acid or cholesterol should be added as chemical markers to UPLC analysis of *C. bovis* samples for quality control and to discriminate natural *C. bovis* sample and its substitutes or some spurious breeds, then normalize the use of natural *C. bovis* and ensure its clinical efficacy.

Keywords: *Calculus bovis* / Principal component analysis / Quantitative determination / Quality control / UPLC-ELSD
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1 Introduction

Calculus bovis (Niuhuang in Chinese and known as cow bezoar, a type of rare Chinese medicinal material) was first reported in *Shennong Bencao Jing* (the Divine Farmer's Herbal Classic, 22–250$_{AD}$, the earliest monograph on medical materials in China) and has been used clinically in China for 2000 years [1]. Among the 365 drugs listed in this book, *C. bovis* was recorded as top grade. It is the dry gallstone of *Bos taurus domesticus* Gmelin, and according to the theory of traditional Chinese medicine has the effect of sedation, relieving fever, diminishing inflammation, immunoregularity, antihyperspasmia, anticancer and anticonvulsive [2–7]. Thousands of Chinese patent medicines and their preparations contain this medicinal material for many clinical demands and goals of treatment [8–11]. Because of limited source of natural *C. bovis*, artificial *C. bovis*, *in vivo* artificial cultivated *C. bovis* and *in vitro* cultured *C. bovis*, even some spurious breeds, such as *C. bovis* powder, *Fellis suis* (*F. suis*) and *Fellis ursi* powder, *etc.*, are always applied as substitutes in the medicinal preparations [12]. But the internal quality of these substitutes or spurious breeds with various sources and preparation technologies is different from or inferior to natural *C. bovis*. The disordered use and abuse result in the loss of original pharmaceutical actions and therapeutic values of this natural product. So it is an important issue to comprehensively evaluate the different species of *C. bovis*, so as to ensure the clinical efficacy of this medicinal material.

Chemical and pharmacological investigations on *C. bovis* resulted in discovering several kinds of bioactive components, *i.e.* bile acids, bilirubin and some inorganic salts [12, 13]. Bile acids are a mixture of steroids, mainly including cholic acid (CA), deoxycholic acid (DCA), hyodeoxycholic acid (HDCA), chenodeoxycholic acid

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Abbreviations: CA, cholic acid; **CDCA,** chenodeoxycholic acid; **CHOL,** cholesterol; **DCA,** deoxycholic acid; **ELSD,** evaporative light scattering detection; **HDCA,** hyodeoxycholic acid; **MeOH,** methanol; **PC,** principal component; **UDCA,** ursodeoxycholic acid; **UPLC-ELSD,** ultra-performance liquid chromatography–evaporative light scattering detection; **TCANa,** sodium taurocholate

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(CDCA) and others [14, 15]. In China Pharmacopoeia, bile acids are the major active components and CA has been used as the chemical marker for the quality control of *C. bovis* and its preparation [2]. But this compound is not a characteristic component of *C. bovis*, as it has also been contained in many other medicinal materials and their preparations with high contents [16, 17]. Moreover, CA alone could not be responsible for the overall pharmacological activities of *C. bovis*. Therefore, CA should also be considered as one of the markers for quality control of *C. bovis*. Consequently, we believe that determinations of more components such as DCA, ursodeoxycholic acid (UDCA) and HDCA in *C. bovis* would be a better strategy for the comprehensive quality control of this medicinal material. Spectrophotometry [18], TLC [19], CE [15], HPLC [20] and other methods are commonly employed to determine the amounts of bile acids in raw materials, Chinese traditional medicine preparations and other bio-samples of *C. bovis* [21–23]. Among them, HPLC with various detectors is most widely used. However, the absence of a chromophore in these bile acids hampers their detection with a UV detector. MS detector or the instrument of NMR is lacking in most laboratories [22, 24]. Evaporative light scattering detection (ELSD) has advantages over other techniques in determining the non-chromophoric compounds in traditional Chinese medicine and it can also detect all analytes less volatile than the solvent [22, 25–27]. The baseline can be maintained stable regardless of the solvent's UV absorbance and the slope of the gradient conditions. HPLC with ELSD method is a widely employed separation technique, and has been successful in pharmaceutical analysis of *C. bovis* [13, 16, 28], but its sensitivity is well known to be unsatisfactory and the running time is much longer. Ultra-performance liquid chromatography (UPLC), which utilizes silica particles 1.7 μm, makes it possible to perform efficient separations for many compositional components in short periods of time. It has the advantages of fast analysis, high peak capacity, great resolution and good sensitivity [29–32].

Hence, in this paper we reported here for the first time the development of a ultra-performance liquid chromatography–evaporative light scattering detection (UPLC-ELSD) method for the simultaneous determination of seven components in the Chinese medicinal material of *C. bovis*. The aims of this study are (i) to simultaneously and quantify rapidly seven components in natural *C. bovis* and its substitutes or spurious breeds; (ii) to discriminate natural *C. bovis* samples and its substitutes or spurious breeds using PCA and (iii) to provide some references for the quality control of this Chinese medicinal material with different sources and preparation technologies.

2 Materials and methods

2.1 Samples, chemicals and reagents

Twenty samples of natural *C. bovis* and its substitutes (cultured *C. bovis*, artificial cultivated *C. bovis*, artificial *C. bovis*) and two spurious breeds (*C. bovis* powder, *F. suis* powder) from various places were collected and are listed in Table 1 and all were authenticated by Professor Xiao-He Xiao (China Military Institute of Chinese Materia Medica, 302 Military Hospital of China, Beijing, P. R. China).

Seven reference compounds CA, DCA, HDCA, CDCA, UDCA, cholesterol (CHOL) and sodium taurocholate (TCANa) were purchased from the National Institute for the

Table 1. Summary of investigated samples

No.	Code	Species	Properties	Site of collection	Date of collection
1	NCB-1	Natural *C. bovis*	Natural	Hebei province	2009.04
2	NCB-2	Natural *C. bovis*	Natural	Hubei province	2008.05
3	ACB-1	Artificial *C. bovis*	*In vitro* prepared	Sichuan province	2008.08
4	ACB-2	Artificial *C. bovis*	*In vitro* prepared	Sichuan province	2008.09
5	ACB-3	Artificial *C. bovis*	*In vitro* prepared	Chongqing city	2009.03
6	ACB-4	Artificial *C. bovis*	*In vitro* prepared	Sichuan province	2009.03
7	ACB-5	Artificial *C. bovis*	*In vitro* prepared	Beijing city	2009.04
8	ACB-6	Artificial *C. bovis*	*In vitro* prepared	Anhui province	2008.09
9	ACB-7	Artificial *C. bovis*	*In vitro* prepared	Shanghai city	2008.03
10	ACB-8	Artificial *C. bovis*	*In vitro* prepared	Sichuan province	2009.02
11	ACB-9	Artificial *C. bovis*	*In vitro* prepared	Jiangxi province	2008.06
12	ACB-10	Artificial *C. bovis*	*In vitro* prepared	Guangdong province	2008.03
13	ACB-11	Artificial *C. bovis*	*In vitro* prepared	Guangdong province	2008.03
14	ACB-12	Artificial *C. bovis*	*In vitro* prepared	Sichuan province	2007.11
15	CCB-1	Cultured *C. bovis*	*In vitro* cultured	Henan province	2008.03
16	CCB-2	Cultured *C. bovis*	*In vitro* cultured	Hubei province	2009.05
17	ACCB-1	Artificial cultivated *C. bovis*	*In vivo* cultivated	Sichuan province	2007.02
18	ACCB-2	Artificial cultivated *C. bovis*	*In vivo* cultivated	Zhejiang province	2009.05
19	CBP	*C. bovis* powder	Natural	Anhui province	2009.02
20	FSP	*F. Suis* powder	Natural	Anhui province	2009.02

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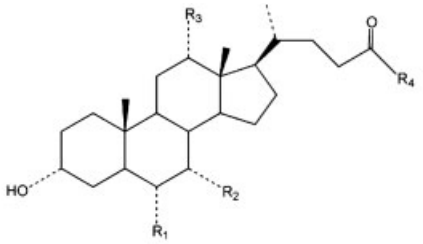

Compounds	R_1	R_2	R_3	R_4	Formula weight
Cholic acid	-H	-OH	-OH	-COOH	408.58
Deoxycholic acid	-H	-H	-OH	-COOH	392.58
Hyodeoxycholic acid	-OH	-H	-H	-COOH	392.58
Chenodeoxycholic acid	-H	-OH	-H	-COOH	392.58
Ursodeoxycholic acid	-H	-OH	-H	-COOH	392.58
Cholesterol	-H	-H	-H	$-(CH_2)CH(CH_3)_2$	386.64
Taurocholate sodium	-H	-OH	-OH	$-CONH(CH_2)_2SO_3Na$	537.68

Figure 1. Chemical structures of seven investigated analytes.

Control of Pharmaceutical and Biological Products of China, Beijing, P. R China. The purity of all compounds is more than 98% and their structures are given in Fig. 1.

Acetonitrile of chromatographic grade was purchased from Fisher Chemicals (Pittsburg, PA, USA). Other solvents, such as methanol (MeOH), formic acid, obtained from Beijing Chemical Factory (Beijing, China), were all of analytical grade. High-purity water was obtained using a Milli-Q water purification system (Billerica, MA, USA).

2.2 UPLC-ELSD analysis

UPLC was performed using a Waters Acquity system equipped with binary solvent delivery pump and an auto sampler. The chromatographic separation was performed on Waters Acquity UPLC® BEH C_{18} column (2.1 × 50 mm, 1.7 μm) at a column temperature of 25°C. The mobile phase consisted of (A) 0.2% aqueous formic acid and (B) acetonitrile with a flow-rate of 0.4 mL/min using a gradient elution of 30% B at 0–2.5 min, 30–35% B at 2.5–3.0 min, 35% B at 2.5–8.6 min, 35–45% B at 8.6–9.6 min, 45–100% B at 9.6–13.0 min.

2.3 HPLC-ELSD analysis

The HPLC separation was also performed using the above equipment with an AQ-C_{18} (250 mm × 4.6 mm, 5 μm) column at a column temperature of 25°C and liquid flow-rate of 1.0 mL/min using (A) 0.2% aqueous formic acid and (B) acetonitrile as mobile phase with the flow-rate of 1.0 mL/min using a gradient elution of 35% B at 0–10.0 min, 35–50% B at 10.0–20.0 min, 50% B at 20.0–30.0 min, 50–95% B at 30.0–50.0 min.

An Alltech 2000ES ELSD (Deerfield, IL, USA) detector was connected to this LC system with a Waters Sat software, and the evaporator temperature for the ELSD was set at 100°C with a nitrogen flow-rate of 1.9 L/min.

2.4 Preparation of mixed standard solution

The mixed standard solutions containing 75.7 μg/mL TCANa, 72.9 μg/mL CA, 61.4 μg/mL DCA, 80.0 μg/mL HDCA, 78.6 μg/mL CDCA, 75.7 μg/mL UDCA and 56.0 μg/mL CHOL were prepared by adding an accurately weighed amount of each standard substance into a volumetric flask and dissolved with MeOH, and then filtered through 0.22 μm millipore membrane to yield the mixed standard solutions.

2.5 Preparation of sample solution

The powdered sample (about 0.05 g) was accurately weighed and extracted in triplicate with MeOH (10 mL each) for 30 min. The extracts were filtered, mixed and concentrated to 10 mL, and then filtered through 0.22 μm millipore membrane and an aliquot of 5 μL of the filtrate was used for the UPLC analysis.

2.6 Calibration curves, LOD and LOQ

The stock solution of mixed standard containing seven analytes was diluted with MeOH to the appropriate concentrations for preparing calibration curves. Six concentrations of the solution were analyzed in duplicate, and then the calibration curves were constructed by plotting the logarithmic values of peak areas and mass for each analyte. The linearity was evaluated by linear regression analysis calculated by the least-squares regression method. The LOD and LOQ under the present chromatographic conditions were determined on the basis of response at a S/N of 3 or 10, respectively.

2.7 Precision, repeatability and accuracy

Intra- and inter-day variations were chosen to determine the precision of the established method. For intra-day variability test, the mixed standards solutions were analyzed for six replicates within 1 day, while for inter-day variability test, the solutions were examined in duplicate for consecutive 3 days. Variations were expressed by RSD. For each calibration curve, the calibration concentrations were back-calculated from the peak area of the analytes. The deviation from the nominal concentration was defined as accuracy.

The repeatability of the established method was evaluated at three levels (0.025, 0.050 and 0.100 g) of the sample NCB-1 in Table 1. The samples of each level were extracted and analyzed in triplicate as mentioned above. The RSD ($n = 3$) for repeatability was calculated.

The recovery was performed by spiking the standards of seven analytes into sample NCB-1 at three concentration levels (approximately equivalent 0.8, 1.0, 1.2 times of the estimated mass in the matrix) with three parallels at each level. Then, the fortified samples were extracted,

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disposed as the sample preparation procedure, analyzed in triplicate. The average recoveries were estimated by the following formula: recovery (%) = (amount found – original amount)/amount spiked × 100% with RSD (%) = (SD/mean) × 100%.

Then this established UPLC-ELSD method was used to determine the contents of seven analytes in all 20 samples.

2.8 Data analysis

2.8.1 PCA on the contents of seven components

Without assuming any previous knowledge of sample class, PCA [31, 33–35], as a non-parametric and unsupervised pattern recognition method, reduces the dimensionality of the original data set by analyzing the correlation among a large number of variables in terms of a small number of underlying factors (principal components or PCs) without losing much information. PCA always results in score plots that provide a visual determination of the similarities and dissimilarities among the samples with respect to their biochemical composition. From the visualization of the data in a reduced dimensional space by this method, the normal and robust samples can be separated and discriminated. The score plots of PC1 *versus* PC2 based on the contents of seven components in these samples were examined for the separation or clusters relating to different groups of natural *C. bovis* and its substitutes or spurious breeds.

2.8.2 Finding chemical markers

The numerical value of a loading of a given variable on a PC shows how much the variable has in common with that component. If the scores plot can discriminate the different groups of samples, the loadings plot can be used to express the components responsible for the separation among samples. The variables having the most influence on the scores plot are those furthest away from the main cluster of variables. In this study, loading plots of PCA were employed to find chemical markers for discrimination among different groups of natural *C. bovis* samples and its substitutes or spurious breeds.

The program of PCA based on the mean-centered with no scaling data is performed using the Unscrambler®, v9.7, CAMO software AS, Norway.

3 Results and discussions

3.1 Comparison of HPLC-ELSD and UPLC-ELSD

For a complex mixture, reliable methods using HPLC for the separation of main components in *C. bovis* samples were relatively time-consuming and also it would result in inefficiency, as they required the use of large amounts of organic solvents and instrument time [13, 17]. Methods for rapid, high resolving and efficient determination of main components in *C. bovis* are of great interest. UPLC method has been introduced to offer greater resolution, good sensitivity and high speed of analysis for the separation of these seven analytes. This could be seen from the comparison of the typical ELSD performance parameters of HPLC and UPLC of mixed standards shown in Table 2 using the four parameters of retention time, USP resolution, tailing factor and plate count as investigation indexes. Compared with the HPLC method, the established UPLC method only needs 16.0 min for analysis, which is only one-third of analysis time of HPLC. With much shorter analysis time, lower solvent consumption, more satisfactory resolution, much smaller USP tailing factor and plate count than HPLC-ELSD, the UPLC-ELSD method was effective for the comprehensive analysis for natural *C. bovis* sample and its substitutes or some spurious breeds.

Table 2. Comparison of system performance of HPLC and UPLC

Analytes	Retention time (min)		Resolution		USP tailing factor		USP plate count	
	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC
TCANa	1.918	7.113	2.65	2.32	0.93	1.01	1762	24 321
CA	6.782	13.321	2.96	2.42	0.77	1.33	29 801	31 632
UDCA	7.457	15.224	2.10	2.03	1.03	1.49	21 932	33 509
HDCA	8.051	16.237	1.31	1.72	0.72	1.31	29 882	34 820
CDCA	12.115	25.521	1.82	1.43	0.99	1.81	51 883	113 084
DCA	12.659	26.743	1.21	2.47	1.02	1.92	55 759	114 280
CHOL	14.406	43.446	2.81	2.06	0.52	1.33	63 218	128 321

3.2 Optimization of UPLC conditions

A series of mobile phases including acetonitrile–water and MeOH–water in combination with acetic acid or formic acid were examined. Finally, it was found that 0.2% aqueous formic acid–acetonitrile system with gradient elution gave the best separation of seven analytes. Representative chromatograms for the seven standard analytes and natural *C. bovis* samples and its substitutes or spurious breeds were shown in Fig. 2. Figure 2A showed that the seven standard analytes were well separated and the resolution between any two compounds was greater than 1.2. The total run time was less than 16.0 min. The chromatographic peaks were identified by comparing their retention time with that of each reference compound, which was eluted in parallel with a series of mobile phases. In addition, spiking samples with the reference compounds showed no additional peaks, which further confirmed the identities of the analytes' peaks.

3.3 Optimization of ELSD parameters

According to the literature [36], controlling the two parameters of ELSD including evaporator tube temperature

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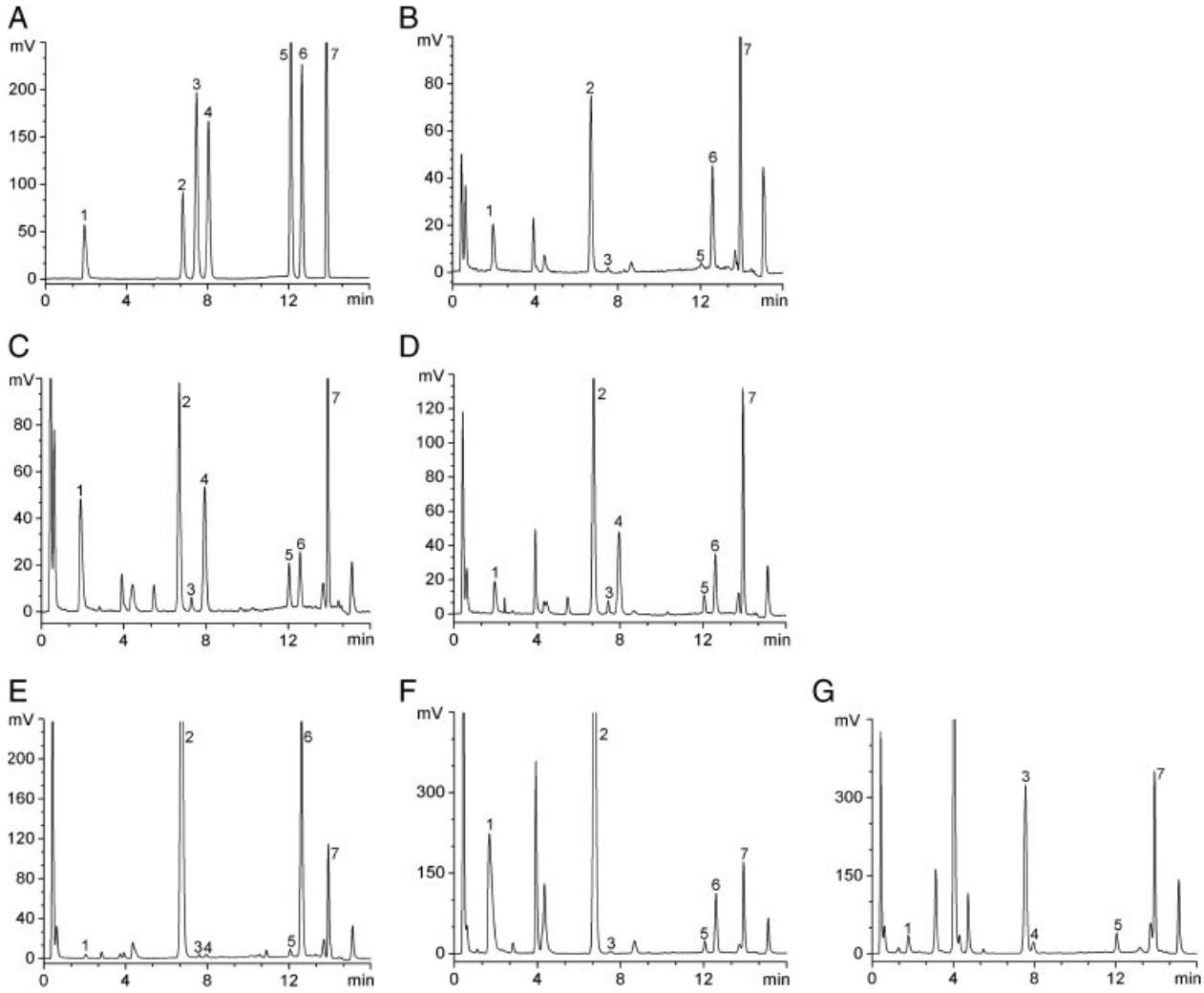

Figure 2. Typical UPLC chromatograms of mixed standards and various samples. Column: Waters Acquity UPLC® BEH C_{18} column (2.1 × 50 mm, 1.7 μm), detector: ELSD. (A) Mixed standards; (B) natural *C. bovis*; (C) artificial *C. bovis*; (D) in *vivo* artificial cultivated *C. bovis*; (E) *in vitro* cultured *C. bovis*; (F) *C. bovis* powder; (G) *F. suis* powder. *Peaks*: (1) TCANa; (2) CA; (3) UDCA; (4) HDCA; (5) CDCA; (6) DCA; (7) CHOL.

and nebulizing flow-rate is important in the analytical procedure for the accuracy and reproducibility. These parameters were optimized to obtain the best signals when ratio of S/N was taken as the observation index. Compound CA was selected as a model component for optimizing ELSD conditions because it was contained in almost all the samples. Temperature of evaporator tube (85–115°C) and flow-rate of nitrogen gas (1.0–3.1 L/min) for the detector were evaluated systematically and respectively. The effect of evaporator tube temperature of ELSD based on S/N of CA showed that the optimal temperature was identified as 100°C. In general, the highest signal should be obtained at the lowest nebulizing gas flow-rate, but in the present study, when the gas flow-rate was too low or high, the baseline was unstable and the S/N became advanced. So, the optimal gas flow-rate was 1.9 L/min according to the effect of gas flow-rate on S/N of CA. Accordingly, the optimized parameters of ELSD were 100°C for evaporator tube temperature and 1.9 L/min for nitrogen gas flow-rate.

3.4 Validation of the developed method

The established UPLC-ELSD method for quantitative evaluation of seven analytes was validated according to International Conference on Harmonization Guidelines [37]. It has been generally observed that the detector response, as measured by peak area, varies exponentially with the mass of analyte, and this behavior can be mathematically expressed in a logarithmic form. Present experimental results showed that the logarithmic value of peak area of each standard analyte was linearly correlated to the logarithmic value of injected mass within an acceptable range (Table 3). All calibration curves showed good linear regression in the range ($r = 0.9987-0.9997$). The

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LODs ($S/N = 3$) and LOQs ($S/N = 10$) for all standard analytes were in the range of 2–11 and 7–33 ng, respectively, indicating that this UPLC-ELSD method is sensitive for the quantitative determination of major active components in *C. bovis* samples. Validation studies of the method proved (Table 4) that this assay had good reproducibility, and the overall intra-day and inter-day variations were less than 3.0% for all analytes. The analytes in the sample solution was stable for 3 consecutive days with an RSD of 0.88–1.99%. As shown in Table 5, the established analytical method had good accuracy with satisfactory recovery in the range of 97.8–101.6% for the concerned analytes. Therefore, the established UPLC-ELSD method is precise, accurate and sensitive for the simultaneous quantitative determination of seven compounds in natural *C. bovis* samples and its substitutes or spurious breeds.

3.5 Quantitation of seven analytes in C. bovis samples

The developed UPLC-ELSD method was applied to analyze the 7 analytes in 20 samples of natural *C. bovis* and its substitutes or spurious breeds and the data were summarized in Table 6. The natural *C. bovis* samples and its substitutes or spurious breeds have various chromatographic profiles and different contents of the seven analytes and the details of which were given in Fig. 2, Tables 1 and 6. Some substitutes of natural *C. bovis*, for example samples Nos. 2, 4, 6, 10 and 14 from the same source (Sichuan province, Table 1), have large differences in the contents of the seven investigated compounds. Table 6 and Fig. 3F showed that HDCA was not detected in *C. bovis* powder, but was detected in most *C. bovis* samples. Table 6 and Fig. 2G also showed that the main components CA and DCA were not detected in *F. suis* power, but were detected in all *C. bovis* samples and *C. bovis* power. All these results showed that there were great variations among the contents of the seven investigated analytes in natural *C. bovis* sample and its substitutes from different preparation technologies, sources, collection and/or storage times and/or locations and the spurious breeds. These factors had large influences on the internal quality of different samples. Also, it was uncertain whether these compounds mentioned above could be used to evaluate the quality among *C. bovis* samples, *C. bovis* powder and *F. suis* powder. This would be studied and validated in the next section.

3.6 Comparison and discrimination of samples

3.6.1 PCA on the 20 samples

In China, the substitutes (artificial *C. bovis*, *in vivo* artificial cultivated *C. bovis*, *in vitro* cultured *C. bovis*), even some spurious breeds, such as *C. bovis* powder, *F. suis* powder

Table 3. Regression equation, LOD and LOQ of investigated analytes.

No.	Analytes	Regression equation[a)]	r	Linear range (μg)	LOD (ng)	LOQ (ng)
1	TCANa	$y = 1.3524x + 6.2360$	0.9991	0.0379–1.1355	3.0	10.0
2	CA	$y = 1.5106x + 6.3771$	0.9995	0.0365–1.458	3.0	11.0
3	UDCA	$y = 1.4181x + 6.7212$	0.9992	0.0151–0.8327	4.0	15.0
4	HDCA	$y = 1.2200x + 6.5832$	0.9997	0.08–1.2	4.0	13.0
5	CDCA	$y = 1.3399x + 6.8252$	0.9994	0.0393–1.179	9.0	31.0
6	DCA	$y = 1.3690x + 6.7909$	0.9993	0.0307–0.921	11.0	33.0
7	CHOL	$y = 1.9663x + 6.7354$	0.9987	0.0224–0.448	2.0	7.0

a) In the regression equation $y = ax + b$; y, x are the logarithmic values of peak area and mass (μg) of the reference analytes, respectively.

Table 4. Results of intra-, inter-day precision, accuracy and repeatability.

Analytes	Accuracy and precision						Repeatability ($n = 6$)	
	Intra-day ($n = 6$)			Inter-day ($n = 6$)			Mean (μg/mL)	RSD (%)
	Mean (μg/mL)	RSD (%)	Accuracy[a)] (%)	Mean (μg/mL)	RSD (%)	Accuracy (%)		
TCANa	45.32	2.24	100.3	47.65	2.42	101.4	51.24	1.89
CA	51.26	0.67	99.9	53.33	0.89	100.3	68.43	1.25
UDCA	60.24	1.33	101.2	61.82	1.64	100.0	66.37	0.97
HDCA	57.87	1.08	100.0	58.56	1.42	99.3	70.29	1.07
CDCA	52.35	0.99	98.7	51.99	1.04	100.6	61.02	0.88
DCA	48.72	1.22	99.9	47.66	1.53	102.3	52.55	1.62
CHOL	41.81	2.11	100.5	43.18	2.07	100.9	47.47	1.99

a) Concentration detected/concentration spiked × 100%.

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Table 5. Recoveries for the assay of seven analytes

Analytes[a)]	Original (μg)	Spiked (μg)	Found (μg)	Recovery[b)] (%)	Mean (%)	RSD (%)
TCANa	0.262	0.208	0.467	98.6	99.8	1.01
	0.262	0.255	0.518	100.4		
	0.262	0.316	0.579	100.3		
CA	0.635	0.511	1.149	100.6	99.7	0.76
	0.635	0.640	1.270	99.2		
	0.635	0.765	1.395	99.4		
UDCA	0.016	0.013	0.029	100.0	101.6	2.72
	0.016	0.018	0.034	100.0		
	0.016	0.021	0.038	104.8		
HDCA	0.349	0.281	0.634	101.4	100.3	1.22
	0.349	0.351	0.702	100.6		
	0.349	0.415	0.760	99.0		
CDCA	0.053	0.040	0.091	95.0	97.8	2.60
	0.053	0.050	0.103	100.0		
	0.053	0.059	0.111	98.3		
DCA	0.103	0.080	0.181	97.5	100.1	2.28
	0.103	0.104	0.209	101.9		
	0.103	0.125	0.229	100.8		
CHOL	0.201	0.159	0.358	98.7	99.6	1.21
	0.201	0.199	0.402	101.0		
	0.201	0.238	0.437	99.2		

a) The notation for analyte refers to Fig. 1. Triplicate assay at each concentration level.
b) Recovery (%) = (amount found − original amount)/amount spiked × 100%.

were widely used as natural *C. bovis*. However, this work showed that the chemical variation was large among different samples even between the two natural *C. bovis* samples. This variation would be sure to result in the differences of internal quality and pharmaceutical actions. Therefore, the exact identity, comparison and discrimination of these samples were assurance of safety and efficacy of medication. In order to evaluate and discriminate these samples, PCA was performed based on the contents of seven investigated analytes (bile acids, CHOL and TCANa) in 20 tested samples.

For the data of 20 samples in Table 6, a six-component model explained 99.33% of the variance, with the first two components explaining 87.04% of the variability. The 2-D scores plot of the first two PCs (Fig. 3A), where each coordinate represented a sample, showed the clear distribution of the natural *C. bovis* samples, the substitutes and spurious breeds. From the scatter points, the 20 samples could be clustered into 3 groups, which were marked as groups I–III. The samples clustered into one group were associated with similar chemical properties/components. The distances among the groups reflected the discrepancy degree of these samples. Group III and groups I, II had large distances, showing samples No. 19 and 20 (*C. bovis* powder and *F. suis* powder) in group III could be significantly distinguished from *C. bovis* samples and its substitutes. Samples No. 15 and 16 (cultured *C. bovis*) in group II could also be discriminated from group I. However, the overlapping clusters could not be avoided among natural *C. bovis* samples and its substitutes, such as in group II, the

Table 6. Contents (mg/g) of investigated analytes in natural *C. bovis* and its substitutes or spurious breeds

Samples	Analytes[a)]						
	CA	UDCA	HDCA	CDCA	DCA	TCANa	CHOL
1	13.59	1.19	–[b)]	1.05	4.85	6.63	5.84
2	11.58	0.62	–	1.70	3.96	0.89	4.16
3	17.58	0.72	7.12	2.55	3.67	15.11	5.43
4	2.22	–	–	1.31	1.52	1.30	5.46
5	24.79	0.71	7.15	1.63	3.81	7.56	6.05
6	22.52	–	9.53	1.13	0.69	–	5.38
7	13.03	–	6.49	1.08	2.02	5.23	5.72
8	2.46	0.83	0.14	0.16	0.77	1.88	4.70
9	12.98	–	7.31	1.06	2.02	4.73	5.77
10	12.52	0.59	2.95	2.30	5.25	4.06	7.14
11	29.53	–	4.02	0.65	2.46	–	7.00
12	6.57	–	0.54	0.32	0.53	0.75	6.45
13	7.06	–	1.38	0.77	0.55	–	4.39
14	13.63	–	1.06	1.22	1.48	1.29	4.37
15	53.73	0.76	0.23	0.21	10.319	1.76	7.96
16	57.27	0.29	0.23	1.27	14.39	2.01	6.71
17	16.79	–	6.98	1.76	3.46	12.83	5.83
18	11.92	–	6.74	0.80	1.14	7.38	5.38
19	70.85	0.44	–	1.94	6.72	11.72	5.29
20	-	3.22	21.61	3.68	–	8.56	6.97

a) The data were presented as average of duplicate.
b) Undetected.

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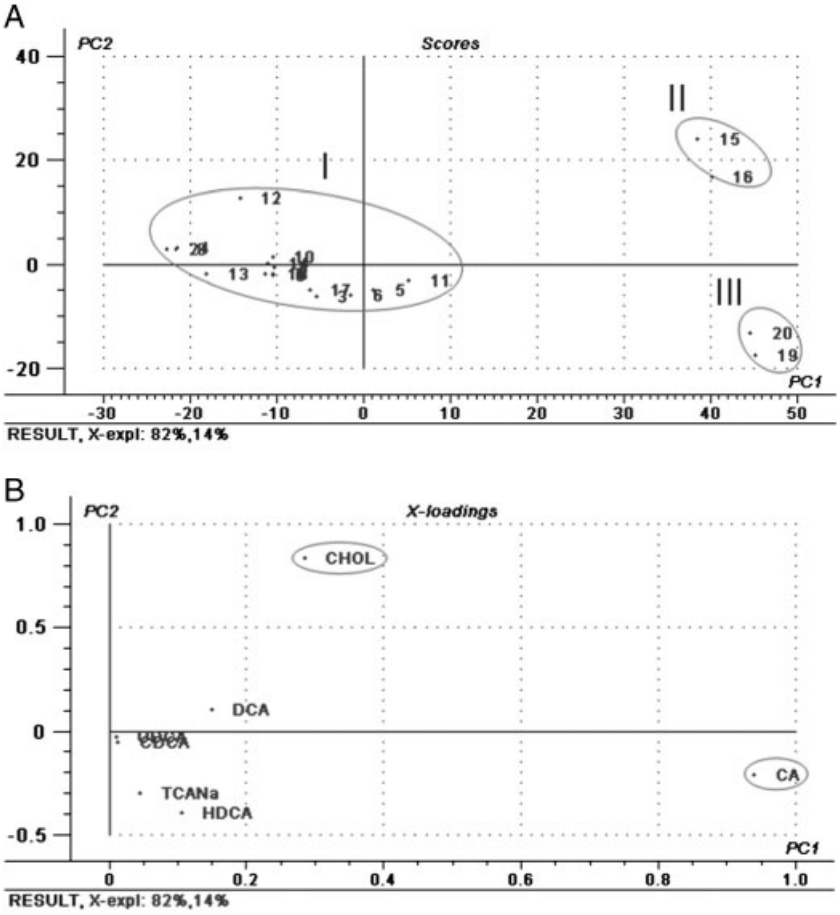

Figure 3. (A) 2-D scores plot of two PCs from PCA on the 20 samples (No. 1–20 in Table 1). PC1 and PC2 are the first two PCs using the mean-centered with no scaling contents of the seven investigated analytes as input data. (B) Corresponding loadings plot of the seven analytes; possible markers are marked with a circle.

substitutes (artificial cultivated *C. bovis*) were crossed with natural *C. bovis* samples and could not be discriminated. The discrimination ability would be evaluated in the next section.

The loadings plot of PCA (Fig. 3B) indicated that compounds of CA and CHOL might have more influence on the discrimination of *C. bovis* samples and the spurious breeds. By controlling the contents of these two compounds, we could control the internal quality of *C. bovis* samples and some spurious breeds, and further discriminate them.

3.6.2 PCA on the 18 samples

The spurious breeds could be easily discriminated from *C. bovis* samples based on the above analysis, but it was relatively difficult to discriminate natural *C. bovis* samples and its substitutes. In order to avoid the overlapping clusters among natural *C. bovis* samples and its substitutes and to well control the internal quality of them, the contents of seven investigated analytes in 18 samples (No. 1–18 in Table 1) were input to the software of Unscrambler 9.7.

For the data of 18 samples in Table 6, a six-component model explained 99.91% of the variance, with the first two components explaining 91.55% and the first three components explaining 95.41% of the variability. The clear separation of natural *C. bovis* samples and its substitutes was observed in the PCA 2-D scores plot (Fig. 4A) of the first two PCs. The plot could be readily divided into four groups: group I for samples of natural *C. bovis*, including samples No. 1 and 2, which were the dried gallstone from *Bostaurus domesticus* Gmelin; group II for samples of artificial *C. bovis*, including samples No. 3–14, which were prepared using the main materials of bilirubin, CA, CHOL and some inorganic salts; group III for samples of *in vitro* cultured *C. bovis*, including samples No. 15 and 16, which were obtained by implanting extraneous materials into the gallbladder of *B. domesticus* Gmelin as the carrier and precipitating the bile on this carrier; group IV for samples of *in vivo* artificial cultivated *C. bovis*, including samples No. 17 and 18, which were obtaining by simulating the generating progress of gallstone from *B. domesticus* Gmelin and preparing the products in manufactory or workshop. Then, we used the 3-D scores plot (Fig. 4B) to further and more intuitively observe the trends of clustering, which gave us a marginally better discrimination plot than Fig. 4A. Clearly, all the 18 samples could be more discriminated according to their preparation technology based on the contents of seven main components in natural *C. bovis* and its substitutes.

The loadings plot (Fig. 4C) illustrated that compounds of CA, DCA and CDCA might have more influence on the discrimination of natural *C. bovis* samples and the substitutes. By controlling the contents of these three compounds,

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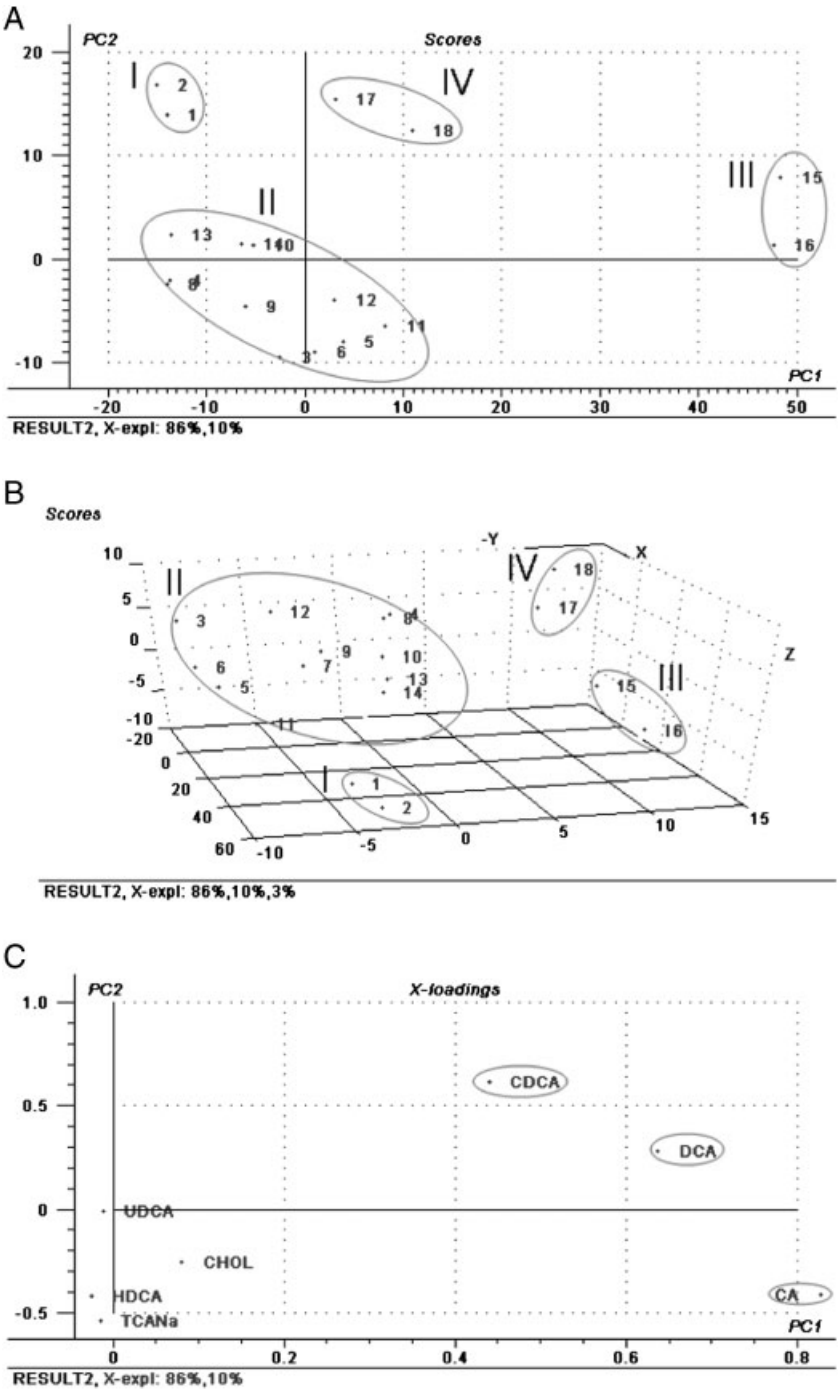

Figure 4. (A) 2-D scores plot of two *PCs* from PCA on the 18 *C. bovis* samples (No. 1–18 in Table 1), (B) 3-D scores plot of three PCs from PCA on the 18 *C. bovis* samples. *X*, *Y* and *Z* are the first three latent variables using the mean-centered with no scaling contents of the 7 investigated analytes in the 18 samples as input data. (C) Corresponding loadings plot of the seven analytes; possible markers are marked with a circle.

the internal quality of natural *C. bovis* samples and the substitutes could be well controlled and these samples could be markedly discriminated.

4 Concluding remarks

The ELSD detector was connected to the Waters UPLC equipment, and then the fast UPLC-ELSD method was applied for the simultaneous determination of seven components in natural *C. bovis* sample and its substitutes or spurious breeds. The results showed that this established method was validated to be sensitive, precise and quick, and has the ability to separate the active components in these samples. The simplicity and universality of the established method allows for application in labs that lack sophisticated analytical instruments, such as LC-MS, GC-MS or NMR.

The results of PCA on the contents of seven investigated analytes suggested that compounds of CA, DCA and

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CDCA or CHOL should be added as chemical markers to the UPLC analysis of *C. bovis* samples for quality control in the future and discriminating the samples of natural *C. bovis* and the substitutes or some spurious breeds, further to normalize the use of natural *C. bovis* medicinal material and ensure its original pharmacodynamic actions and clinical efficacy. This work provided some references for the quality control of *C. bovis* and other valuable Chinese medicinal materials. Future work would be focused on the different pharmacodynamic actions of these natural *C. bovis* samples and the substitutes or some spurious breeds.

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