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Identification and Quantitation of Bile Acids in Bear Bile by HPLC

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ABSTRACT

A method for simultaneous quantitation of tauroursodeoxycholic acid (TUDC), taurocholic acid (TC) and taurochenodeoxycholic acid (TCDC) using high performance liquid chromatography with a Vydac reversed phase C_{18} column is described. Peak area ratios versus concentration calibration curve for TUDC, TC and TCDC showed linearity over the range of 50-2000 μg/mL with correlation coefficient (r) of 0.9999, 0.9989 and 1.0000, respectively. Intraday and interday precisions range from 0.08 to 5.63% for TUDC, 0.28 to 6.70% for TC and 0.14 to 4.82% for TCDC. Quantitative HPLC analysis was performed on 93 Ursidae bile salts. Average concentrations were 3087 μg/mL for TUDC, 212.6 μg/mL for TC and 1968 μg/mL for TCDC. Our data suggests that North American and polar bears contain a higher concentration of TC (relative to TUDC and TCDC), while the relative concentration of TC in Asiatic bears (wild or farmed) is much lower. Analytical results of 217 gall bladder (11 cases) items submitted as criminal evidence from customs officials indicate: 147 (67.74%) were of domestic pig origin; 42 (19.36%) were from Asiatic bears, 14 (6.45%) were from goat gall bladders and 14 (6.45%) were absent of animal bile.

Key words: bear gall bladder, identification, quantitation, HPLC

INTRODUCTION

In traditional Chinese medicine, bear gall is considered a bitter, cold medicine that enters the liver, gall bladder, spleen, and stomach channels to clear heat and detoxify various forms of fire[(1)]. It is used for delirium from extensive burns, high fevers and convulsions. Topically, it alleviates skin pain, and is used for reducing swelling and trauma from sprains and fractures.

Both the gall bladder and bile salts of bears *(Ursidae)* are used in traditional and patented oriental medicines. Gall bladders used in traditional oriental medicine come from the Asiatic black bear *(Ursus thibetanus)* and Tibetan brown bear *(Ursus arctos pruinosus)*[(2)]. However, recent information indicates that medicinal gall bladders also came from American black bears *(Ursus americanus)* and two Asian bear species, the Sun bear *(Helarctos malayanus)* and Himalayan brown bear *(Ursus arctos isabelinus)*[(3)]. Exploitation of the remaining Asian bear species, the Giant panda *(Ailuropoda melanoleuca)*, has not been reported. The Peoples' Republic of China has developed a procedure to extract bile fluid from the gall bladders of living bears[(4)]. More than 12,000 bears are now kept in captivity in bear farms for the purpose of "milking" their bile for medicinal use.

Bile is a fluid secreted by the liver, stored and concentrated in the gall bladder and is secreted into the small intestine via the bile ducts. Due to its bicarbonate content, bile is alkaline and has a bitter taste. Its color ranges from golden brown to greenish yellow. Conjugated bile salts and phospholipids normally dissolve cholestrol in a mixed micellar solution. There exists about 112 different types of bile salts. The purpose of this study was not to identify all bile salts, but to infer, from the bile salts, that a given gall bladder or bile sample was produced by the *Ursus* family. Presence of the following bile salts are indicative of the *Ursus* family: tauroursodeoxycholic acid (3α, 7β-dihydroxy-5β-taurocholic acid, TUDC), taurochenodeoxycholic acid (3α, 7α-dihydroxy-5β-taurocholic acid, TCDC) and taurocholic acid (TC). Tauroursodeoxycholic acid is the medically active substance in bear bile. Bears are the only animal known to produce this bile acid in significant quantity[(5)]. The purpose of this study was to determine whether (a) bear gall bladders and bile can be distinguished from gall bladders and bile from other animals, and (b) quantitative information of TUDC, TCDC, and TC can be used to differentiate different bear species.

MATERIALS AND METHODS

I. *Reagents*

All solvents and reagents were HPLC grade and were purchased from Baker Inc. (Phillipsburg, NJ). Tauroursodeoxycholic acid (TUDC), taurocholic acid (TC), taurochen-

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odeoxycholic acid (TCDC), taurodeoxycholic acid (TDC), taurolithocholic acid (TLC), glycodeoxycholic acid (GDC), glycolithocholic acid (GLC), glycochenodeoxycholic acid (GCDC), glycocholic acid (GC), and monobasic potassium phosphate were purchased from Sigma Chemical Co. (St. Louis, MO). Taiwan black bear and Polar bear bile salt were courtesy of the Taipei City Zoo. Bovidae and Suidae gall bladders were obtained from local sources.

II. *Sample Preparation*

Samples were prepared by either weighing out 20 mg of crystallized bile salts or by pipetting 200 µL of fresh bile and then transferring to a 10 × 75 mm test tube. Two mL of methanol was added. The test tubes were placed on a horizontal shaker for 15 minutes and then centrifuged for 5 minutes. One mL of the supernatant was transferred to a 1.8 mL autosampler vial which contained 50 µL of 4-methylphenol (1 mg/mL) serving as the internal standard.

III. *High Performance Liquid Chromatography*

The HPLC procedure used for the separation of bile acids is based on that reported by the US National Fish and Wildlife Forensic Laboratory[6]. A Hewlett Packard 1090 HPLC equiped with a diode array detector was used for analysis. The analytical column was a Vydac reversed phase C_{18} column, 25 cm by 4.6 mm I.D., and 5 µm particle size,

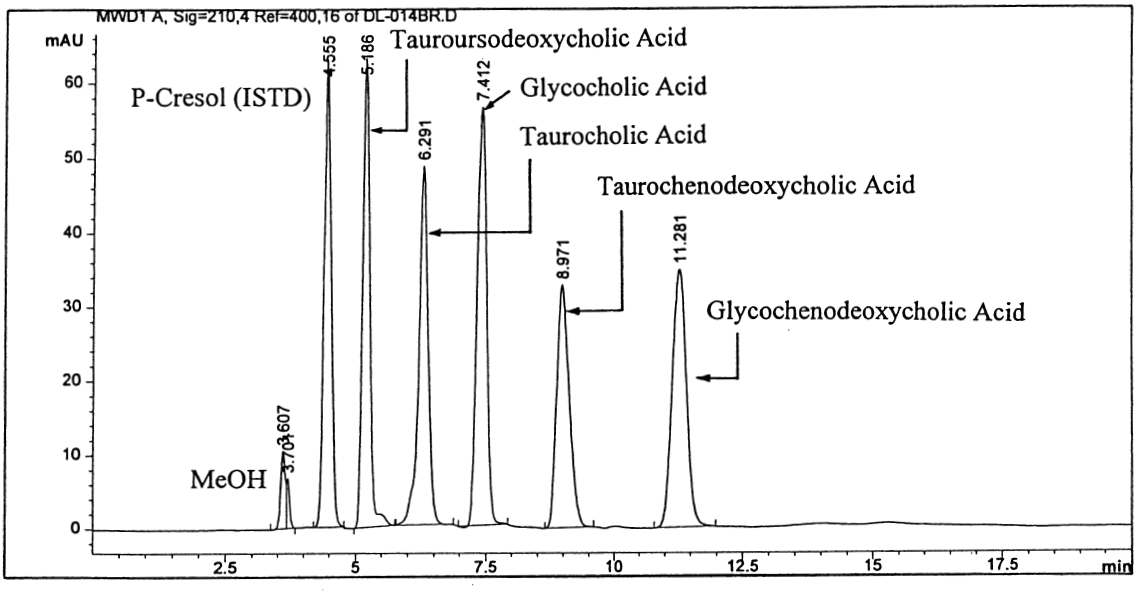

Figure 1. HPLC chromatogram of conjugated bile acids: P-Cresol (I.S.); tauroursodeoxycholic acid (TUDC); taurocholic acid (TC); glycocholic acid (GC) taurochenodeoxycholic acid (TCDC) and glycochenodeoxycholic acid (GCDC).

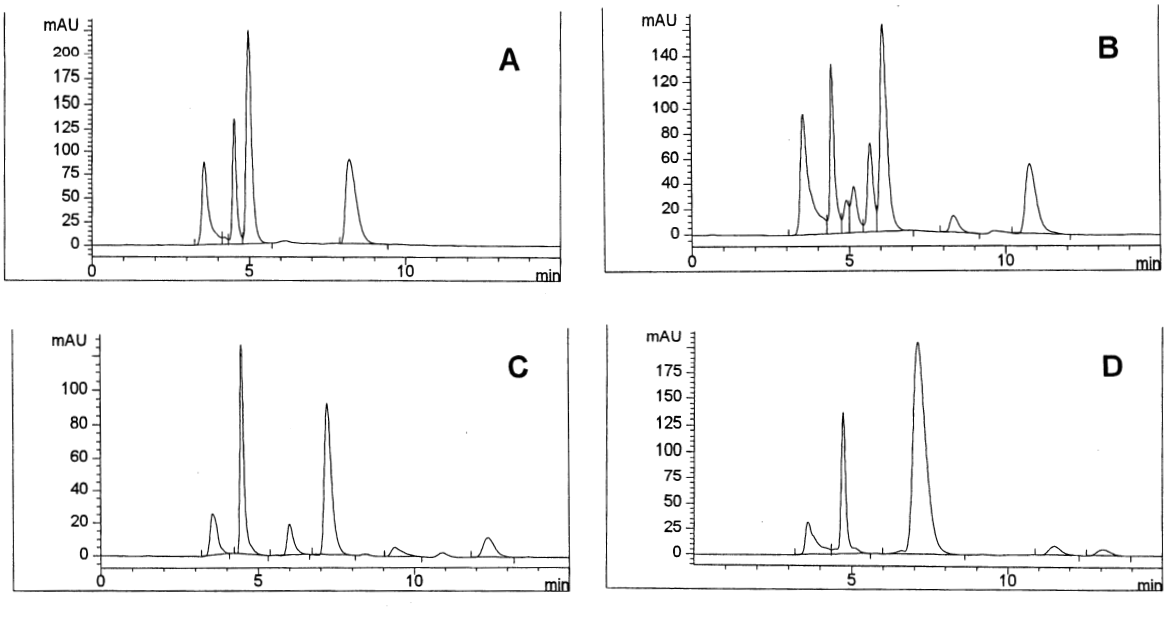

Figure 2. HPLC chromatogram of animal bile salts. A: Asian bear; B: Pig; C: water buffalo; D: goat.

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85:15 (v/v) of 25 mM KH_2PO_4/K_2HPO_4 buffer (apparent pH 5.45) in methanol:water was used as the elution solvent (0.75 mL/min, isocratic). The analytical wavelength was 210 nm with a reference wavelength of 400 nm. Tentative peak identifications were made by comparing the relative retention times with those of known standards.

RESULTS AND DISCUSSION

I. *Identification of Bear Bile Products*

Figure 1 illustrates the separation efficiency of this system for a complex mixture of bile acid standards. With a run time of under 15 min, satisfactory resolution was obtained for all common natural conjugated bile acids. Characteristic features of HPLC chromatograms of bile extract from Asian bear, pig, water buffalo and goat are shown in Figure 2. A typical HPLC chromatogram of standards of three Asian bear bile acids (Figure 2A) shows well resolved peaks, with reproducible retention times. Bile acids derived from goat and water buffalo also contain TC, however, the HPLC pattern of goat and water buffalo lack TUDC components and can be easily recognized. Quantitative HPLC data is not needed for excluding goat and water buffalo bile products.

Representative HPLC chromatograms of bile extracts from Asian wild bear, Asian farmed bear, North American black bear and polar bear are shown in Figure 3. HPLC chromatograms of bear products (Figure 3A-3B) show a relative TUDC/TCDC/TC content pattern similar to that reported[6] for farmed bear-derived products, i.e., "a decreased presence of [TC] (<10%) and a dramatic increase in the percent composition of [TUDC] (>50%) and [TCDC] (>20%)". Studies reported by Chinese scientists also failed to show significant differences in the composition of bile salts between wild and farmed Asian bear species[7,8].

It appears that the low content of TC is common among all Asian bear species (wild or farmed) analyzed. This could be due to metabolic differences between the American and Asiatic black bear. It is possible that Asiatic black bear does not produce secondary salts.

II. *Calibration and Linearity*

A series of standard solutions were prepared using TUDC, TC and TCDC in the following concentrations: 50, 125, 250, 500, 1000, 2000 μg/mL. Each standard solution also contain 50 μL of the internal standard (1 mg/mL). A calibration curve based on the peak response ratios of TUDC,

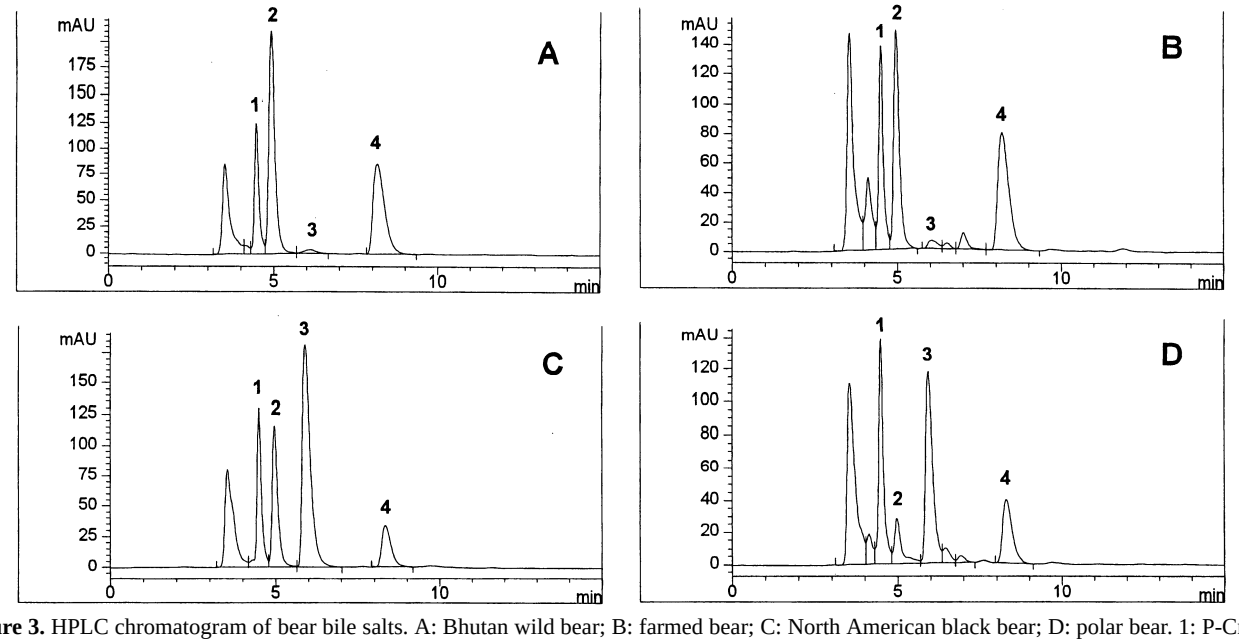


Figure 3. HPLC chromatogram of bear bile salts. A: Bhutan wild bear; B: farmed bear; C: North American black bear; D: polar bear. 1: P-Cresol (I.S.); 2: tauroursodeoxycholic acid (TUDC); 3: taurocholic acid (TC); 4: taurochenodeoxycholic acid (TCDC).

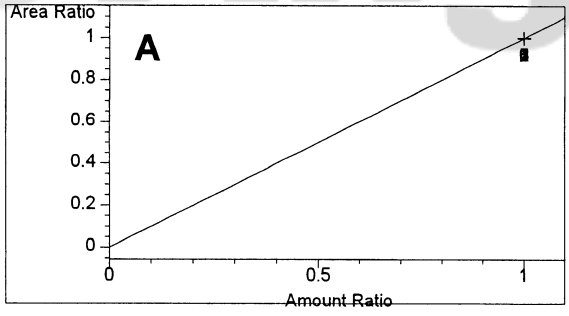
Table 1. Intraday and interday analytical precisions for tauroursodeoxycholic acid (TUDC)

Conc. (μg/mL)	n	Intraday mean ± S.D. (R.S.D.)	Interday mean ± S.D. (R.S.D.)
50	3	51.84 ± 2.92 (5.63%)	51.09 ± 1.99 (3.90%)
125	3	128.19 ± 1.69 (1.32%)	126.65 ± 3.10 (2.45%)
250	3	251.46 ± 3.23 (1.28%)	256.77 ± 4.60 (1.79%)
500	3	506.36 ± 3.33 (0.66%)	510.34 ± 7.03 (1.38%)
1000	3	981.15 ± 2.78 (0.28%)	982.10 ± 9.26 (0.94%)
2000	3	2007.30 ± 1.52 (0.08%)	2006.26 ± 3.56 (0.18%)

Table 2. Intraday and interday analytical precisions for taurocholic acid (TC)

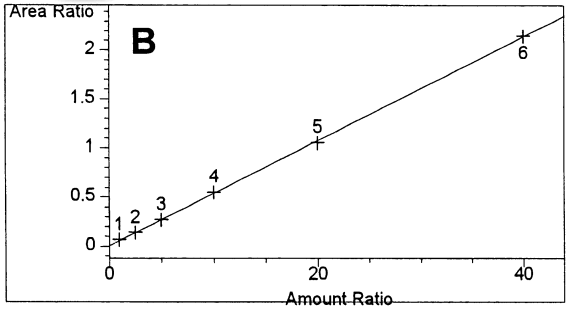
Conc. (μg/mL)	n	Intraday mean ± S.D. (R.S.D.)	Interday mean ± S.D. (R.S.D.)
50	3	46.28 ± 3.10 (6.70%)	59.97 ± 1.37 (2.28%)
125	3	134.13 ± 2.39 (1.78%)	134.09 ± 2.75 (2.05%)
250	3	267.15 ± 1.78 (0.67%)	262.85 ± 3.76 (1.43%)
500	3	530.00 ± 2.54 (0.48%)	524.25 ± 7.45 (1.42%)
1000	3	992.79 ± 15.89 (1.60%)	988.06 ± 4.62 (0.47%)
2000	3	2002.29 ± 5.62 (0.28%)	1997.76 ± 8.73 (0.47%)

TC and TCDC to the 4-methylphenol internal standard versus TUDC, TC and TCDC concentration was constructed to determine the linearity of the chromatographic response. Peak area ratio versus concentration ratio plots (Figure



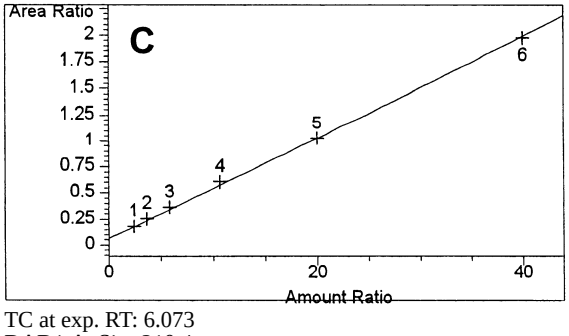
P-Cresol at exp. RT: 4.481
DAD1 A, Sig=210,4

Correlation:	1.00000		
Residual Std. Dev.:	0.00000		
Formula:	y=mx+b		
m:	1.00000	b:	0.00000
x:	Amount Ratio	y:	Area Ratio



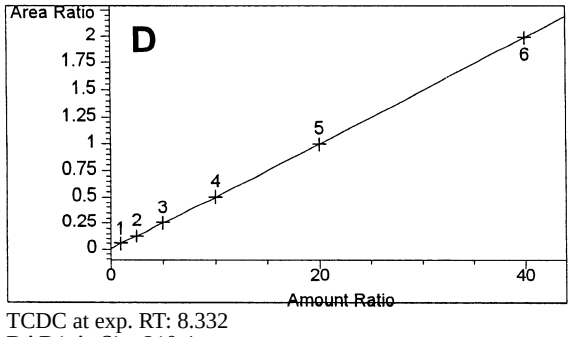
TUDC at exp. RT: 4.990
DAD1 A, Sig=210,4

Correlation:	0.99990		
Residual Std. Dev.:	0.01202		
Formula:	y=mx+b		
m:	5.34452e-2	b:	3.87927e-3
x:	Amount Ratio	y:	Area Ratio



TC at exp. RT: 6.073
DAD1 A, Sig=210,4

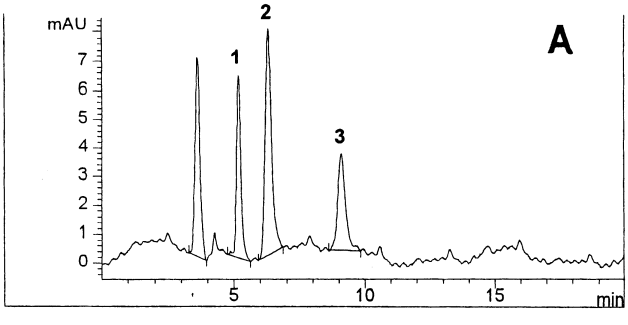
Correlation:	0.99896		
Residual Std. Dev.:	0.03399		
Formula:	y=mx+b		
m:	4.81607e-2	b:	6.10112e-2
x:	Amount Ratio	y:	Area Ratio

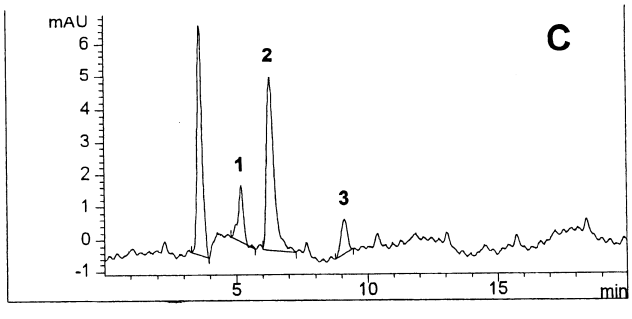


TCDC at exp. RT: 8.332
DAD1 A, Sig=210,4

Correlation:	1.00000		
Residual Std. Dev.:	0.00242		
Formula:	y=mx+b		
m:	4.96308e-2	b:	1.97504e-3
x:	Amount Ratio	y:	Area Ratio

Figure 4. Calibration curve of conjugated bile acids analysis. A: P-Cresol (I.S.); B: tauroursodeoxycholic acid (TUDC); C: taurocholic acid(TC); D: taurochenodeoxycholic acid (TCDC).





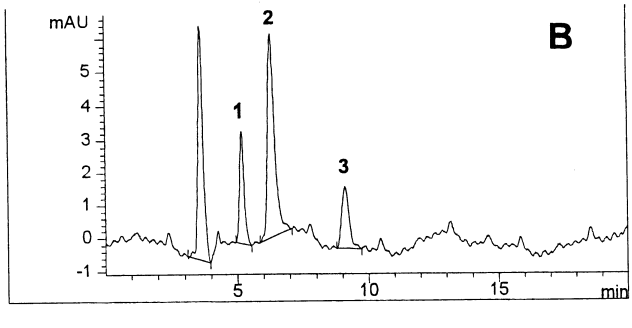


Figure 5. HPLC chromatogram of bear bile salts. A: 25 µg/mL; B: 10 µg/mL; C: 5 µg/mL. 1: tauroursodeoxycholic acid (TUDC); 2: taurocholic acid (TC); 3: taurochenodeoxycholic acid (TCDC).

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4) showed good linearity. Linear regression equations and correlation coefficients (r) were y = 0.05344x + 0.00388, r = 0.99990 for TUDC; y = 0.04816x + 0.06101, r = 0.99896 for TC; y = 0.04963x + 0.00197, r = 1.0000 for TCDC, respectively. The limits of detection for TUDC, TC and TCDC were less than 10 µg/mL (Figure 5).

III. *Precision and Accuracy*

To evaluate the precision of the system, standard solutions of TUDC, TC and TCDC at concentrations of 50, 125, 250, 500, 1000 and 2000 µg/mL were injected into the system three times in one day and three times on three consecutive days. The results are shown in Table 1, 2 and 3. Intraday precisions range from 0.08 to 5.63% for TUDC, 0.28 to 6.70% for TC and 0.45 to 4.82% for TCDC. Interday precisions range from 0.18 to 3.90% for TUDC, 0.47 to 2.28% for TC and 0.14 to 3.92% for TCDC.

IV. *Identification and Differentiation of Bear Bile Used in Taiwan*

Samples, in the form of crystalized bile salts cut from dried whole/partial gall bladders, were obtained from Chinese medicine shops located in different parts of Taiwan. The origin of the 183 samples analyzed were found as follows: 118 (64%), bile salts or gall bladders of domestic pig; 56 (31%), bile products of Asiatic bear; 4 (2.2%), Asiatic bear mixed with pig bile salts; 3 (1.6%), goat gall bladders; 1 (0.55%), water buffalo bile salts; and 1 (0.55%), pig bile salts mixed with water buffalo bile salts[9].

The Investigation Bureau Forensic Laboratory has received 217 gall bladders (11 cases) from the Council of Agriculture Executive Yuan. Analysis of these 217 gall bladder samples submitted as criminal evidence, showed that 147 (67.74%) were from domestic pigs, 42 (19.36%) from Asiatic bears, 14 (6.45%) from goats and 14 (6.45%) did not contain animal bile components[10].

V. *Quantitation of TUDC, TC and TCDC in Bear Bile Salts*

This HPLC method was further used to analyze the main biliary components, specifically, the bile acids TUDC, TC and TCDC in bear bile salts and gall bladder samples. Concentration of bile salts from 95 Ursidae gall bladders analyzed by this method are shown in Table 4. The average concentration (for 93 Asiatic bear bile salts), standard deviation were 3087 ± 1626 µg/mL (range, 1389-14260) for TUDC; 212.6 ± 154.1 µg/mL (range, 37.63-992.5) for TC and 1968 ± 678.1 µg/mL (range, 331.3-4112) for TCDC.

It appears that low content of TC is common among all Asiatic bear species (wild or farmed). Thus, TC content cannot be used for the differentiation of wild and farmed bear bile as suggested in an earlier report[6]. Data shown in Table 4 also suggests that North American and Polar bears contain higher TC concentrations (relative to TUDC and TCDC), whereas the relative concentration of TC in the Asiatic bear (wild or farmed) is much lower. Thus, the relative TC concentration may potentially be useful for the differentiation of Asiatic from North American and Polar bear products. However, a definite conclusion cannot be made due to the small size (n=1) of the North American and Polar bear samples. More comprehensive studies using a statistically significant number of samples from all geographical sources are needed.

Table 3. Intraday and interday analytical precisions for taurochenodeoxycholic acid (TCDC)

Conc. (µg/mL)	n	Intraday mean ± S.D. (R.S.D.)	Interday mean ± S.D. (R.S.D.)
50	3	50.44 ± 2.43 (4.82%)	48.47 ± 1.90 (3.92%)
125	3	126.63 ± 2.81 (2.22%)	123.08 ± 4.11 (3.34%)
250	3	249.44 ± 5.87 (2.35%)	251.90 ± 2.56 (1.02%)
500	3	505.65 ± 2.28 (0.45%)	506.92 ± 7.34 (1.45%)
1000	3	1008.49 ± 10.29 (1.02%)	991.94 ± 14.89 (1.50%)
2000	3	1993.91 ± 9.39 (0.47%)	1995.79 ± 2.73 (0.14%)

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Table 4. Concentration (∝g/mL) of conjugated bile salts in bear biles

Samples source	n	TUDC mean ± S.D. (range)	TC mean ± S.D. (range)	TCDC mean ± S.D. (range)
Bear gall bladders (Chinese medicine stores)	21	3022.1 ± 1012.2 (1388.8 – 4779.4)	200.8 ± 144.3 (53.1 – 682.9)	2046.2 ± 781.9 (331.3 – 3336.4)
Farmed bear biles (Chinese medicine stores)	35	2686.3 ± 741.8 (1726.7 – 5781.9)	180.5 ± 114.3 (37.6 – 480.0)	1876.6 ± 520.6 (868.2 – 2799.0)
Bear gall bladders (Council of Agriculture)	37	3495.9 ± 1626.6 (1394.4 – 14259)	249.1 ± 185.7 (44.4 – 992.5)	2004.2 ± 678.1 (364.3 – 4112.2)
Total	93	3087.8 ± 1626.5 (1388.8 – 14259)	212.6 ± 154.1 (37.6 – 992.5)	1967.5 ± 678.1 (331.3 – 4112.2)
North American black bear	1	1735.9	4332.1	973.1
Polar bear	1	382.5	2113.7	983.1

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以高效液相層析法鑑別及定量熊膽內膽酸成分

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摘　要

本研究利用高效液相層析法，鑑別各種動物膽汁成分並同時定量分析熊膽內之TUDC, TC及TCDC之含量，採用Vydac C_{18} 4.6 × 250 mm逆相層析管柱，25 mM KH_2PO_4/K_2HPO_4甲醇溶液：水（85: 15, v/v）為移動相，流速為0.75 mL/min，檢測波長為210 nm，並以P-Cresol為內標準品。三種成分之定量曲線於50-2000 μg/mL濃度範圍內，均呈現良好的線性關係（r>0.998）。同日間及異日間試驗之相對標準偏差，TUDC為0.08　5.63%，TC為0.20　6.70%，TCDC為0.14　4.82%。應用上述高效液相層析法，定量分析93個亞洲熊膽檢體，TUDC平均濃度為3087 μg/mL、TC為213.6 μg/mL、TCDC為1968 μg/mL，並發現TC含量的多寡是鑑別亞洲熊與北極熊、北美黑熊之關鍵，而非用來鑑別亞洲野生熊膽與飼養引流熊膽。分析市售183個熊膽檢體，56（30.6%）個為亞洲熊膽，118（64.5%）個為豬膽；分析農委會查扣217個熊膽檢體，42（19.4%）為亞洲熊膽，147（67.7%）個為豬膽。

關鍵詞：熊膽，鑑別，定量，高效液相層析法