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International Trade in Bear Gall Bladders: Forensic Source Inference

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ABSTRACT: Fresh and desiccated gall bladders of the Ursidae family (bears) obtained as criminal evidence were characterized by analysis of the principal biliary components, mainly ursodeoxycholyl-taurine, cholyl-taurine and chenodeoxycholyl-taurine using TLC and HPLC. This bile acids profile appears to be an Ursidae family characteristic. Results show that of the samples from Asia only 3% were from the Ursidae family and 18% were from "farmed bears." Samples seized in the U.S.A. and Canada showed that 22.6% and 85% respectively, were from Ursids. The remaining samples were consistent with bile from the domestic pig (Suidae).

KEYWORDS: criminalistics, forensic source inference, illegal trade, bear gall bladder

Asian civilizations have used bear gall bladders for medicinal purposes for centuries. They are used to reduce fever, to aid in "liver detoxification," and to reduce swelling and pain [*1*].

The slaughter of bears for their gall bladders has been recently documented in the popular press [*2*]. The trade in gall bladders is such a lucrative business that the number of gall bladders found in the black market has increased dramatically. Currently, desiccated gall bladders represent the most common form of international trafficking. Desiccation turns the gall bladders dark brown and shrinks them to a teardrop shape, the size of a fig. The bile salts harden to a mass of molasses-colored crystals. A desiccated gall bladder will sell for $15 in Idaho, $1500 in Hawaii and will command $55,000 in Korea [*3,4*]. These prices have created an illegal international black market for bear gall bladders by individuals involved in criminal wildlife commercialization. The trade in bear parts has placed the Asiatic bears in danger of extinction and the demand has now turned to North America.

The strong demand for bear bile in China and Korea has led to the development of bear farms where brown and black Asiatic bears are housed in small cages and their bile acids are removed daily. These captive animals have had a catheter surgically implanted into their gall bladders so that the bile can be easily "milked" [*5*].

Ursodeoxycholic acid, the deconjugated form of a major bear bile acid, has found

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extensive use in modern medicine for the dissolution of gall stones [6]. Actigall [7] is an approved pharmaceutical product whose active component is a semi-synthetic ursodeoxycholic acid.

The main objective of this work is to determine whether bear gall bladders can be distinguished from gall bladders from other species. Classical methods for characterizing the origin of unknown gall bladders have relied on immunological techniques applied to gall bladder tissue. This assay works well with fresh gall bladders but gives spurious results once the tissues have been desiccated. Nuclear magnetic resonance has been used to identify bile acids amidated with the amino acids taurine and glycine [8], but family identification is not fully resolved. We have characterized both fresh and desiccated gall bladders of the Ursidae family by analyzing the main components of the bile salts, specifically ursodeoxycholyl-taurine, cholyl-taurine and chenodeoxycholyl taurine using thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC).

Experimental

Materials

HPLC grade methanol, chloroform and isopropanol were purchased from Fisher Scientific Co. Deionized water was distilled and filtered through a 0.45 μm membrane filter (Millipore). Tauroursodeoxycholic acid, taurocholic acid, glycocholic acid, glycochenodeoxycholic acid and potassium phosphate monobasic were purchased from Sigma Chemical Co. Glycohyodeoxycholic, glycohyocholic and taurochenodeoxycholic acids were obtained from Dr. Hagey. The Prep Torr solid phase vacuum system and the Solid Phase Extraction (SPE) Reversed Phase C_{18} columns were purchased from Fisher.

Bear and nutria gall bladders were obtained courtesy of the U.S. Fish and Wildlife Service Law Enforcement agents and selected zoos. Lyophilized bear bile salts were obtained courtesy of Dr. Theis from the University of California, Davis Campus. Bovidae and Suidae gall bladders were obtained from local sources. Raccoon, Canidae and Felidae gall bladders were obtained courtesy of Ashland Veterinary Hospital.

Sample Preparation

Ten milligrams of crystallized bile salts were removed through a small incision at the base of desiccated gall bladders and 0.1 mL of fluid samples from fresh gall bladders were removed by syringe. The samples were transferred to a test tube and one milliliter of 0.1 N NaOH was added. Samples were sonicated for eight minutes followed by C_{18} solid phase extraction (SPE) columns. The extracted bile samples were eluted thrice using 0.33 mL of methanol. The eluted samples were then transferred to glass collection vials for analysis.

TLC

The TLC method of Batta et al. [9] was used to confirm the presence of bile acids detected by HPLC. Bile samples were prepared as described and 10 μL was spotted onto Fisher silica TLC plates. The plates were developed twice in chloroform:isopropanol:acetic acid:deionized water, 30:30:4:1,v/v. The plates were air dried and then sprayed with 20% v/v sulfuric acid in water followed by 3.5% w/v phosphomolybdic acid in isopropanol and heated at 110°C for 2 min.

HPLC

A Hewlett Packard 1090 HPLC with 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 five μm particle size. The HPLC tubing was 0.21 mm I.D. The analytical wavelength was 210 nm with a reference wavelength of 250 nm. Separations were obtained using a modification of Rossi et al. [*10*]. The methods used an isocratic 25 mM KH_2PO_4–K_2HPO_4 buffer, apparent pH 5.45 in methanol:water,67.4:33.6, with elution at 0.75 mL/min. Peaks were assigned by comparison of the relative retention times with those of known standards because the ultraviolet spectra of all bile acids tested were similar and did not offer resolution.

Results

Gall bladders from 289 individuals of the Ursidae family were analyzed using HPLC and confirmed by TLC. Quantitative HPLC analysis was performed on 242 samples. Species represented include six grizzly bears (*Ursus arctos*), 35 North American black bears (*Ursus americanus*), three polar bears (*Thalarctos (Ursus) maritimus*), 34 farmed bears (*Ursus thibetanus* and *Ursus arctos* suspected [*5*]), and 124 North American Ursidae gall bladders of unknown species.

The main bile acids of the Ursidae family consist of ursodeoxycholyl-taurine (3α,7β-dihydroxy-5β-cholanoic taurine), its 7 α hydroxy epimer chenodeoxycholyl taurine (3α,7α-dihydroxy-5β-cholanoic taurine) and cholyl-taurine (3α,7α,12α-trihydroxy-5β-cholanoic taurine) as well as minor components characterized by Hagey [*11*]. A typical HPLC chromatogram of standards of these 3 bile acids (Fig. 1) shows well resolved peaks, with reproducible retention times. Characteristic TLC retention factors (R_f) for the bile acids of interest are found in Table 1 [*9*]. Two bears from a total of the 289 individuals did not appear initially to contain ursodeoxycholyl-taurine as a main component by HPLC analysis, although it was detected subsequently by increasing the sample size from 10 to 20 mg. All samples from known Ursids however, contained the bile

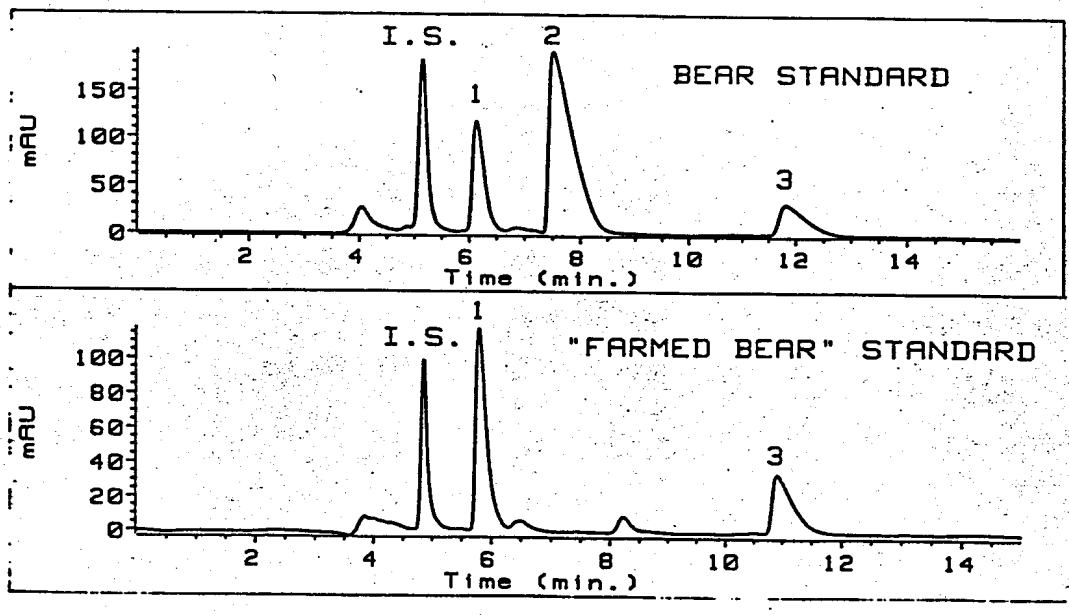

FIG. 1—*HPLC chromatogram of wild and farmed bear. 1: Ursodeoxycholyl-taurine; 2: Cholyl-taurine, and 3: Chenodeoxycholyl taurine.*

TABLE 1—*R_f values of conjugated bile acids.*

Compound	R_f
Cholyl-taurine	0.18
Deoxycholyl taurine	0.34
Chenodeoxycholyl taurine	0.37
Ursodeoxycholyl taurine	0.43
Lithocholyl taurine	0.53
Cholyl-glycine	0.47
Deoxycholyl glycine	0.73
Chenodeoxycholyl glycine	0.76
Ursodeoxycholyl glycine	0.81
Lithocholyl glycine	0.87

acids chenodeoxycholyl-taurine, cholyl-taurine and ursodeoxycholyl taurine when analyzed by TLC.

Concentration and percentage of the bile salts from 164 Ursidae gall bladders analyzed by HPLC are represented in Table 2. The identity of the individual species of these gall bladders is unknown but all were obtained from known Ursids. This data is consistent with that from five black bears analyzed by MacDonald et al. [*12*] for the relative concentration of the three principal bile acids. Table 2 presents the average, median, interquartile range, standard deviation and standard error as major statistical descriptors.

The median was selected for statistical analysis purposes so that nonparametric methods could be applied [*13*] since an initial analysis of the data showed that the bile acid concentrations do not fit a gaussian distribution. Because the range of bile acid concentration exceeded two orders of magnitude, the interquartile range is presented to demonstrate the variability of the data (Fig. 2 and Table 2). The interquartile range is the distance between the 25th and the 75th percentile. The standard error of the mean is defined as the standard deviation divided by the square root of the sample size and represents the average deviation from the mean.

The ratio of dihydroxy (ursodeoxycholyl-taurine and chenodeoxycholyl taurine) to trihydroxy (cholyl-taurine) bile salts is not normally distributed. A negative correlation ($P = -0.91$) was observed between the percentage of ursodeoxycholyl-taurine and cholyl-taurine ($R^2 = 83.53\%$, SE = 8.17).

Concentration and percentage of the bile salts of known black bears ($n = 35$), brown bears ($n = 6$), polar bears ($n = 3$) and suspected farmed bears ($n = 34$) is also represented in Table 2.

The qualifier "suspected" farmed bears is used because we have not obtained reliable standards from bear farms.[3] A concerning caveat is that within the last 3 years we have not been able to obtain a known sample of Asiatic black bear gall bladder for analysis and it is possible, although unlikely, that the data we are presenting is due to a species difference. The main bile acids of the farmed bears consist of ursodeoxycholyl-taurine, chenodeoxycholyl taurine and little or no cholyl-taurine (Fig. 1). Quantitative information and summary statistics of 34 bile samples from bear farms is represented in Fig. 2 and Table 2. Our data is consistent with that of Guanlin and Guanzhu [*14*] who in their analysis of bile of three farmed bears found high percentages of ursodeoxycholyl-taurine (31 to 42%), chenodeoxycholyl taurine (46 to 56%) and low percentage of cholyl-taurine (6 to 13%).

[3]The farmed bear samples were obtained from asian medicinal containers that stated that the provenance of the bile salts were from bear farms.

TABLE 2—*Concentration (μg/mL) and percentage of conjugated bile salts in four species of bear.*

		Ursodeoxycholyl-taurine	Cholyl-taurine	Chenodeoxycholyl taurine
N.A. Bears (n = 164) Species Unknown	Average	659	1852	213
	Standard Deviation	1051	2509	224
	Median	303 [15%]	771 [69.7%]	174 [12%]
	Interquartile Range	121–549 [9–34%]	578–1458 [50–80%]	121–246 [4–19%]
	Standard Error	82.08 [1.56%]	195.93 [1.70%]	17.54 [0.69%]
N.A. Black Bear (n = 35)	Average	1634	4835	202
	Standard Deviation	1705	3505	87
	Median	985 [16%]	4857 [78%]	209 [3%]
	Interquartile Range	504–2247 [7–45%]	1429–6570 [49–89%]	143–246 [2–4%]
	Standard Error	288.27 [4.70%]	592 [4.72%]	14.81 [0.60%]
Farmed Asiatic Black Bear (n = 34)	Average	3464	383	1413
	Standard Deviation	1765	269	648
	Median	3767 [70%]	286 [4%]	1336 [25%]
	Interquartile Range	1971–4566 [47–80%]	128–1184 [0–6%]	1001–1822 [18–46%]
	Standard Error	302.78 [3.65%]	58.89 [1.98%]	111.20 [2.91%]
Grizzly Bear (n = 6)	Average	1014	4122	312
	Standard Deviation	973	1902	207
	Median	554 [13%]	3440 [80%]	255 [5%]
	Interquartile Range	475–1118 [10–15%]	2516–6036 [80–85%]	153–361 [4–5%]
	Standard Error	397.59 [6.20%]	776.81 [7.39%]	84.81 [1.21%]
Polar Bear (n=3)	Average	174	76	13
	Standard Deviation	214	4	7
	Median	65 [16%]	614 [75%]	16 [8%]
	Interquartile Range	35–422 [5–18%]	351–2459 [73–80%]	5–19 [7–13%]
	Standard Error	124.07 [4.20%]	662.94 [2.22%]	4.20 [2.04%]

Discussion

The Kolmogorov-Smirnov (K-S) is a nonparametric test that tests differences between two distributions [*18*] and allows to test the hypothesis (HO) that there is no difference between the distribution of the total bile acids content, where $P < 0.05$ represents the smallest value that would lead to rejection of the hypothesis. The distribution of the total bile acids found in both farmed bear and black bear, was compared by the K-S test statistic, with $P < 0.05$ considered significant. Total bile acids were estimated by adding the individual bile salts. The calculated Kolmogorov-Smirnov test statistic was D = 0.426 (significant level 3.81×10^{-3}) where D represents the maximum absolute deviation between the two cumulative distributions. Since the significant level falls below 0.05, the distributions of wild black bears and farmed bears are significantly different from

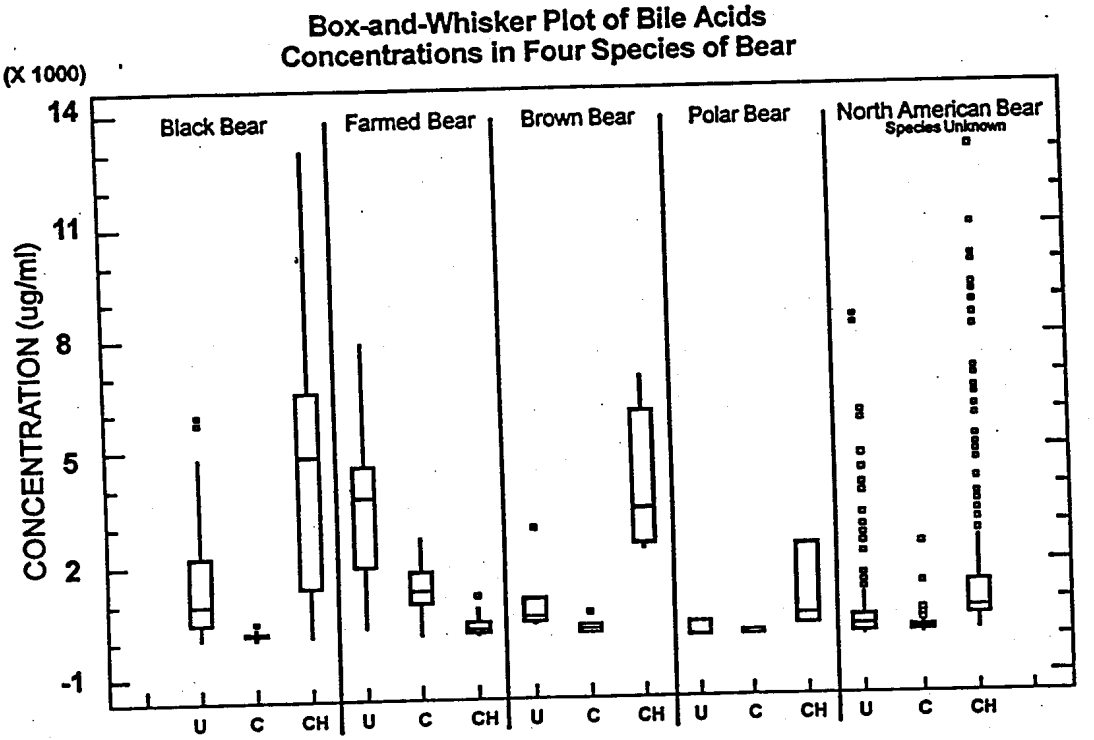

FIG. 2—*Box and Whisker plot of all samples tested. The box covers the interquartile range and the horizontal line within represents the median. The "whiskers" extend to the extremes and the outlier points are those values that exceed 1.5 times the interquartile range. U = Ursodeoxycholyl-taurine; C = Chenodeoxycholyl taurine; CH = Cholyl-taurine.*

each other at the 5% level but the total bile acids production (not the distribution) of the farmed bear falls within the range of the black bears (Fig. 2). We conclude that the distributions of the sum of the bile acids are significantly different but the total production is similar in both populations.

The distributions of ursodeoxycholyl-taurine, chenodeoxycholyl taurine and cholyl-taurine in farmed bear and black bear were also compared by the K-S test statistic with $P < 0.05$ considered significant. The calculated K-S test statistic for ursodeoxycholyl-taurine was D = 0.538 (significant level 9.00×10^{-5}); the calculated K-S test statistic for chenodeoxycholyl taurine was D = 0.912 (significant level $\geq 1.00 \times 10^{-6}$); the calculated K-S test statistic for cholyl-taurine was D = 0.838 (significant level $\geq 1.96 \times 10^{-8}$). In all three cases the significant level falls below 0.05, and we conclude that distributions of these bile acids in black bears and farmed bears are significantly different from each other at the 5% level (Fig. 2).

It is notable that the decreased production of cholyl-taurine and the concurrent increase in both ursodeoxycholyl-taurine and chenodeoxycholyl taurine appears unique to farmed bears. It is possible to speculate that within the Ursidae family ursodeoxycholic acid, chenodeoxycholic acid and cholic acid are primary bile acids and that the bile acids detected in farmed bears reflect a constant synthesis of the dihydroxy components. The observed decrease in cholic acid synthesis might represent a suppression from a feedback system. Kurozumi et al. [*15*] analyzed bile from five bears whose origin were uncertain. Samples claimed to have originated from Himalayan bears also exhibited a profile of low concentration of cholyl conjugates and high concentrations of chenodeoxycholyl and ursodeoxycholyl conjugates. Until known standards are obtained, we can not discount species differences as an explanation for the marked decreased or absence of cholyl

taurine in farmed bears (Table 2). Future investigations will answer the question if these changes are due to the farming process, i.e. the constant drainage of the bile, or due to diet or species.

Hagey [*11*] has additionally investigated the bile acid composition of sun bears (Helarctos malayanus), sloth bears (Melursus ursinus) and spectacled bears (Tremarctos ornatus), and has found that ursodeoxycholyl-taurine, chenodeoxycholyl-taurine and cholyl-taurine are also the principal bile acids detected. Therefore, it can be inferred that these bile acids are an *Ursidae family characteristic* and that no other species among 600 analyzed [*11*] share this unique bile salt profile.

Gall bladders from the domestic pigs (Suidae) and a selected rodent, the nutria (Myocastor coypus) were also chemically characterized. Bile salts from six nutrias did not contain any taurine amidated bile acids but did contain ursodeoxycholyl glycine, chenodeoxycholyl glycine and 7-ketolithocholyl glycine as reported by Tint et al. [*16*]. The principal bile salts identified in domestic pig gall bladders were hyocholyl-glycine, hyodeoxycholyl-glycine, cholyl-glycine, and chenodeoxycholyl-glycine and other minor components. Gall bladders from various species of cat, snake and fish as well as raccoon, dog and cow were analyzed by TLC and HPLC. Although the bile chemistry from these sources was not characterized, the bile profiles were distinct and different from that of the Ursidae family.

Based on this data, the following criteria was established for bile or gall bladder source inference: I) If ursodeoxycholyl-taurine, chenodeoxycholyl-taurine and cholyl-taurine were detected by HPLC and confirmed by TLC, it was concluded that the bile salts were from the Ursidae family. II) If quantitative HPLC analysis revealed high levels of chenodeoxycholyl-taurine (>20%), ursodeoxycholyl-taurine (>50%) and low levels of cholyl-taurine (<10%) and TLC analysis confirmed the presence of ursodeoxycholyl-taurine, chenodeoxycholyl-taurine and *cholyl-taurine*, it was inferred that the bile salts were from the Ursidae family and its origin consistent with bear farms. III) The absence of the Ursidae species characteristic bile salts were indicative that the source of the bile did not belong to the Ursidae family.

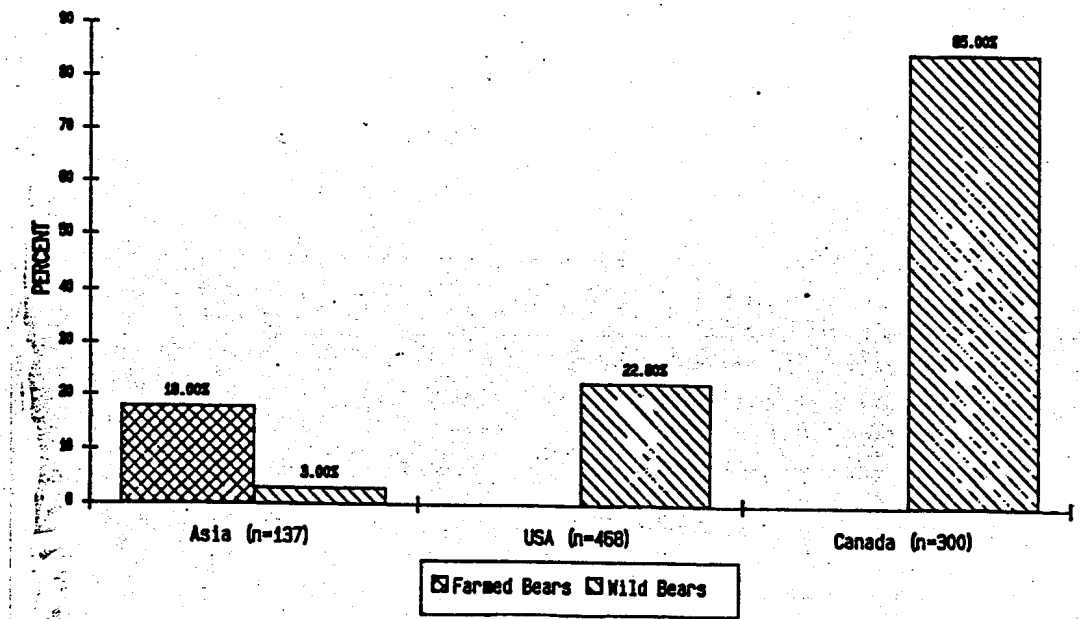

FIG. 3—*Bar graph shows the percentage of bear evidence bile or gall bladders seized from Asia, U.S.A. and Canada. Diagonal lines represents wild bears and the crossing lines represent farmed bears.*

Bile salts and gall bladders received as criminal evidence from Asia (Hong Kong, Taiwan and Malaysia), Canada and the U.S.A. were analyzed by HPLC and TLC. Analysis showed that of the samples from Asia (n = 137) three gallbladders (3%) from were from the Ursidae family, 25 bile samples (18%) were from farmed bears and the remainder were consistent with those of domestic pigs. Samples seized in the U.S.A. (n = 468) showed that 106 gallbladders (22.6%) were from the Ursidae family and 77.3% were from pigs. Samples seized in Canada (n = 300) revealed that 255 gallbladders (85%) were from the Ursidae family (Fig. 3). The widespread substitution of pig gall bladders for those of bear and the fraudulent nature of the trade in gall bladders and bile salts is not surprising since Namba et al. [*17*] documented fraud as early as 1982.

Conclusion

HPLC and TLC were used to analyze and characterize the provenance of bile salts and gall bladders. The principal bile salts detected in North American bears are ursodeoxycholyl-taurine, cholyl-taurine and chenodeoxycholyl taurine. This bile salt profile appears to be an Ursidae family characteristic and it is not shared by 600 species tested [*11*]. Farmed bears are characterized by a decreased presence of cholyl-taurine (<10%) and a dramatic increase in the percent composition of ursodeoxycholyl-taurine (>50%) and chenodeoxycholyl taurine (>20%). Other families such as Suidae, as well as individual species such as nutria do not exhibit the same profile of bile salts as the Ursidae family.

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References

[*1*] Bensky, D. and Gamble, A., *Chinese Herbal Medicine, Materia Medica*, Estland Press, Seattle, 1986, pp. 91–93.

[*2*] Poten, C. J., "A Shameful Harvest," *National Geographic* Vol. 180, No. 3, 1991, pp. 106–132.

[*3*] Fitzgerald, S., *International Wildlife Trade: Whose Business Is It?* World Wildlife Fund, Washington, D.C., 1986.

[*4*] Mills, J. and Servheen, C., *The Asian Trade in Bears and Bear Parts*, World Wildlife Fund, Washington, D.C., 1991.

[*5*] Mills, J., "Milking the Bear Trade," *International Wildlife*, May/June, 1992, pp. 38–45.

[*6*] Bachrach, W. H. and Hofmann, A. F. Ursodeoxycholic Acid in The Treatment of Cholesterol Cholelithiasis: A Review. *Dig. Dis. Sci.*, 27, 1982, pp. 833–856.

[*7*] *Physicians Desk Reference*, Medical Economics Data, E. R. Barnhart, Pub., New Jersey, 1991, pp. 2180–2182.

[*8*] Theis, J. H., deRopp, J. S., Schwab, R. G., Banks, J., and Levine, K., "Nuclear Magnetic Resonance to Differentiate Bear, Pig, and Cow Bile for Forensic Investigations," *Wildlife Society Bulletin*, Vol. 16, 1988, pp. 430–433.

[*9*] Batta, A., Shefer, S., and Salen, G. "Thin Layer Chromatographic Separation of Conjugates of Ursodeoxycholic Acid from Those of Litho-, Chenodeoxy-, Deoxy, and Cholic Acids," *Journal of Lipid Research*, Vol. 22, 1981, pp. 712–714.

[*10*] Rossi, S. S., Converse, J. L., and Hofmann, A. F., "High Pressure Liquid Chromatography Analysis of Conjugated Bile Acids in Human Bile: Simultaneous Resolution of Sulfated and Unsulfated Lithocholyl Amidates and the Common Conjugated Bile Acids," *Journal of Lipid Research*, Vol. 28, 1987, pp. 589–595.

[*11*] Hagey, L. R., *Bile Acid Biodiversity in Vertebrates: Chemistry and Evolutionary Implications*, Dissertation, University of California, San Diego, 1992.

[12] MacDonald, A. C. and Williams, C. N., "Studies of Bile Lipids and Bile Acids of Wild American Black Bears in Nova Scotia, Showing a High Content of Ursodeoxycholic Acid," *Journal of Surgical Research*, Vol. 38, 1985, pp. 173–179.
[13] Sokal, R. R. and Rohlf, F. J., *Biometry*, W. H. Freeman and Co. New York, 1981.
[14] Guanlin, J. and Guanzhu, J., "Experimental Research on Collecting Live Bear Biles," *Chinese Journal of Zoology*, Vol. 23, No. 2, 1988, pp. 27–30.
[15] Kurozumi, K., Harano, T., Yamasaki, K., and Ayaki, T., "Studies on Bile Acids in Bear Bile," *Journal of Biochemistry*, Vol. 74, No. 3, 1973, pp. 489–495.
[16] Tint, G. S., Bullock, J., Batta, A. K., Shefer, S., and Salen, G., "Ursodeoxycholic acid, 7-Ketolithocholic Acid, and Chenodeoxycholic Acid Are Primary Bile Acids of The Nutria (Myocastor coypus), *Gastroenterology*, Vol. 90, 1986, pp. 702–109.
[17] Namba, T., Nunome, S., Hattori, M., Hogashidate, S., and Maekubo, T., "Fundamental Studies on the Evaluation of Crude Drugs. VII. On Animal Gall (1)," *Yakugaku Zasshi*, Vol. 102, 1982, pp. 760–767.
[18] Kraft, C. H., and Van Eeden, C., *A Nonparametric Introduction to Statistics*, The MacMillan Company, New York, 1968, pp. 172–173.

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