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Identification and Differentiation of Bear Bile Used in Medicinal Products in Taiwan

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ABSTRACT: One hundred eighty-three suspect bear bile used in medicinal products, collected in Taiwan as gall bladders or dried powder forms, were analyzed using FTIR, HPTLC, and HPLC techniques to identify whether they are indeed bear bile. Those confirmed were further examined to determine whether the observed analytical parameters can be reliably used for source inference, i.e., differentiating products among North American black bear, farmed Asiatic black bear, polar bear, etc. Our data suggested that North American and polar bears contain a higher concentration of TC (relative to TUDC and TCDC), whereas the relative concentration of TC in Asiatic bears (wild or farmed) is much lower. Thus, the relative concentration of TC can potentially be used for differentiating Asiatic bear bile from North American and polar bear products, but it cannot be used for the differentiation of wild and farmed bear bile as suggested in an earlier report by Espinoza et al. The origin of the 183 samples analyzed were found to be as follows: 118 (64%), bile salts, or gall bladders were 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.

KEYWORDS: forensic science, bear bile, bear gall bladder, identification, illegal trade

Bear bile is highly valued and has been used as an important ingredient in Chinese medicinal remedies for thousands of years. Its medicinal functions were widely recorded in ancient Chinese medicinal and pharmaceutical documents (1) and in recent Chinese herbal medicine publications (2). Bear bile is considered a "cold" medicine effective in clearing "heat" and detoxifying various forms of "fire", which can be manifested externally as burns, or internally as liver disease. "Cold" medications fight fever, reduce inflammation and swelling, reduce pain, and detoxify (2).

Asiatic black bear (*Selenarctos thibetanus*) and Tibetan brown bear (*Ursus arctos pruinosus*) are the original sources of gall bladders used for medicinal purposes (2). However, recent information indicated that medicinal gall bladders also came from American black bear (*Ursus americanus*) and from two Asian bear species, Sun bear (*Helarctos malayanus*) and Himalayan brown bear (*Ursus arctos isabelinus*) (3). The exploitation of the remaining Asian bear species, Giant panda (*Ailuropoda melanoleuca*), has not been reported.

International trades of bear gall bladders are regulated by The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The Asiatic black bear, sloth bear, sun bear, and certain Asian populations of the brown bear are included in CITES Appendix I which prohibites commercial trade. All remaining Asian populations of brown bear and American black bear are listed in CITES Appendix II. Trading of products from these latter species is regulated by the issuance of permits. With diminishing bear population and strict regulations, the Peoples' Republic of China has developed a procedure to extract bile fluid from the gall bladders of living bears (4). More than 10,000 bears are now kept in captivity in bear farms for the purpose of "milking" their bile for medicinal use.

Bear bile found in traditional Chinese medicine stores can be divided into three categories: (a) true complete bear gall bladder; (b) farmed bear bile in dried powder form; and (c) fraudulent bear products, e.g., pig, water buffalo, goat, etc., as whole gall bladder or dried bile powder forms. Each of these categories can also be further subdivided. For example, true bear gall bladders are divided into golden-silk gall bladders, black oil ink gall bladders, or named according to their countries of origin. The farmed bear bile can be 100% pure or a mixture of various amounts of biles from other animals, such as pig, water buffalo, goat, and others. Fraudulent bear gall bladders, the trade names like "cheap bear gall bladder", "common bear gall bladder", "various gall bladder" and "miscellaneous gall bladder", are not derived from bear and their animal origins are unknown. Traditional Chinese medicine stores adopt certain identification procedures for these products. These procedures, however, are not based on modern chemical analyses methods (5).

Because the cost of bile derived from farmed bear is very competitive, it has recently taken over the market of wild bear gall bladder in Taiwan. The sale of fraudulent products is also common. The main objective of this study is to determine whether: (a) bear gall bladders and bile can be distinguished from gall bladders and bile from other animals, and (b) gall bladders and bile from different bear species can be differentiated. This study was conducted with reference to the HPTLC and HPLC procedures and the findings reported by the US National Fish and Wildlife Forensic Laboratory (6). We further employed fourier transform infrared spectroscopy (FTIR) for this investigation and found it extremely useful.

Materials and Methods

Reagents

All solvents and reagents were HPLC grade and were purchased from Baker Inc. (Phillipsburg, NJ). Tauroursodeoxycholic

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acid (TUDC), taurocholic acid (TC), taurochenodeoxycholic 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 obtained courtesy of the Taipei City Zoo. Bovidae and Suidae gall bladders were obtained from local sources.

Sample Preparation

Bile-containing samples were named using two different systems in the text and figures: those from known sources were designated with words or letters, e.g., TCDC and pig; those collected from Chinese medicine shops were designated with numbers (even if they might have been considered authentic), e.g., 80-4.

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 by 75 mm test tube. Two mL of methanol was added. The test tube was placed on a horizontal shaker for 15 min and then centrifuged for 5 min. 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.

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 1050 HPLC equipped with a multi-wave length detector was used for analysis. The analytical column was a Vydac reversed phase C18 column, 25 cm by 4.6 mm I D, and 5 μm particle size, 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.

Thin Layer Chromatography

The HPTLC and viewing procedures reported by the US National Fish and Wildlife Forensic Laboratory (6) were modified and used to confirm the presence of conjugated bile acids detected by HPLC. Bile samples were spotted (5 μL) on a HPTLC aluminum sheet silica gel 60 F_{254} plate (10 by 20 cm) (E. Merck: Darmstadt, Germany). The plates were developed three times in the chloroform/isopropanol/glacial acetic acid/water (30:30:0.4:0.1, v/v) solvent system. The plates were developed up to 9.5 cm from the origin (approximately 1 h) and then were removed and dried under hot air. After the final development, the plate was sprayed with 20% sulfuric acid in water followed by 3.5% w/v phosphomolyblic acid in isopropanol and heated at 100°C for 5 min.

Fourier Transform Infrared Spectroscopy

A model FTS-40 fourier transform infrared spectrometer (Digilab: Cambridge, MA) was used for this study. Bile samples (0.1–0.2 mg) were mixed with approximately 100 mg of dry, powdered potassium bromide by thoroughly grinding in a smooth agate mortar. The mixture was pressed into a transparent disc with dies under a pressure of 12,000 lb/in^2. Spectra were obtained in transmittance mode with 16 scans over the range of 400 to 4,000 cm^{-1} at 4 cm^{-1} resolution.

Results and Discussion

Identification of Bear Bile Products

Major bile acids are TUDC, TCDC, and TC (Fig. 1) which include the taurine constituent characterized by the presence of a SO_3^- functional group. This functional group exhibits strong IR absorption at 1082 cm^{-1} and 1208 cm^{-1} (Fig. 2). Computerized search of an unknown IR spectrum against an in-house generated library composed of bile acids from various animal has proven to be a very effective for identifying a bear bile product.

This approach, however, is limited in two aspects. First, bile acids derived from goat also contain TC, thus may be falsely recognized as bear bile by its IR spectrum alone. (Approximately 93 bear and 12 goat samples that were from known sources or considered authentic products from Chinese medicine shops have been tested with this computerized searching process. All were correctly identified.) Furthermore, chromatographic procedures (HPTLC and HPLC) are needed to provide basis for potential differentiation of bear gall bladder products originating from various geographical regions.

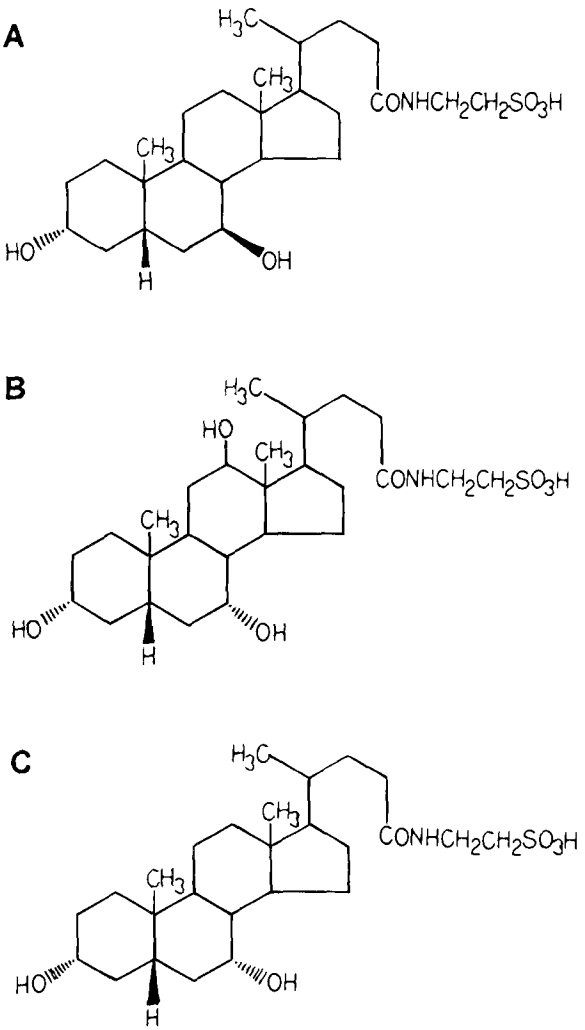

FIG. 1—*The structure of conjugated bile acids. A: tauroursodeoxycholic acid (TUDC); B: taurocholic acid (TC); C: taurochenodeoxycholic acid (TCDC).*

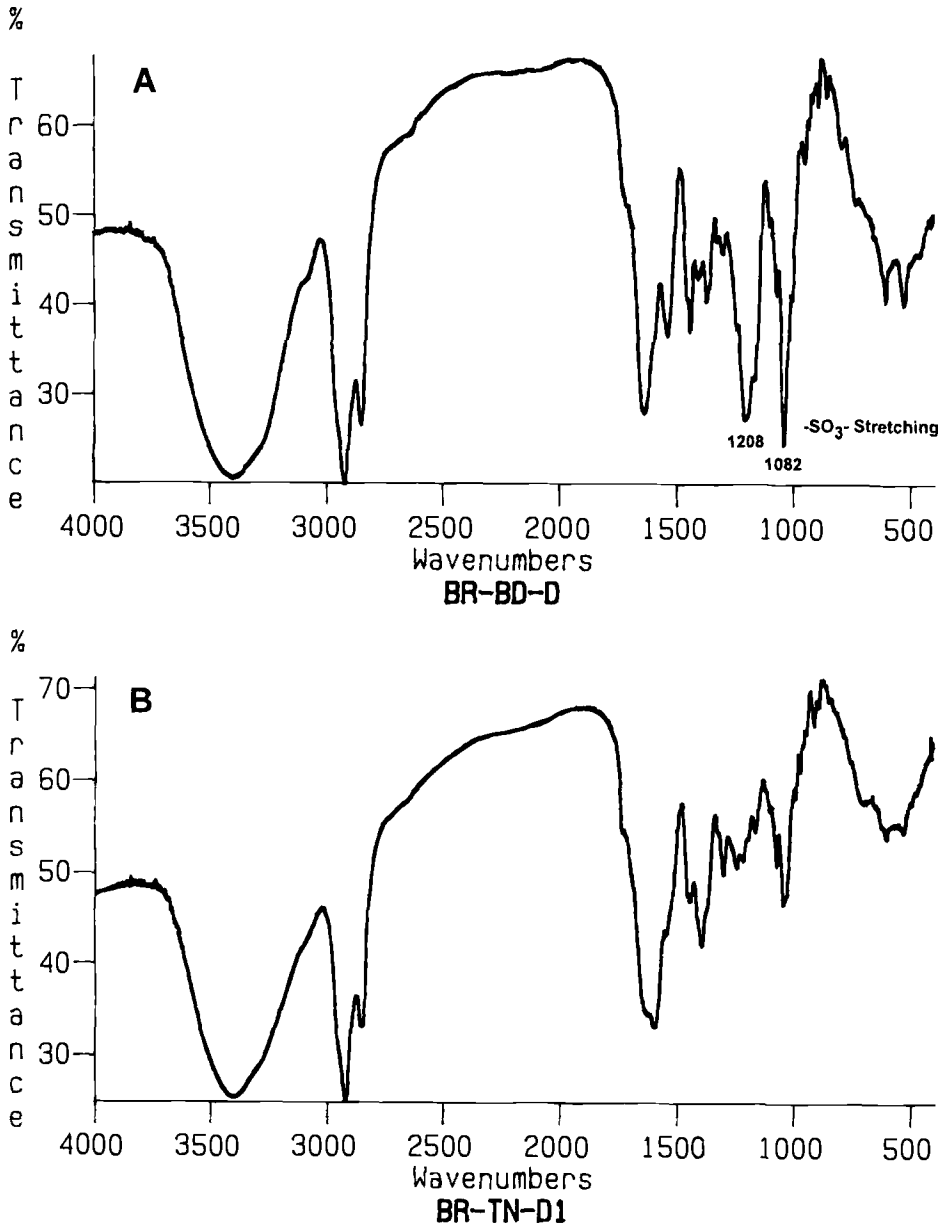

FIG. 2—*FTIR spectrum of bear and pig bile salts. A: bear; B: pig.*

Chromatographic Differentiation of Various Bear Bile Products

Characteristics features of HPTLC and HPLC chromatograms of bile extracts from goat, pig, and water buffalo are shown in the right-hand section of Fig. 3A and Fig. 4, respectively. Although not as convenient as the computerized IR spectrum searching process, these chromatograms provide characteristic patterns that may help identify the animal origins of gall bladder-derived products.

HPTLC retention factors (R_f) and HPLC retention time data of common bile products, (purchased from Sigma) are shown in Table 1. HPTLC patterns of several standard compounds and various bile products are shown in Fig. 3. Representative HPLC chromatograms of wild and farmed bile extracts are shown in Fig. 5.

Differentiation of Goat and Bear Bile Products—The TC component in goat bile acid confounds its differentiation from bear bile by IR analysis. However, the HPTLC pattern of a goat bile lacks TCDC, TUDC, and GC components and can be easily recognized (far-right in Fig. 3A). Quantitative HPLC data are not needed for excluding goat bile products.

Differentiation of Bear Gall Bladder Products from Various Regions—Representative HPTLC patterns obtained from: (a) wild bear gall bladders smuggled from Bhutan (BD-A, BD-B, BD-C), (b) Taiwan black bear bile (from Taipei City Zoo), and (c) farmed bear bile (30-1, 40-13, 40-18) are shown in the left-hand section of Fig. 3A. HPLC chromatograms of these three categories of bear products (Figs. 5A–5C) show a relative TUDC/TCDC/TC content pattern similar to that reported (6) for farmed bear-derived products, i.e., "Characterized by 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 bear products (7,8).

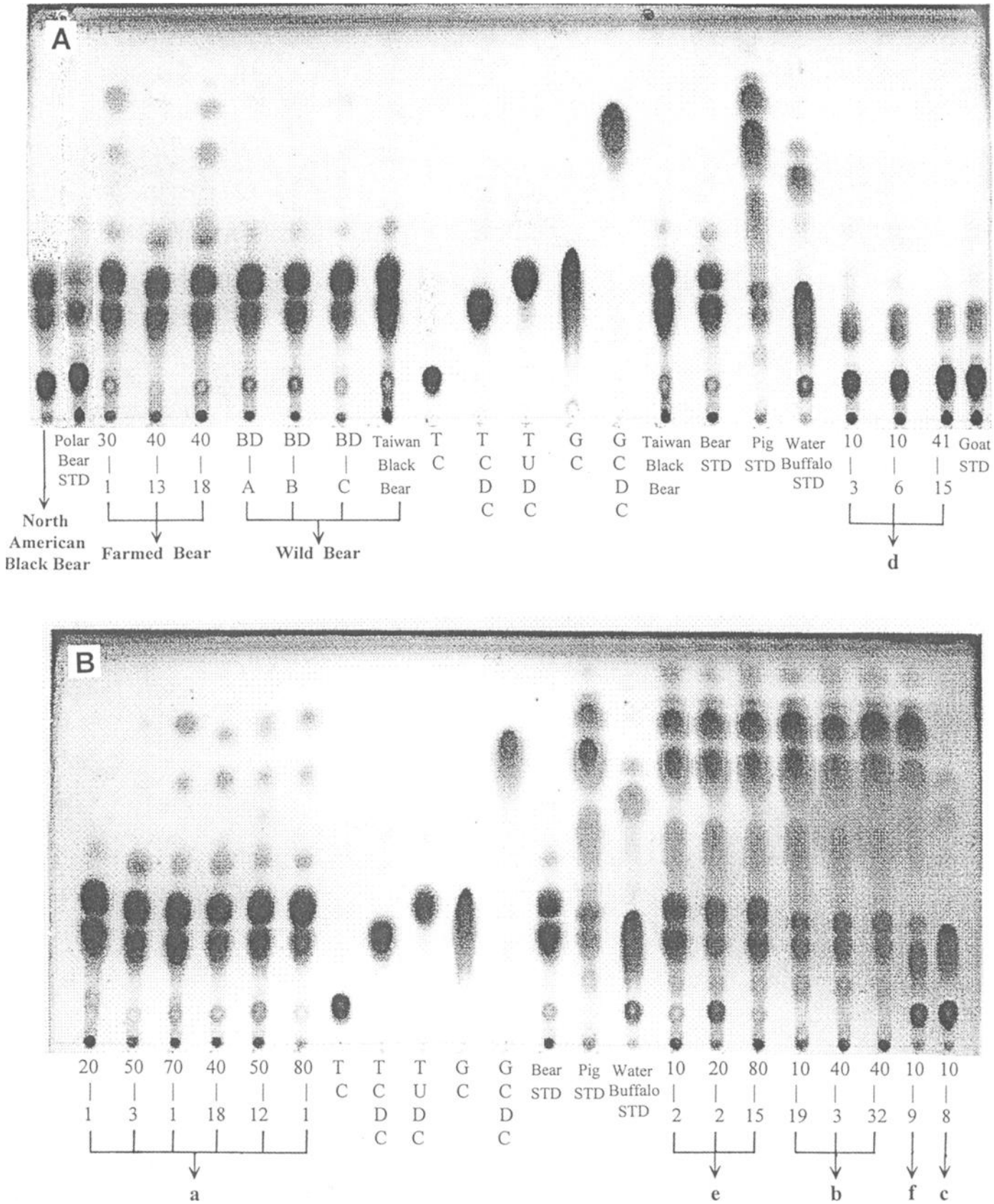

FIG. 3—*HPTLC chromatogram of bear bile salts from Chinese medicine shops. Six types: (A) true bear bile; (B) pig bile; (C) water buffalo bile; (D) goat bile; (E) mixture of bear and pig bile; and (F) mixture of water buffalo and pig bile.*

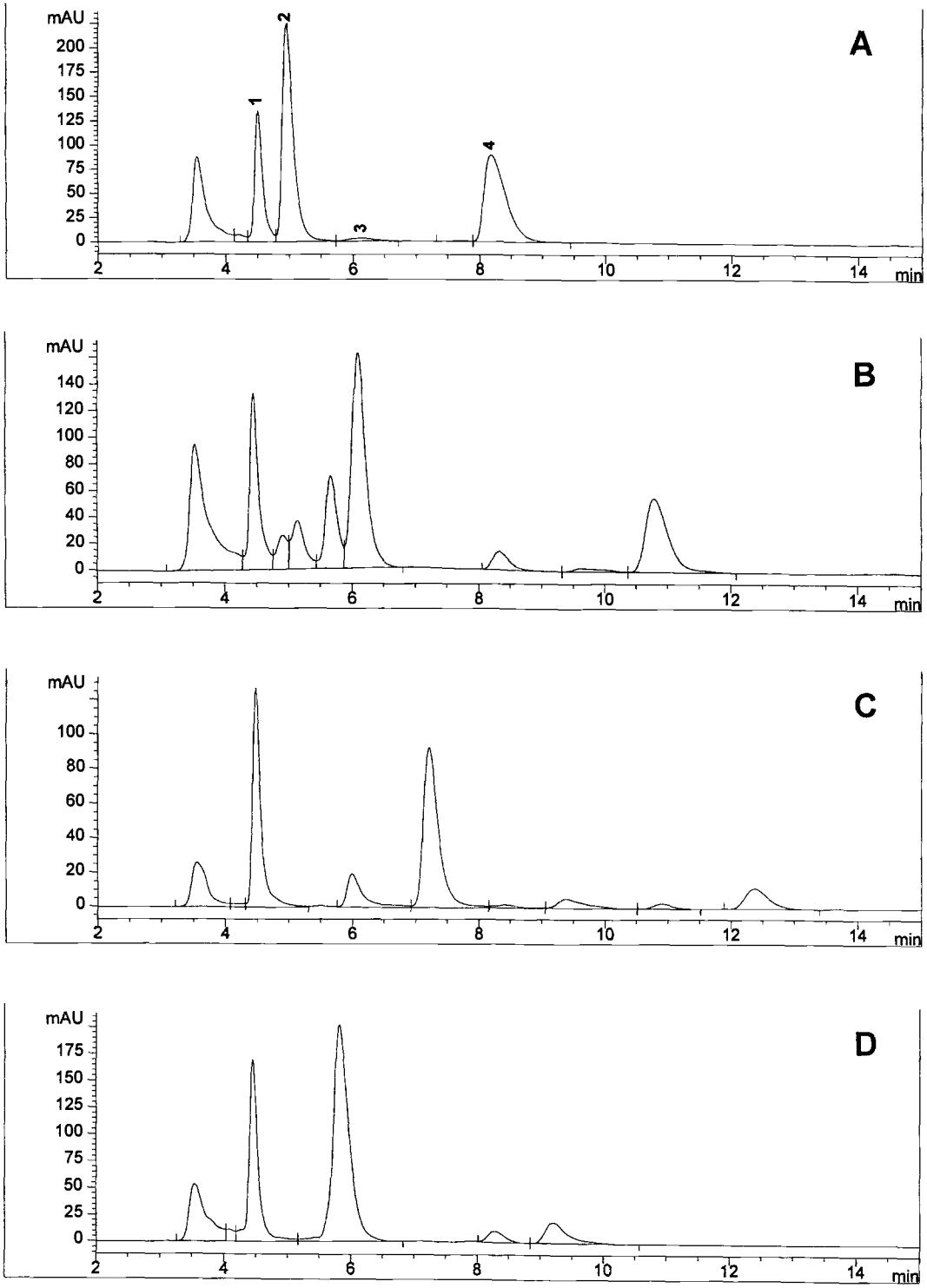

FIG. 4—*HPLC chromatogram of animal bile salts. A: Asian bear; B: pig; C: water buffalo; D: goat.*

It appears that the low content of TC is common among all Asian bear species (wild or farmed) analyzed, with the exception of those derived from Polar and North American black bears (Figs. 5E and 5D). It is thus suggested that high content of TC may serve as an indicator for identifying Polar and North American bears (far left in Fig. 3A), but decreased TC can not be used to identified farmed bear bile, as suggested by Espinoza et al. (6). More comprehensive studies are needed to clarify bile salts distributions among bear gall bladder products from geographical sources.

Source Survey of "Bear" Bile Products in Taiwan's Chinese Medicine Store Market

The results of TLC analyses on 183 samples collected from Chinese medicine shops throughout Taiwan indicate that alleged bear bile products sold in the market can be categorized into the following six types: (a) true bear bile (Fig. 3B: 20-1, 50-3, 70-1, 40-18, 50-12, 80-1); (b) pig gall bile (Fig. 3B: 10-19, 40-3, 40-32); (c) water buffalo bile (Fig. 3B: 10-8); (d) goat bile (Fig. 3A:

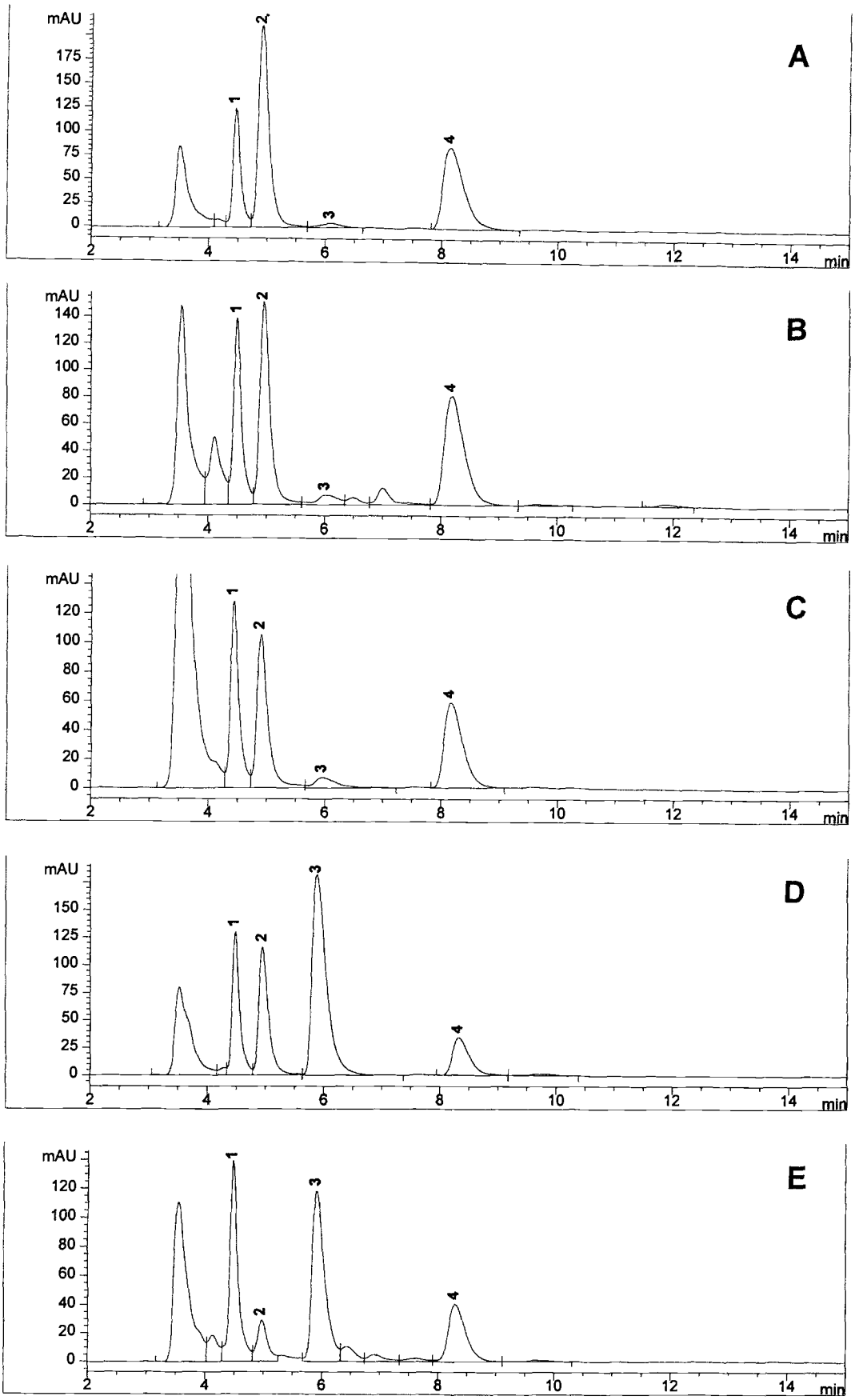

FIG. 5—*HPLC chromatogram of bear bile salts. A: Bhutan wild bear; B: farmed bear; C: Taiwan black bear; D: North American black bear; E: polar bear. 1: P-Cresol (I.S.); 2: tauroursodeoxycholic acid (TUDC); 3: taurocholic acid (TC); 4: taurochenodeoxycholic acid (TCDC).*

TABLE 1—*HPTLC R_f values and HPLC retention time of conjugated bile acids.*

Compound	HPTLC Rf	HPLC Retention Time (Min)
p-Cresol (HPLC retention time reference)	—	4.48
Taurocholic acid (TC)	0.08	6.09
Taurodeoxycholic acid (TDC)	0.21	—
Taurochenodeoxycholic acid (TCDC)	0.25	8.18
Glycocholic acid (GC)	0.29	7.21
Tauroursodeoxycholic acid (TUDC)	0.32	4.95
Taurolithocholic acid (TLC)	0.44	—
Glycodeoxycholic acid (GDC)	0.66	—
Glycochenodeoxycholic acid (GCDC)	0.71	10.78
Glycolithocholic acid (GLC)	0.91	—

10-3, 10-6, 41-15); (e) mixture of bear and pig bile (Fig. 3B: 10-2, 20-2, 80-15); and (f) mixture of water buffalo and pig bile (Fig. 3B: 10-9). Both types (a) and (e) contain bear bile salts. Type (e) includes pig bile components shown as spots at R_f 0.7 and 0.8 (Fig. 3B: 70-1, 40-18, 50-12, 80-1). It is possible that a pig gall bladder sac that was filled with bear bile, but one cannot be certain. Type (a) does not show any bile components of other animals indicating unadulterated Asian bear (wild or farmed) bile product.

The origin of the 183 samples analyzed were found to be as follows: 118 (64%), bile salts or gall bladders were 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.

Conclusion

FTIR spectra of an unknown sample can be used (through a computerized search algorithm against an in-house generated library) as an effective screening tool to determine its alleged authenticity. Samples that pass this preliminary screen can be further analyzed by HPTLC and HPLC procedures to determine whether it is a goat- or a bear-derived product, and if the latter category, differentiate polar and North American bears from Asian bears. Polar and North American bears appear to have higher TC content than the Asiatic bear species. Data collected in this study do not support an earlier proposal in using the relative TC content as the basis for the differentiation of wild and farmed bear biles.

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