



RESEARCH ARTICLE

Differentiation of *Xenarthra* (Mammalia) species through the identification of their fecal bile acid patterns: An ecological tool

Diferenciación de especies de *Xenarthra* (Mammalia) a través de la identificación de sus patrones de ácidos biliares fecales: Una herramienta ecológica

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ABSTRACT

The analysis of feces is a fundamental tool for field work, especially to identify the presence of certain species in an area. Fecal bile acids and their relative concentration follow patterns that are species-specific, and can be characterized by Thin Layer Chromatography (TLC). This technique has been used for differentiating feces of several mammal species; however it has never been used for *Xenarthra* species. In this work, 96 feces of *Xenarthra* species were analyzed by TLC to determine the bile acid pattern. The species were: *Zaedyus pichiy* (n = 10), *ChaetophRACTUS vellerosus* (n = 5), *ChaetophRACTUS villosus* (n = 57), *DasyPUS hybridus* (n = 4), *Priodontes maximus* (n = 2), *Tamandua tetradactyla* (n = 14) and *Myrmecophaga tridactyla* (n = 4). There were differences between the bile acid patterns of all the species, but not between males and females, nor between wild and captive animals of the same species. We found seven known bile acids, cholesterol and seven unidentified compounds (X₁-X₇). All the species had taurocholic, glycochenodeoxycholic and lithocholic acids, and cholesterol. Only *C. villosus* had deoxycholic acid (R_f: 0.30 ± 0.01). *Z. pichiy*, *C. vellerosus* and *C. villosus* had two or three bands of dehydrocholic acid (R_f between 0.29 ± 0.06 and 0.45 ± 0.02), while the other species had one or two. *Z. pichiy* had two unidentified bile acids, X₆ (R_f: 0.85 ± 0.06) and X₇ (R_f: 0.93 ± 0.03), that were almost indistinguishable in other species. *D. hybridus* differed from *Z. pichiy*, *C. vellerosus* and *C. villosus* because it did not have chenodeoxycholic acid and X₂. *T. tetradactyla* was the only species without cholic acid and it differed from *M. tridactyla* because it had dehydrocholic acid. *D. hybridus* was the species with the lowest number of compounds (seven), and differed from the others because it did not have the X₁ and X₅ unidentified compounds. These results are the first for *Xenarthra* and would be very important for future studies about the conservation and the ecophysiology of the group.

Key words: conservation, fecal bile acids, TLC, *Xenarthra*.

RESUMEN

El análisis de las heces es una herramienta fundamental para el trabajo de campo, especialmente para identificar la presencia de una determinada especie en un área. Los ácidos biliares fecales y su concentración relativa siguen patrones que son especie-específicos, y pueden ser caracterizados por Cromatografía en Capa Fina (TLC). Esta técnica ha sido utilizada para diferenciar heces de varias especies de mamíferos, pero nunca en *Xenarthra*. En este trabajo se analizaron 96 heces de especies de *Xenarthra* a través de TLC, para determinar el patrón de ácidos biliares fecales. Las especies fueron: *Zaedyus pichiy* (n = 10), *ChaetophRACTUS vellerosus* (n = 5), *ChaetophRACTUS villosus* (n = 57), *DasyPUS hybridus* (n = 4), *Priodontes maximus* (n = 2), *Tamandua tetradactyla* (n = 14) y *Myrmecophaga tridactyla* (n = 4). Se encontraron diferencias entre los patrones de ácidos biliares para todas las especies, pero no entre machos y hembras, ni entre animales de cautiverio y silvestres de la misma especie. Se encontraron siete ácidos biliares conocidos, colesterol y siete compuestos aun no identificados (X₁-X₇). Todas las especies tuvieron los ácidos taurocólico, glicoquenodeoxicólico y litocólico, y colesterol. Solo *C. villosus* tuvo ácido deoxicólico (R_f: 0.30 ± 0.01). *Z. pichiy*, *C. vellerosus* y *C. villosus* tuvieron dos o tres bandas de ácido dehidrocólico (R_f entre 0.29 ± 0.06 y 0.45 ± 0.02), mientras que las otras especies tuvieron una o dos. *Z. pichiy* tuvo dos compuestos no identificados, X₆ (R_f: 0.85 ± 0.06) y X₇ (R_f: 0.93 ± 0.03), que fueron casi indistinguibles en las otras especies. *D. hybridus* difirió de *Z. pichiy*, *C. vellerosus* y *C. villosus* porque no tuvo ácido quenodeoxicólico y X₂. *T. tetradactyla* fue la única especie que no presentó ácido cólico y difirió de *M. tridactyla* por la presencia de ácido dehidrocólico. *D. hybridus* fue la especie con el menor número de compuestos (siete) y difirió del resto porque no presentó los compuestos X₁ y X₅. Estos resultados son los primeros para *Xenarthra* y podrían ser muy importantes para futuros estudios acerca de la conservación y ecofisiología del grupo.

Palabras clave: ácidos biliares fecales, conservación, TLC, *Xenarthra*.

INTRODUCTION

Xenarthra are one of the most characteristic groups of mammals from South America. Including armadillos, anteaters and sloths, they comprise a group with 31 species, 38 % of them threatened by extinction (Aguiar & Da Fonseca 2008). Despite all the intriguing features about Xenarthrans, they seem understudied relative to many other mammalian groups (Vizcaíno & Loughry 2008). Rarely conspicuous or easily found, many species are nocturnal burrowers, while others are lifelong residents of the high forest canopy (Aguiar & Da Fonseca 2008). For this reason, the study of Xenarthra indirect evidences plays a particularly relevant role.

The analysis of feces is a fundamental tool for field work, especially to identify or to confirm the presence of certain species in an area. The identification can be done by external physical characteristics such as size, shape, odor and color, or through specific signals associated with the deposition of feces, for example tracks and scrapes (Fernández et al. 1997, Cazón & Sühring 1999). However, this technique is sometimes useless because of the difficulties that exist in the correct identification of feces. Often, this sort of evidence is not present mainly because many of these external characteristics are sensitive to environmental conditions such as heat, desiccation or fast decomposition in humid and rainy regions, and can be affected by another type of factors: health, diet, size and age of the individual (Capurro et al. 1997, Chame 2003). Because of these reasons is that other techniques become necessary. Fecal bile acids and their relative concentration follow patterns that are species-specific (Haslewood 1967). These patterns can be characterized by thin layer chromatography (TLC) (Cazón & Sühring 1999).

Although various separation techniques are commonly applied for the determination of bile acids, TLC offers practical advantages, mainly its simplicity, economical equipment needed, ease of operation, short analysis time and high efficiency in analyzing simultaneously a large number of samples (Palamarev et al. 2004, Berezkin et al. 2005). It enables reliable separation and analysis of a wide variety of compounds from different types of biological samples. Moreover, this technique is versatile

because it can be modified using different types of mobile and stationary phases and developers (Sherma & Fried 2005, Dolowy 2007).

TLC of bile acids has been used to differentiate feces of several mammal species, mainly carnivores such as the lesser grisson (*Galictis cuja*) (Molina, 1782), guinea cat (*Leopardus guigna*) (Molina, 1782), red fox (*Lycalopex culpaeus*) (Molina, 1782), grey fox (*Lycalopex griseus*) (Gray, 1837), pampas's fox (*Lycalopex gymnocercus*) (Fischer, 1814), puma (*Puma concolor*) (Linnaeus, 1771), jaguar (*Panthera onca*) (Linnaeus, 1758), snow leopard (*Panthera pardus ciscaucasica*) (Satunin, 1914), pandas and different species of bears (Hagey et al. 1993a, Picton & Kendall 1994, Jiménez et al. 1996, Capurro et al. 1997, Fernández et al. 1997, Taber et al. 1997, Guerrero et al. 2006, Solá et al. 2006, Khorozyan et al. 2007). It had also been applied to a wide variety of other species: manatees (Kuroki et al. 1988), sperm whales (Hagey et al. 1993b), storks and herons (Hagey et al. 2002) among others. However, TLC of bile acids has never been applied to differentiate Xenarthra feces.

Because fecal bile acid composition is species-specific among several mammal species, we propose that the analysis of those compounds could also be useful to differentiate feces of Xenarthra species. Particularly, some armadillo species are sympatric and use similar habitats (Ciuccio et al. 2007), being difficult to correctly identify their feces. If those feces can be recognized, they could be used as an indicator of the presence of that species in a certain area.

The aim of this work was to identify, by TLC, the fecal bile acid patterns of some Xenarthra species and to test if they are useful to differentiate those species. Through the identification of those patterns, we expect to find an important tool for future ecological, conservation and distribution studies of Xenarthra species in the wild.

METHODS

Sample collection and treatment

Samples were collected in different areas of Argentina. Those of wild animals were mainly collected in a

private field 17 km southwest from Bahía Blanca's city (Buenos Aires province), El Rey National Park (Salta province), Calilegua National Park (Jujuy province) and El Copo National Park (Santiago del Estero province). Those from captive animals were obtained in the Bioterio of Dpto. de Biología, Bioquímica y Farmacia, UNS, Bahía Blanca (Buenos Aires province), Buenos Aires Zoo (Ciudad Autónoma of Buenos Aires), La Plata Zoo (Buenos Aires province) and Finca Las Costas (Salta province).

Ninety-six feces of the following species were analyzed: common pichi or "pichi común", *Zaedyus pichi* (Desmarest, 1804) (n = 10), lesser hairy armadillo, crying armadillo or "pichi llorón", *Chaetophractus vellerosus* (Gray, 1865) (n = 5), large hairy armadillo or "peludo", *Chaetophractus villosus* (n = 57), southern lesser long-nosed armadillo or "mulita", *Dasybus hybridus* (Desmarest, 1804) (n = 4), tamandúa, *Tamandua tetradactyla* (Linnaeus, 1758) (n = 14), giant anteater, *Myrmecophaga tridactyla* (Linnaeus, 1758) (n = 4) and giant armadillo or "tatú carreta", *Priodontes maximus* (Kerr, 1792) (n = 2). Each sample was dried in oven at 30° C for one day and stored in hermetic flasks in a dry and dark place.

TLC analysis

Feces were crushed and sieved. One gram of each sample was extracted with 20 mL of benzene: methanol (1:1 v/v). The extracts were filtered and concentrated to a final volume of 5 ml. Each sample extract and standards for the most common mammal bile acids were spotted on silicagel 60F₂₅₄ plates with aluminum base of 20 x 20 cm, 0.2 mm (Merck). The standards used were: lithocholic acid, taurocholic acid, glycocholic acid, cholic acid, chenodeoxycholic acid, deoxycholic acid, dehydrocholic acid, glycochenodeoxycholic acid and cholesterol. Bile acid standard stock solutions were prepared in methanol at a concentration of 0.1 %. Different sample (75, 90, 105, 120, 150 and 180 µl) and standard (7.5, 15, 22.5 and 30 µl) quantities were spotted on the plates, so as to standardize the optimal concentrations for a better visualization.

To test and find the best eluant, plates were eluted in a glass developing tank with: ciclohexane, petroleum ether and three concentrations of a solution of toluene: acetic acid: water (5:5:1.5 v/v; 1.5:5:5 v/v and 5:5:0 v/v). All runs were performed under saturated mobile phase vapor conditions at ambient temperature.

Bile acid spots were visualized by spraying the plates with a revealing solution of anisaldehyde: glacial acetic acid: sulphuric acid (0.5:50:1 v/v). Plates were heated in oven at 150 °C for 15 minutes.

Data analysis

The bile acid pattern of each species was determined by the comparison of R_f values (relation between distance travelled by the compound and distance travelled by the eluent), color and intensity (concentration) of the compounds with those of standard solutions. R_f mean (X) \pm standard deviation (SD) of each compound were calculated for each species.

Similarity in the bile acid composition for all species was assessed through the Jaccard index (Krebs 1989). Comparisons among different species should give values smaller than 1 (partial similarity).

RESULTS

Bile acid pattern

A total of 15 compounds were detected; seven corresponded to bile acid standards, seven were unidentified compounds ($X_1 - X_7$) which did not coincide with any of the standards, and cholesterol (Table 1). Glycocholic acid was the only compound which was totally absent in all the species.

In the chromatographic plates, standard bile acids had a characteristic color and R_f value (mean \pm SD), helping in the right identification of the compounds. Dehydrocholic acid differed from the other standards because it showed two and, in some cases, three bands, and a wide variation between its R_f values. The standards which showed more intense colors were lithocholic acid (dark green), taurocholic acid (blue-grey) and cholesterol (dark pink). The rest of the compounds had less intense colors, being dehydrocholic acid distinctively orange (Table 2).

Differences among the bile acid patterns of all the studied species but not between males and females of the same species were found (Table 1, Fig. 1). All species had three known bile acids: taurocholic, glycochenodeoxycholic and lithocholic acids, cholesterol (Fig. 2) and two unknown compounds, X_4 and X_6 .

The primary bile acids, cholic and chenodeoxycholic, appeared in six and in four of the seven species, respectively; the secondary bile acids, deoxycholic and lithocholic, appeared in one and in all species respectively. The compound which appeared with the lowest frequency was deoxycholic acid (Fig. 2).

D. hybridus had the lowest number of compounds (7), followed by *M. tridactyla* with 11, *T. tetradactyla* and *C. vellerosus* with 12, *P. maximus* with 13, *Z. pichi* with 14 and *C. villosus* which had all the compounds found (15/15).

C. villosus was the only species which showed deoxycholic acid (R_f : 0.30 \pm 0.01). *Z. pichi* and *C. vellerosus* differed because *Z. pichi* had X_3 (R_f : 0.57 \pm 0.02) and X_7 (R_f : 0.93 \pm 0.03).

Z. pichi had two unidentified bile acids, X_6 (R_f : 0.85 \pm 0.06) and X_7 (R_f : 0.93 \pm 0.03) which, although were found in other species, they

were almost indistinguishable. *Z. pichiy* differed from *D. hybridus*, *P. maximus*, *T. tetradactyla* and *M. tridactyla* because it had more compounds. *D. hybridus* differed from *Z. pichiy*, *C. vellerosus* and *C. villosus* because it

did not have chenodeoxycholic acid and X₂; it differed from *T. tetradactyla* because it had cholic acid (R_f: 0.13 ± 0.03), and from *P. maximus* and *M. tridactyla* because it did not have X₃ (Table 1).

TABLE 1

Presence and/or absence of each compound for all species expressed as mean R_f value (± SD). Note: Blank boxes mean the compound is not present in that species.

TCA: taurocholic acid; GCDCA: glycochenodeoxycholic acid; CA: cholic acid; GCA: glycocholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; DHCA: dehydrocholic acid; LCA: lithocholic acid and CHOL: cholesterol.

Presencia y/o ausencia de cada compuesto para todas las especies expresado como media de los valores de R_f (± DE). Nota: los espacios en blanco significan que el compuesto no está presente en esa especie. TCA: ácido taurocólico; GCDCA: ácido glicoquenodeoxicólico; CA: ácido cólico; GCA: ácido glicocólico; CDCA: ácido quenodeoxicólico; DCA: ácido deoxicólico; DHCA: ácido dehidrocólico; LCA: ácido litocólico y CHOL: colesterol.

	<i>D. hybridus</i>	<i>Z. pichiy</i>	<i>C. vellerosus</i>	<i>T. tetradactyla</i>	<i>C. villosus</i>	<i>M. tridactyla</i>	<i>P. maximus</i>
TCA	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
GCDCA	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.03	0.04 ± 0.01	0.05 ± 0
CA	0.13 ± 0.03	0.15 ± 0.03	0.17 ± 0.01		0.18 ± 0.03	0.20 ± 0	0.19
GCA							
CDCA		0.25 ± 0.04	0.23 ± 0.04		0.27 ± 0.01		0.27
DCA					0.30 ± 0.01		
DHCA 1		0.29 ± 0.06	0.34 ± 0.08	0.26 ± 0.02	0.29 ± 0.08		
DHCA 2		0.43 ± 0.04	0.45 ± 0.02		0.45 ± 0.08		0.42
LCA	0.52 ± 0.05	0.52 ± 0.03	0.50 ± 0.03	0.55 ± 0.03	0.53 ± 0.04	0.56 ± 0.05	0.51
CHOL	0.54 ± 0.04	0.56 ± 0.03	0.53 ± 0.03	0.54 ± 0.03	0.56 ± 0.04	0.57 ± 0.04	0.53 ± 0.01
X ₁		0.38 ± 0.03	0.38 ± 0.02	0.39 ± 0.03	0.40 ± 0.04	0.45 ± 0	0.37 ± 0.01
X ₂		0.41 ± 0.03	0.43 ± 0.02	0.45 ± 0.02	0.45 ± 0.03	0.49 ± 0.01	
X ₃		0.57 ± 0.02		0.58 ± 0.02	0.59 ± 0.03	0.63 ± 0.02	0.56
X ₄	0.58 ± 0.03	0.62 ± 0.03	0.58 ± 0.03	0.63 ± 0.03	0.61 ± 0.04	0.65 ± 0.06	0.60 ± 0.02
X ₅		0.73 ± 0.04	0.66 ± 0.07	0.81 ± 0.04	0.74 ± 0.07	0.69 ± 0.05	0.73 ± 0.06
X ₆	0.91 ± 0.05	0.85 ± 0.06	0.87 ± 0.04	0.93 ± 0.04	0.92 ± 0.03	0.91 ± 0.04	0.87
X ₇		0.93 ± 0.03		0.96 ± 0.01	0.92 ± 0.02		0.93

TABLE 2

Color and mean R_f values (± SD) of the standard bile acids. Note: in some cases more than one band of the same acid appeared in the chromatographic plates.

Color y media de los valores de R_f (± DE) de los ácidos biliares estándares. Nota: en algunos casos aparece más de una banda del mismo ácido en las placas.

Standard	Color	Mean R _f ± SD
Taurocholic acid (TCA)	Blue-grey	0 ± 0
Glycochenodeoxycholic acid (GCDCA)	Light-violet	0.02 ± 0
Glycocholic acid (GCA)	Violet	0.11 ± 0.04
Cholic acid (CA)	Grey-greenish	0.11 ± 0.03
Chenodeoxycholic acid (CDCA)	Light violet	0.26 ± 0.03
Deoxycholic acid (DCA)	Green	0.29 ± 0.03
Dehydrocholic acid (DHCA) (2 bands)	Orange-reddish	0.33 ± 0.03; 0.41 ± 0.05
Lithocholic acid (LCA)	Dark green	0.49 ± 0.06
Cholesterol (CHOL)	Dark pink	0.56 ± 0.04

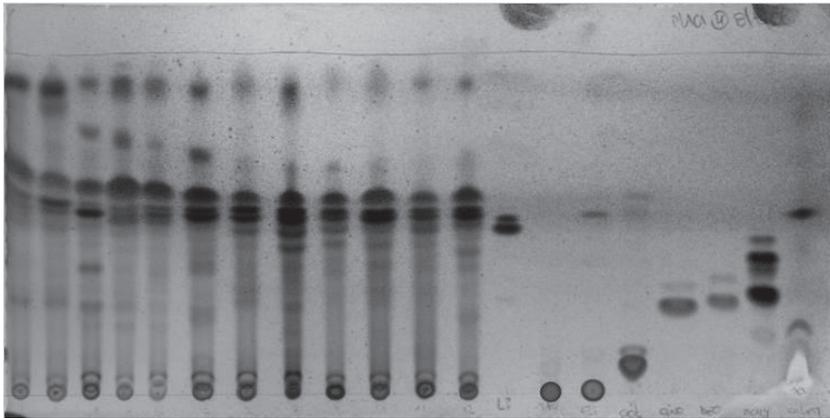


Fig. 1: Developed chromatographic plate showing the different bile acids as differentiated spots. Note: on the right the standard bile acids are shown.

Placa cromatográfica revelada que muestra los diferentes ácidos biliares como bandas diferenciadas. Nota: a la derecha se muestran los ácidos biliares estándares.

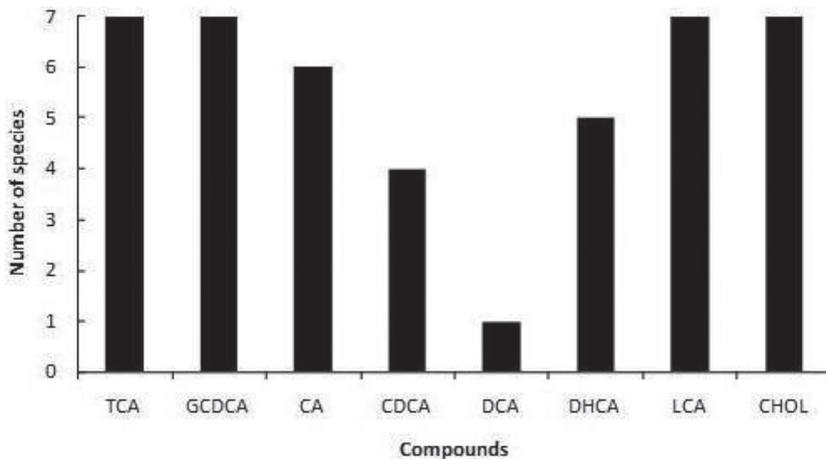


Fig. 2: Frequency of appearance of the seven identified bile acids and cholesterol in relation to number of species. TCA: taurocholic acid; GCDCA: glycochenodeoxycholic acid; CA: cholic acid; GCA: glycocholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; DHCA: dehydrocholic acid; LCA: lithocholic acid and CHOL: cholesterol.

Frecuencia de aparición de los siete ácidos biliares identificados y colesterol en relación al número de especies. TCA: ácido taurocólico; GCDCA: ácido glicoquenodeoxicólico; CA: ácido cólico; GCA: ácido glicocólico; CDCA: ácido quenodeoxicólico; DCA: ácido deoxicólico; DHCA: ácido dehidrocólico; LCA: ácido litocólico y CHOL: colesterol.

P. maximus did not have X₂ and it had one band of dehydrocholic acid (R_f: 0.42); it differed from *T. tetradactyla* and *M. tridactyla* because it had chenodeoxycholic acid (R_f: 0.27). *T. tetradactyla* was the only species without cholic acid; it did not have chenodeoxycholic acid, and it differs from *M. tridactyla* because that one had one band of dehydrocholic acid (R_f: 0.26 ± 0.02). *M.*

tridactyla had cholic acid, and *P. maximus* had cholic, chenodeoxycholic and dehydrocholic acids; it lacked X₂ (Table 1).

Only three species had more than one band of dehydrocholic acid: *C. villosus* (R_f: 0.29 ± 0.08 and 0.45 ± 0.08), *C. vellerosus* (R_f: 0.34 ± 0.08 and 0.45 ± 0.02) and *Z. pichiy* (R_f: 0.29 ± 0.06 and 0.43 ± 0.04) (Table 1).

Vegetal pigments

During plate running colored bands were observed; they corresponded to plant pigments which were yellow or orange for captive animals and green for wild ones. For *Z. pichiy*, *C. vellerosus* and *D. hybridus* samples, these bands were less green and less intense than for *C. villosus* samples, which showed the most intense bands among armadillo species. On the other hand, *M. tridactyla*, *T. tetradactyla* and *P. maximus* showed brownish bands.

Comparison of eluents and sample concentration

The eluent which showed the best resolution in the separation of the compounds was toluene: acetic acid: water, in a proportion of 5:5:1.5 v/v; petroleum ether and ciclohexane were not able to separate them.

Samples from captive animals had more concentrated extracts than those from wild ones, showing more intense bands in the chromatographic plates. For standard solutions the optimal quantity for a good visualization was 15-20 µl; and for fecal samples, it depended upon the condition, 90 µl for captive individuals and 150-180 µl for wild ones.

Interesting, in one sample from a wild individual of *C. villosus* which was two years older than the rest of the samples, the bands were perfectly visualized and identified.

Similarity among species

Jaccard index for comparisons among all species varied between 0.44 and 0.94 (Table 3). Relative similarity in the bile acid pattern of some pairs of species, did not agree with phylogenetic relatedness among Xenarthra species. The bile acid pattern for *D. hybridus* was more comparable to that of *Z. pichiy* or *C. vellerosus* than to *C. villosus*. On the other hand, the profile of *Z. pichiy* was more comparable to *C. vellerosus* and *C. villosus* than to any other species, showing the greatest degree of similarity with *C. villosus*. The lowest degree of similarity occurred between *D. hybridus* and *C. villosus*. Jaccard values were also low for the pairs *T. tetradactyla*-*D. hybridus*, *P. maximus*-*D. hybridus*, *M. tridactyla*-*C. villosus*, *T. tetradactyla*-*C. vellerosus* and *T. tetradactyla*-*P. maximus*. Jaccard values were also higher for the pairs of species *Z. pichiy*-*C. vellerosus* and *Z. pichiy*-*P. maximus* (Table 3).

DISCUSSION

As a non-invasive method, the analysis of feces is a fundamental tool for field work in ecological studies, not only to identify the presence of certain species in a particular area (Ciuccio et al. 2007), but also for studying threatened species or animals difficult to observe and trap. For the identification, the original fecal shape must be maintained;

TABLE 3

Jaccard index for comparisons of fecal bile acid pattern among *C. villosus*, *C. vellerosus*, *D. hybridus*, *Z. pichiy*, *T. tetradactyla*, *M. tridactyla* and *P. maximus*.

Índice de Jaccard para la comparación de los patrones de ácidos biliares entre *C. villosus*, *C. vellerosus*, *D. hybridus*, *Z. pichiy*, *T. tetradactyla*, *M. tridactyla* y *P. maximus*.

	<i>D. hybridus</i>	<i>Z. pichiy</i>	<i>C. vellerosus</i>	<i>T. tetradactyla</i>	<i>C. villosus</i>	<i>M. tridactyla</i>	<i>P. maximus</i>
<i>D. hybridus</i>	1	0.47	0.54	0.46	0.44	0.64	0.54
<i>Z. pichiy</i>		1	0.87	0.80	0.94	0.73	0.87
<i>C. vellerosus</i>			1	0.67	0.81	0.71	0.73
<i>T. tetradactyla</i>				1	0.75	0.77	0.67
<i>C. villosus</i>					1	0.69	0.81
<i>M. tridactyla</i>						1	0.71
<i>P. maximus</i>							1

however, as several factors can corrode it through time, visual identification is not always reliable (Chame 2003, Khorozyan et al. 2007). Particularly, feces from *Xenarthra* are sometimes difficult to identify in the wild because they are, commonly, total or partially mixed with the substrate.

The chromatographic determination of fecal bile acids has become a more precise method to identify unknown feces from the wild. The comparison of the whole pattern of fecal bile acids between field-collected scats and scats with known origin allows identifying the species from fecal material (Khorozyan et al. 2007), avoiding capture and manipulation of animals.

Even if TLC of fecal bile acids offers practical advantages such as simplicity and ease of operation (Perwaiz et al. 2001, Sherma & Fried 2005), like any laboratory technique it needs much of practical knowledge and skills (Khorozyan et al. 2007) and it requires a careful and detailed analysis from the researcher. Several characteristics of the bands such as color, intensity and R_f of each bile acid had to be carefully analyzed in the chromatographic plates to determine the whole pattern of each species. The usefulness of TLC to identify feces from *Xenarthra* species was demonstrated in this work as it allowed the extraction, visualization and identification of fecal bile acids from individuals of all the studied species.

Moreover, we showed the sensitivity of TLC because some pairs of bile acids with very similar R_f values, chenodeoxycholic-deoxycholic acids, and cholic-glycocholic acids, were discriminated. Although some standard R_f values partially overlapped, it was possible to establish a R_f range for each compound, and together with their color and intensity, the spots were correctly identified. The relative concentration of the compounds was proportional to the intensity of each band in the chromatographic plate, as determined visually. Specific colors obtained through the oxidation by anisaldehyde for each bile acid allowed the identification of the spots in the plates, for example green for lithocholic acid and violet for cholesterol. Therefore, we stress the importance of spotting standard solutions together with the samples in each chromatographic plate.

For several years, TLC has been used to identify wild-collected feces, mainly for species with low contents of vegetal material in their diets, such as carnivores (Major et al. 1980, Johnson et al. 1984, Fernández et al. 1997, Cazón & Sühling 1999). Nevertheless, Jiménez et al. (1996) were not able to differentiate feces from *Lycalopex culpaeus* and *L. griseus* in Chile; they argued that there was too much variability in the spot pattern even among feces from the same individual. In spite of that, Capurro et al. (1997) could discriminate feces from both species. Moreover, Guerrero et al. (2006) demonstrated that bile acid patterns were specific for some threatened carnivore species in Chile.

In the present study, although there were some variations among samples of the same species, especially between wild and captive animal feces, all the studied species could be identified, being *D. hybridus* the species with the lowest number of compounds in its pattern and *C. villosus* the species with the biggest number of compounds; the rest of the species had between 11 and 16, including seven unidentified compounds and cholesterol.

Variations between some samples of the same species found in this study and also reported before for other species (Jiménez et al. 1996, Capurro et al. 1997, Fernández et al. 1997) may be due to different factors.

The first one is the concentration of the sample; samples from captive animals showed more intense bands, while samples from wild animals had little concentration of some bile acids, making the correct identification sometimes difficult. However, as it was said before, we could find the optimal concentration, i.e., the concentration that allowed the detection of the compounds in the chromatographic plates, for wild and captive animals, being higher for wild ones.

The second factor is the effect of the type of diet, which has been considered in several investigations to interpret the results. It is possible that the presence of some chemical substances in the feces, as products of the diet, has an effect on the detectability of bile acids, masking the spots (Jiménez et al. 1996) as it was observed for coyotes (Quinn & Jackman 1994).

Xenarthras here studied are in general omnivores, and some of them carnivores-

omnivores (Redford 1985), including invertebrates, small vertebrates, carrion, plant roots, tubers and seeds in their diets (Casanave et al. 2003, Dalponte & Tavares-Filho 2004; Soibelzon et al. 2007, McDonough & Loughry 2008). The color differences of extracts among all species found in this work may reflect those variations in their diet composition, mainly due to vegetal pigments. Although we found some plant material in the feces, they had only small amounts; and as it was established, there was no evidence that any of the natural prey items taken by these species interfere with bile acids during the chromatographic run; even more, spots were able to run above pigments line.

Another factor to discuss is age of wild-collected scats and thus, weathering, which plays an important role in the ability of bile acid TLC to identify species from their scats (Taber et al. 1997, Khorozyan et al. 2007). Some authors (Major et al. 1980, Ray 1996, Fernandez et al. 1997, Khorozyan et al. 2007) suggest that feces weathering can lower the concentration of bile acids in scats, leading to their erroneous identification. In our study, it was demonstrated that bile acids were clearly identified even in old feces, as it was the case of *C. villosus*. Further, small sample size can worsen the TLC performance (Ray 1996, Taber et al. 1997, Ray & Sunquist 2001); however, this was not the case of this study since we used sufficient quantity of dry pulverized fecal material to allow a good detection of the compounds (Cazón & Sühring 1999).

The precision in the use of TLC is another important factor to be considered. For example, the spraying with the visualizing agent is not always uniform and some areas of the chromatographic plate may be uncolored, resulting in an incorrect interpretation of the bands. The choice of the correct stationary and mobile phases is also important as it depends upon the type of sample and the objectives of the study. The stationary phase is usually polar and the mobile phase is non-polar; nowadays, silica is the first choice for the stationary phase. Thus, the greatest problem in using TLC in specific cases is the selection of suitable mobile phases (Palamarev et al. 2004). We probed that the solution composed of toluene: acetic acid: water (5:5:1.5 v/v) was the most suitable mobile phase for our purpose,

allowing a good separation of the compounds for *Xenarthra* feces.

From our results, and because TLC of fecal bile acids has proved to offer robust data to establish habitat use and to study food habits of other sympatric mammal species (Taber et al. 1997, Guerrero et al. 2006), we assume that this technique would also be useful for future ecological studies in *Xenarthra*. In addition, studies about the presence of possible variations in the bile acid composition due to other factors such as age and environmental conditions, as those done for other vertebrate species (Hagey et al. 1993, Morozov & Vysotskaya 2007, Lundell & Wikvall 2008) are needed for *Xenarthra*.

Finally, considering the scarcity of available information about some ecological and biological aspects of *Xenarthra*, these results, the first ones on the application of TLC for the identification of their feces, could be very important for future studies about the conservation, distribution and eco-physiology of this group.

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