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Inability of thin-layer chromatography to distinguish feces from congeneric foxes by their bile acid contents

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We used thin-layer chromatography of fecal bile acids in an attempt to separate feces of culpeo fox *Pseudalopex culpaeus* (Molina, 1782) from those of chilla fox *P. griseus* (Gray, 1837). We tested the method with pure bile acids and with feces from known fox species on two different plate types. Results differed according to the plate type used. The technique failed to distinguish scats from the two fox species. We found variability in the spot pattern of bile acids within species, and within individuals, likely associated to the diet. The location of the spots (*Rf* values) also varied with the concentration of the sample, and the color and location changed with different plate types. We warn that the thin-layer chromatography of fecal bile acids is still unreliable for distinguishing feces between sympatric carnivores. Hence, we propose to rely on alternative techniques.

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Introduction

Carnivore diets have been extensively studied by analyzing prey remains found in feces. However, scats of canids are highly variable morphologically and when species are sympatric, their feces are difficult to distinguish (Murie 1974). Fecal diameter (Weaver and Fritts 1979, Danner and Dodd 1982, Jiménez 1993) and pH (Green and Flinders 1981) are unreliable characters for separating sympatric canids. The bile acids present in the feces of carnivores, however, are species--specific (Lin *et al.* 1978 cited in Major *et al.* 1980) and when dry are very stable (Major *et al.* 1980, Johnson *et al.* 1984). Thus, it is feasible to differentiate feces of some species by using the thin-layer chromatography technique (TLC) of fecal bile acids (Kritchevsky *et al.* 1963, Chavez and Krone 1976). Capurro *et al.* (in press) found that this technique can be used to separate feces of five South

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American canids. Here, we test the TLC of fecal bile acids as a method for distinguishing feces from the sympatric and congeneric culpeo *Pseudalopex culpaeus* (Molina, 1782) and chilla fox *P. griseus* (Gray, 1837) in north-central Chile (Jiménez 1993).

Material and methods

We followed the TLC technique described by Major *et al.* (1980). Initially, we used Macherey--Nagel (Düren, Germany) pre-coated TLC plates SIL G-25 UV_{254}. Later, we used Merck (Germany) pre-coated TLC plates silica gel 60 F_{254}, because of the initial failure to distinguish species-specific bile acid spot patterns with the SIL G-25 UV_{254} plates. Both the color of the spots and the distances migrated by the samples were recorded under a UV light. The solvent front was recorded immediately after the run.

The *Rf* value is the ratio of the distance travelled by the sample relative to the distance moved by the solvent front. It measures the relative migration ability of the compound on the plate. Individual compounds in a mixture segregate by their differential movement ability.

Ten samples were run in each of 12 plates. Samples were deposited on the plates with a Hamilton syringe (with a 1 µl error). Ten, 20, 30, 40, 60, and 90 µl of 5 mg/ml concentration of the standards were run in order to standardize the optimal concentrations for each bile acid. For standardizing the methods, we proceeded by steps.

We ran different bile acid standards in the same plate types and the same standards in different plate types. As standard bile acids, we used purified cholic, dehydrocholic, deoxycholic, lithocholic, cholic methyl ester, and chenodeoxycholic acid from SIGMA (bile acid kit Sigma 091H9001, USA). A mixture of all standards was also tested using 20, 40, and 60 µl of 5 mg/ml concentration. Thereafter, separate standards were run in the same plates with feces of known chillas and culpeos. Different feces from the same fox were also used.

We used culpeo and chilla feces from captive animals in the Santiago Zoo, from free-ranging culpeos in Fray Jorge National Park (30°38'S, 71°40'W, 400 km N of Santiago), where only culpeo is present, and from foxes trapped at the Chinchilla National Reserve (at Aucó, 31°30'S, 71°06'W, 300 km N of Santiago; Jiménez 1993). As a test, we ran known samples with unknown feces collected at Aucó.

Feces from conspecific captured foxes that had remains of different prey types (eg feces made up of fruit remains vs mammal remains vs insect remains) provided insights for addressing the effect of prey type in the pattern of TLC spots obtained.

Results

Using the standard bile acids, different results were obtained with different plates. *Rf* values (Rank sum test, Hollander and Wolfe 1973; $W \geq 3$, $p < 0.05$), colors, and numbers of spots changed substantially (Table 1). *Rf* values also changed with the concentration of the sample. *Rf* values decreased inversely with the concentration of the sample used (Table 1; eg for chenodeoxycholic acid on Macherey-Nagel plates, $r = -0.993$, $n = 5$, $p = 0.0008$, not shown). Even at the same concentration, and for the same sample and plate type used, there was high variability in the *Rf* values (as an example see results on Merck plates in Table 1). Standards when run together showed different spot pattern and *Rf* values than if they were run separately.

Table 1. Colors under UV light and *Rf* values of spots for different amounts (µl of 5 mg/ml concentration) of standard bile acids. Results are from two different TLC plate brands. Data shown here are representative of several other runs. The symbol "–" indicates that the test was not run for that concentration.

Bile acid	Macherey-Nagel SIL G-25 UV_{254} plates							Merck 60 F_{254} plates		
	Color	µl						Color	µl	
		10	20	30	40	60	90		30	30
Lithocholic	Blue	–	–	0.880	–	0.867	–	Fluorescent	0.813	0.736
	Blue	–	–	0.741	–	0.741	–	Fluorescent	0.761	0.684
Deoxycholic	Yellow	–	–	0.590	–	0.566	–	Yellow	0.497	0.491
Chenodeoxycholic	Blue	–	0.513	0.506	0.500	0.488	–	Yellow	0.465	0.459
								Fluorescent	0.115	0.127
Cholic	Yellow	–	–	0.491	–	0.487	0.484	Yellow	0.136	0.151
Cholic methyl ester	Brown	–	0.306	0.269	–	0.270	–	Yellow	0.209	0.205
Dehydrocholic	Brown	0.171	0.171	0.171	0.171	–	–	Yellow	0.545	0.503
								Fluorescent	0.487	0.454

Feces of chilla and culpeo could not be distinguished by the bile acids pattern using TLC (Table 2). There was no clear pattern for the spots produced by feces from either known chillas or culpeos. Further, there were important differences in the spot patterns of different feces from the same individual (Table 2). The results presented in Table 2 were consistent with several other runs with different plates and combinations of fox species and individuals. By running several samples from conspecific foxes with known diets, we found that foxes (probably different individuals) fed on different diets produced different spot patterns of bile acids.

Table 2. Colors and *Rf* values of spots from control culpeo fox (CU) and chilla fox (CH) feces run on the same Merck plate. Different numbers represent feces from different individuals. Different superscripts ($^{a, b}$) indicate separate feces from the same individual. The symbol "–" indicates that no spot was detected.

Color	CU1[a]	CU1b	CU2	CH1[a]	CH1b	CH2
Red	0.891	0.858	0.892	0.901	0.893	0.890
Fluorescent	–	0.768	0.759	–	0.774	0.816
Fluorescent	0.655	0.652	0.639	0.677	0.667	–
Yellow	0.485	0.529	0.494	0.497	0.516	0.497
Fluorescent	–	0.452	–	0.429	0.440	0.436
Fluorescent	0.346	0.400	–	–	–	–
Fluorescent	–	0.277	–	0.255	0.270	–
Fluorescent	–	–	–	0.193	0.201	–
Fluorescent	0.121	0.129	0.121	0.118	0.126	0.129

Fox feces containing fruits of *Porlieria* produced larger spots with lower *Rf* in addition to those produced by feces with vertebrate remains only.

Discussion

Because results varied with the plate used, the plate type is obviously important. This point is relevant to highlight, because the plate type and brand are not usually described in the literature. Thus, *Rf* values from other studies may not be used as standards for comparisons. Another aspect that needs to be considered is the concentration of the sample used. For individual studies, it is necessary to standardize the method to render it useful. Further, because of the high variability among samples, which is generally not reported in the literature, several runs must be made from the same sample to obtain an average *Rf* value. The lack of clear separation of standards when run together may reflect interference among bile acids as indicated by Major *et al.* (1980). Also, too much concentration of the sample may produce interference and hence changes in *Rf* value.

The effect of fecal contents (ie diet type) or contamination from anal glands products has not been considered in previous research for interpreting similar results (but see Quinn and Jackman 1994). Feces that contain fatty acids and esters as products of the diet (ie certain fruits such as *Porlieria chilensis* in the feces of Aucó foxes) will produce a distinctive large dark spot, which will mask those from bile acids. Therefore, by keeping the diet constant, some patterns may emerge. However, this is not possible to do with feces from unknown free-ranging carnivores. Indeed, it has been shown in coyotes *Canis latrans* that diet type ("previous diet experience") alone can change the bile acids spot patterns (Quinn and Jackman 1994). These authors cautioned that differences in diet might thus render the technique useless. Our results on *Pseudalopex* feces support their claim. In addition, several biochemists at the Catholic University of Chile (ie P. Bull, M. Bronfman, and F. Nervi, pers. comm.) concurred that qualitative differences in these conservative bile acids of congeneric vertebrates should not be expected.

The quantification with gas chromatography of the amount of different bile acids in the feces might distinguish feces of closely related species. But even by using this method, it is difficult to distinguish congeneric felids (Johnson *et al.* 1984: 242). In addition, for this purpose, the use of gas chromatography is prohibitively expensive. Tagging individuals with species-specific radio-isotopes is another alternative for separating feces from sympatric congeneric carnivores (Conner 1982) or by combining feces diameter and the location of the feces within known radio-collared fox territories (Jiménez 1993).

Contrary to the findings of Capurro *et al.* (in press), we conclude that the TLC cannot be used for distinguishing feces from chilla and culpeo. There is too much variability in the spot pattern, even among feces produced by the same individual.

Part of this variability, which may override interspecific differences, might be explained by the type of food consumed as found by Quinn and Jackman (1994) for coyotes. Consequently, we warn that thus far, the use of bile acid to distinguish feces from sympatric free ranging carnivores is still an unsuitable technique.

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