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Extreme Variability of Steroid Profiles in Cow Feces and Pig Slurries at the Regional Scale: Implications for the Use of Steroids to Specify Fecal Pollution Sources in Waters

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ABSTRACT: Thirty-five samples of cow feces (cowpat and cow manure) and pig slurries subjected to different treatment processes and different storage times before land spreading were extracted and analyzed by gas chromatography−mass spectrometry to determine their fecal stanol profiles. The fresh pig slurry data presented here increase considerably the classical range of values obtained for steroid ratios, resulting in an overlap with the range for cow feces. These results lead to the inability to distinguish species source of feces on the basis of steroid ratios alone. The cause of these differences is not known, although it appears likely to be related to differences in the metabolism of animals in relation to their age and/or variations in diet, rather than to secondary mechanisms of steroid degradation during storage or/and treatment of the feces. Nevertheless, the specificity of steroids to serve as a tool to differentiate cow feces from pig slurries is restored by considering the fecal stanol profile, notably, the six most diagnostic stanol compounds, which are 5β-cholestan-3β-ol (coprostanol), 5β-cholestan-3α-ol (epicoprostanol), 24-methyl-5α-cholestan-3β-ol (campestanol), 24-ethyl-5α-cholestan-3β-ol (sitostanol), 24-ethyl-5β-cholestan-3β-ol (24-ethylcoprostanol), and 24-ethyl-5β-cholestan-3α-ol (24-ethylepicoprostanol). In this study, chemometric analysis of the fingerprint of these six stanols using principal components analysis (PCA) distinguished pig slurries from cow feces. The application of PCA to the stanol profiles, as developed in this study, could be a promising tool for identifying the animal source in fecal contamination of waters.

KEYWORDS: fecal stanols, animal feces, fecal contamination, molecular markers, principal component analysis

INTRODUCTION

Animal manures are valuable fertilizers that provide macro- and micronutrients as a low-cost alternative to mineral fertilizers.[1,2] However, excessive application of manure to soil could ultimately contaminate surface water with fecal matter, causing risks to humans through possible exposure to pathogenic bacteria, viruses, and (or) protozoa.[3−6]

Different methods grouped together under the generic term of "microbial source tracking" (MST) have been developed to identify fecal contamination sources in water, with the aim of obtaining a full characterization of the contamination (i.e., source, timing, severity, etc.).[7−10] Among animal-specific markers, steroids have the potential to discriminate between different animal sources.[11−18] Steroid profiles in animal feces depend on three factors: (i) the animal's diet, (ii) the animal's ability to biosynthesize sterols, and (iii) the presence/absence of anaerobic bacteria able to biohydrogenate sterols into stanols of various isomeric configurations.[11] For example, herbivore feces are dominated by steroids with 29 carbon atoms such as 24-ethylcholest-5-en-3β-ol (sitosterol), 24-ethylcoprostanol, and sitostanol, whereas omnivore feces mainly contain steroids with 27 carbon atoms such as coprostanol, cholest-5-en-3β-ol (cholesterol), 5α-cholestan-3β-ol (cholestanol), and epicoprostanol.[11−18]

Insofar as fecal steroids undergo dilution during their transfer from soil to water, steroid ratios rather than absolute steroid compound concentrations are used for tracking purposes. Several ratios have been proposed as biomarkers suitable to differentiate between fecal matters of different animal origin.[11,16−18]

However, the limits of steroid ratios as fecal source indicators were previously pointed out by Jardé and co-workers.[14] They reported values of 5β-stanols/C_{27} ratios ranging from 3.8 to 8.3 for five samples of fresh pig slurry originating from Brittany, France. The sources of variation in the relative steroid concentrations within a given animal feces type can have several origins. They can arise from exogenous factors such as different physicochemical treatments and storage conditions of the animal feces prior to land application. In areas of intensive farming such as Brittany, the problem of tracking fecal contamination sources is particularly acute because of the production of large amounts of animal feces.[14] Here, the feces are often stored in tanks for extended periods of time to comply with regulations that limit land application to only certain periods of the year. Various physicochemical treatments are often utilized during this long-term storage, including centrifugation, aerobic digestion, and anaerobic denitrification. All of these treatment processes, particularly those intended to stimulate microbial degradation of the animal feces, can create variations in the steroid concentration profiles and thus also in the steroid ratios. These changes are a result of differences in the lability of steroid compounds to microbial degradation or/and microbial biosynthesis.[17−21] For example, it is well-established that the activity of aerobic bacteria is responsible for relative losses of 5β-stanols.[17,22−25] Therefore, it has become essential to quantify the potential increase in the variability of steroid ratios caused by storage and treatment processes and distinguish it from the variations primarily created

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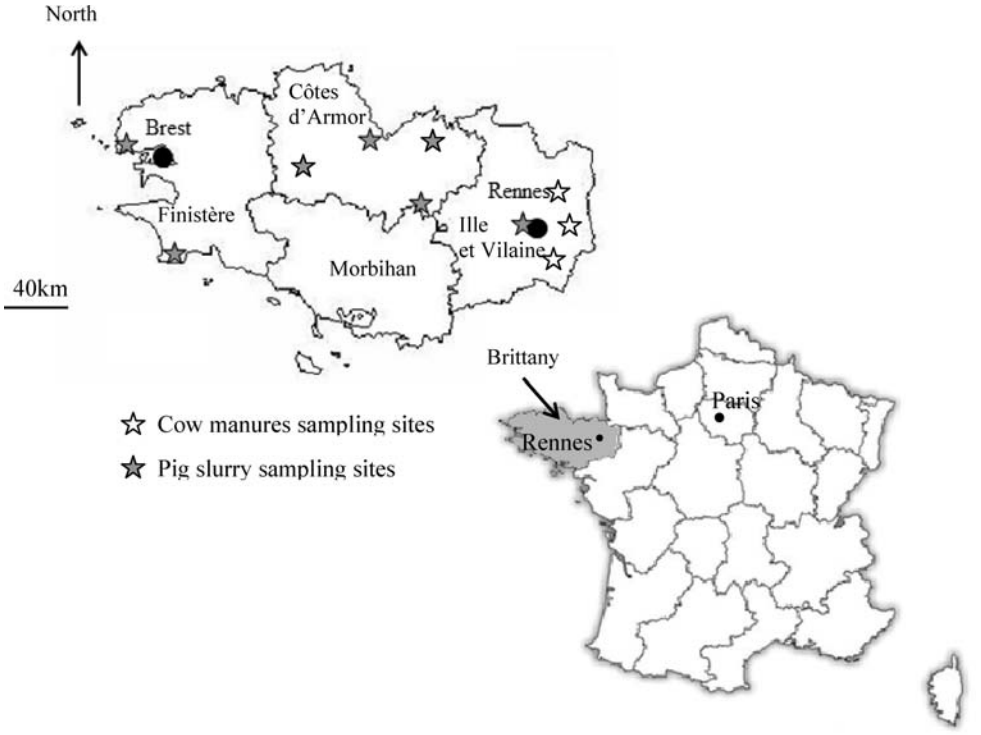

Figure 1. Sample location map. Open stars, cow manures; solid stars, pig slurries.

by differences in animal diet and animal metabolism. To assess the secondary source of variability, the present study reports the analysis of fecal steroids in 35 samples of pig slurries and cow feces subject to different treatment processes and following storage for different periods of time. Pig slurry and cow manure were preferentially selected because they represent the two most intensively spread types of manure in Brittany, the target region of this study. Our aims were to (i) quantify the extent of changes in steroid profiles that can be encountered within each animal feces type at the regional scale as a result of the combined effects of variations in animal diet and metabolism, in addition to feces storage time and treatment, and (ii) establish whether fecal steroids can still be used to distinguish water contamination by pig slurries or by cow feces, despite the variability generated by these primary and secondary variation sources.

MATERIALS AND METHODS

Samples and Sample Preparation. Nineteen samples of cow feces and sixteen samples of pig slurries were collected and analyzed. All samples came from Brittany, northwestern France. Figure 1 shows the locations of the farms where the samples were collected.

The 19 samples of cow feces were collected at 10 different dairy farms. Each sample was a composite of feces produced by a group of 50 animals. In detail, the samples included the following specific wastes: (i) six fresh cowpats, (ii) nine fresh cow manures (i.e., cow feces mixed with straw), and (iii) four aged cow manures. The different samples collected are representative of the cow feces that were either directly deposited on meadows (cowpats) or spread onto agricultural soils (cow manures). Table 1 summarizes the main characteristics of the studied cow samples, including farm location, sampling date, storage time, and animals' diet.

The 16 samples of pig slurry were collected from a pig fattening experimental farm and from six different piggeries containing sows associated in some cases with piglets. Each sample was a composite of feces produced by a group of 100 sows. Brittany supports more than half of the total French pig livestock and generates 8−10 million tons of pig slurry each year. To limit contamination of soils and waters by pathogens, nitrogen, and phosphorus, the French regulations require storage and treatment of the slurry before land application. In Brittany, between 50% and 100% of the raw pig slurry is treated, primarily by physical treatment (physical separation of urine from solid feces by centrifugation), followed in some cases by chemical treatment (aerobic digestion and anaerobic denitrification). The storage period before treatment ranges from 2 to 8 weeks. Once treated, the slurries are again stored before land application. This second storage is carried out in anaerobic tanks and usually lasts from 3 to 9 months. Among the 16 collected pig slurry samples, 12 were used to investigate the combined effects of storage and treatment. These samples included (i) four fresh raw pig slurries collected in a pig fattening farm, (ii) four aged raw pig slurries, (iii) four physically treated pig slurries, and (iv) four chemically treated pig slurries. Groups ii−iv were collected from sow farrowing/nursery farms. Table 2 summarizes the main characteristics of the studied pig slurry samples, including farm location, sampling date, animal diet, manure treatment type, and storage time.

About 5 kg of raw manure was collected for each sample. After homogenization, about 0.5 kg was frozen for 2 days, then freeze-dried for 5 days, and finally crushed (<250 μm) before chemical extraction and subsequent steroid analysis.

Reagent and Chemicals. Organic solvents were of high-performance liquid chromatography (HPLC) grade. Dichloromethane (DCM) was purchased from Carlo-Erba SDS (Val de Reuil, France), and methanol and cyclohexane were purchased

Table 1. Description of Cow Feces Samples

livestock farming type	farm location	feces type and sample number by type	storage time	animal diet	sampling date
dairy farming	Ille-et-Vilaine	cowpat (1)	fresh	pasture and a corn silage	Apr 2007
		cow manure[a] (1)	4 months		
		cow manure (1)	6 months		
		cowpat (1)	fresh	pasture	Apr 2007
		cow manure (1)	5 months		
		cowpat (1)	fresh	pasture and corn	Apr 2007
	unknown	cow manure (1)	fresh	grass and/or corn silage, food supplements (protein), and pasture	Apr 2007
		cow manure (1)	fresh	grass and/or corn silage, food supplements (protein), and pasture	Apr 2007
		cow manure (3)	fresh	unknown	Apr 2008
		cow manure (1)	fresh	unknown	Feb 2007
		cowpat (1)	fresh	pasture	Apr 2007
		cow manure (1)	fresh		
		cow manure (1)	4 months		
		cowpat (1)	fresh	grass and/or corn silage, food supplements (protein), and pasture	Feb 2007
		cow manure (1)	fresh		
		cowpat (1)	fresh	grass and/or corn silage, food supplements (protein), and pasture	Apr 2007
		cow manure (1)			

[a] All cow manure samples contained straw.

Table 2. Description of Pig Feces Samples

livestock farming type	farm location	feces type (including treatment type) and sample number by type	storage time	animal diet	sampling date
pig fattening	Ille-et-Vilaine	raw pig slurries (4)	fresh	wheat and soybean (80% and 20%)	Jun 2009
				wheat and soybean (90% and 10%)	
				wheat and soybean (90% and 10%)	
				wheat, soybean and rapeseed (70%, 10% and 20%)	
sow and piglet	Côtes-d'Amor	raw pig slurries (4)	1 month	mixture of cereals and food supplements[a]	Apr 2007
sow	Côtes-d'Amor	physically treated	fresh	mixture of cereals and food supplements[a]	Mar 2009
		pig slurries (2)	4 months		
		physically treated pig slurry (1)	6 months	mixture of cereals and food supplements[a]	Mar 2009
		chemically treated pig slurry (1)	9 months		
		chemically treated pig slurry (1)	8 months	mixture of cereals and food supplements[a]	Apr 2009
	Finistère	physically treated pig slurry (1)	fresh	mixture of cereals and food supplements[a]	Mar 2009
		chemically treated pig slurry (1)	6 months		
		chemically treated pig slurry (1)	8 months	mixture of cereals and food supplements[a]	Apr 2009

[a] Mineral salts, vitamins, micronutrients, and antibiotics.

from VWR (West Chester, PA). A mixture of N,O-bis-(trimethylsilyl)trifuoroacetamide and trimethylchlorosilane (99/1, v/v) (BSTFA + TMCS) was purchased from Supelco (St. Quentin Fallavier, France). Coprostanol, cholestanol, stigmasterol (24-ethylcholesta-5,22-dien-3β-ol), sitosterol and 5α-cholestane were purchased from Sigma (St. Quentin Fallavier, France). Epicoprostanol, epicholestanol (5α-cholestan-3α-ol), campesterol (24-methylcholest-5-en-3β-ol), and sitostanol were purchased from Steraloids (Newport, RI). Cholesterol was purchased from Aldrich (St. Quentin Fallavier, France).

Calibration Solutions of Sterol and Stanol Compounds. Solutions of individual compounds (coprostanol, cholestanol, epicoprostanol, epicholestanol, sitostanol, cholesterol, campesterol, stigmasterol, sitosterol) were dissolved in DCM at 1000 μg/mL. 5α-Cholestane was used as an internal standard and was dissolved in DCM at 40 μg/mL. The determination of the limits of detection was performed using three solutions containing coprostanol, cholestanol, epicoprostanol, epicholestanol, sitostanol, cholesterol, campesterol, stigmasterol, and sitosterol at 5, 10, and 15 ng/mL. The calibration was performed by the internal standard method using five-point calibration curves (0.1, 0.5, 1, 5, and 10 μg/mL) with a constant internal standard concentration of 4 μg/mL.

Extraction−Fractionation. The extraction protocol used in our laboratory is modified from Li et al.[26] No recovery standard was used to account for losses in extraction. The organic extractions of the animal feces were performed using an Accelerated Solvent Extractor (ASE200 from Dionex) with dichloromethane

Table 3. IUPAC and Trivial Names, Compound Numbers, *m/z* Values Used for the Identification and Quantification, and Information on Quantification Compounds (Name, Linearity, and Limit of Detection)

			m/z		quantification characteristics		
IUPAC name	trivial name	number	identification	quantification	standard	linearity	LD[a] (ppb)
cholest-5-en-3β-ol	cholesterol	1	255, 353, 368	129	cholesterol	0.995	5
5β-cholestan-3β-ol	coprostanol	2	257, 355, 370	215	coprostanol	0.996	5
5β-cholestan-3α-ol	epicoprostanol	3	257, 355, 370	215	epicoprostanol	0.996	5
5α-cholestan-3β-ol	cholestanol	4	257, 355, 384	215	cholestanol	0.995	10
24-methylcholest-5-en-3β-ol	campesterol	5	255, 353, 382	129	campesterol	0.997	5
24-methyl-5α-cholestan-3β-ol	campestanol	6	369, 398, 484	215	coprostanol	0.996	5
24-ethylcholesta-5,22(*E*)-dien-3β-ol	stigmasterol	7	255, 355, 394	129	stigmasterol	0.997	5
24-ethylcholest-5-en-3β-ol	sitosterol	8	255, 357, 396	129	sitosterol	0.998	10
24-ethyl-5α-cholesta-22(*E*)-dien-3β-ol	stigmastanol	9	215, 383	215	coprostanol	0.996	5
24-ethyl-5β-cholesta-22-en-3β-ol	5β-stigmastanol	10	257, 353, 486	215	coprostanol	0.996	5
24-ethyl-5β-cholesta-22-en-3α-ol	5β-epistigmastanol	11	257, 486	215	coprostanol	0.996	5
24-ethyl-5α-cholestan-3β-ol	sitostanol	12	383, 398, 473	215	sitostanol	0.989	10
24-ethyl-5β-cholestan-3β-ol	24-ethylcoprostanol	13	257, 383, 398	215	coprostanol	0.996	5
24-ethyl-5β-cholestan-3α-ol	24-ethylepicoprostanol	14	257, 283, 398	215	coprostanol	0.996	5

[a] LD = limit of detection.

(DCM). Between 1 and 2 g of freeze-dried sample was used, and the three extractions performed on each sample were pooled. The extractions were carried out under the following conditions: 11 mL of cells, with 5 min heating to 100 °C and 130 bar, followed by two cycles of 5 min each, completed with a 150% flush and 200-s purge with nitrogen.

The organic extracts were then fractionated by two-step liquid chromatography into aliphatic, aromatic, and polar compounds. In the first step, alumina retained high-molecular-weight polar compounds, whereas hydrocarbons and low-molecular-weight polar molecules were eluted with DCM. High-molecular-weight polar compounds were then eluted with a mixture of DCM/methanol (1/1, v/v). After solvent exchange of cyclohexane for dichloromethane, hydrocarbons and low-molecular-weight polar molecules were fractionated on a silica column by successive elutions with cyclohexane, followed by cyclohexane/dichloromethane (2/1, v/v) and then methanol/dichloromethane (1/1, v/v). The two polar fractions thus obtained were then pooled together, dried under a gentle flux of nitrogen, and finally weighed for quantification. The present study focused on the steroids present in the polar fraction.

Gas Chromatography−Mass Spectrometry (GC−MS). Polar fractions were analyzed after derivatization using BSTFA + TMCS [N,O-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane]. One microliter of the derivatized sample was injected onto a Shimadzu QP2010+MS gas chromatograph/mass spectrometer (Shimadzu, Tokyo, Japan). The injector used was in splitless mode and maintained at a temperature of 310 °C. The chromatographic separation was performed on a fused silica SLB-5 ms capillary column (from Supelco, length = 60 m, diameter = 0.25 mm, film thickness = 0.25 μm) under the following temperature program: 70 °C (held for 1 min) to 130 at 15 °C min^{-1}, then 130 to 300 °C (held for 15 min) at 3 °C min^{-1}. The helium flow was maintained at 1 mL min^{-1}. The chromatograph was coupled to the mass spectrometer by a transfer line heated to 250 °C. The analyses were performed in SIM mode (selective ion monitoring). Quantification was based on the internal standard 5α-cholestane (CDN isotope, CIL Cluzeau, Sainte-Foy-la-Grande, France), which was added to the sample postextraction and prior to derivatization. The quantification method utilizes a five-point calibration curve (0.1, 0.5, 1, 5, and 10 μg/mL) with a constant internal standard concentration of 4 μg/mL. The limit of detection (LD) of each compound was estimated by calculating the signal-to-noise ratio (S/N) for three solutions containing the target compounds at 5, 10, and 15 ng/mL. Each solution was analyzed 10 times. The LD was defined as the concentration at which S/N > 3. Table 3 presents the different steroid compounds quantified in this study and information on the linearity, limits of detection, and *m/z* fragments used for identification and quantification of the steroids analyzed.

Statistical Analysis. PCA, a descriptive multivariate method based on a geometric model,[27,28] was used to analyze and quantify the statistical relationships between the different steroid compounds at the sample population scale. This method is useful for revealing correlation patterns in complex databases such as the steroid profile matrix obtained in this study. Based on a rectangular data matrix containing the values of p quantitative variables having n units (also called individuals), PCA provides geometric plots of these variables and individuals. The different plots allow for the identification of the relationships between the individuals. In the same way, a representation of the variables (i.e., correlation circle) can be used to highlight the linear correlation of the considered variables.[29]

In this study, we considered 35 individuals (i.e., the 35 investigated samples of animal feces) and 10 variables (relative abundances, in percentages, of the 10 stanols quantified in this study). A first PCA was performed using those variables. The results of this PCA showed redundancy between two variables (5β-epistigmastanol and 24-ethylepicoprostanol) and a low contribution (<5%) of three variables (cholestanol, stigmastanol, and 5β-stigmastanol) to the F1 axis. After elimination of cholestanol, stigmastanol and 5β-stigmastanol, and 5β-epistigmastanol, a second PCA was performed using only the six most significant variables (i.e., coprostanol, epicoprostanol, 24-ethylcoprostanol, 24-ethylepicoprostanl, campestanol, and sitostanol).

The PCA and relative statistical tests were performed with XLSTAT (Addinsoft, 2010), using nonparametric tests for small samples of unknown distribution (Mann−Whitney).

RESULTS AND DISCUSSION

Differences in Steroid Profiles between Cow Feces and Pig Slurries and the Effect of Diet Variation and Animal Metabolism. Figure 2 shows the average relative distribution profiles (in weight percentage) of steroids in the samples of fresh cowpat, fresh cow manure, and aged cow manure.

Cow feces samples were characterized by high proportions of C_{29} steroids, representing between 63% and 68% of the total steroids, compared to only 4−11% and 21−31% for the C_{28} and C_{27} steroids, respectively. Of the C_{29} steroids, 24-ethylepicoprostanol (no. 14, 18−20%) and sitostanol (no. 12, 14−15%) were the most abundant, with approximately twice the proportion of the C_{27} steroids. Cholesterol (no. 1, 8%) and cholestanol (no. 4, 7−9%) dominated the C_{27} steroids. Relatively little variation was observed between the states of cow manures. In fact, the only notable difference was an approximately 2-fold increase ($p < 0.05$) of the campesterol (no. 5), 24-ethylcholesta-5,22(*E*)-dien-3β-ol (stigmasterol, no. 7), and sitosterol (no. 8) abundances in the cow manure samples (fresh and aged) compared to the cowpat samples.

Figure 3 shows the average relative distribution profiles (in weight percentage) of steroids in the samples of fresh raw pig slurry, aged raw pig slurry, physically treated pig slurry, and chemically treated pig slurry. Compared to cow manure, the pig slurry profiles were marked by relatively higher proportions of 3β,5β-stanols [coprostanol (no. 2), 24-ethylcoprostanol (no. 13)] and relatively lower proportions of sitostanol (no. 12) and 24-ethylepicoprostanol (no. 14). Those two features were already noted in previous studies by Leeming et al.,[11] Tyagi et al.,[12] and Jardé et al.[14] Another difference is the lower relative proportion of C_{29} steroids (between 41% and 52%) as compared to C_{27} steroids (between 32% and 53%). Coprostanol (no. 2, 15−24%) was the most abundant C_{27} steroid in samples of raw (both fresh and aged) slurry. In contrast, proportions of coprostanol (no. 2) and epicoprostanol (no. 3) in treated samples were similar (11−14%) for both chemically and physically treated slurries. Moreover, two trends were revealed between the profiles of raw (fresh and aged) slurry samples and those of physically and chemically treated slurry samples, namely, treated samples showed (i) an increase ($p < 0.05$) of the relative proportion of stigmasterol (no. 7) and (ii) a decrease ($p < 0.05$) of the relative proportion of 3β,5β-stanols [coprostanol (no. 2) and 24-ethylcoprostanol (no. 13)], accompanied by an increase ($p < 0.05$) of the relative proportion of the corresponding 3α,5β epimers [epicoprostanol (no. 3) and 24-ethylepicoprostanol (no. 14), respectively].

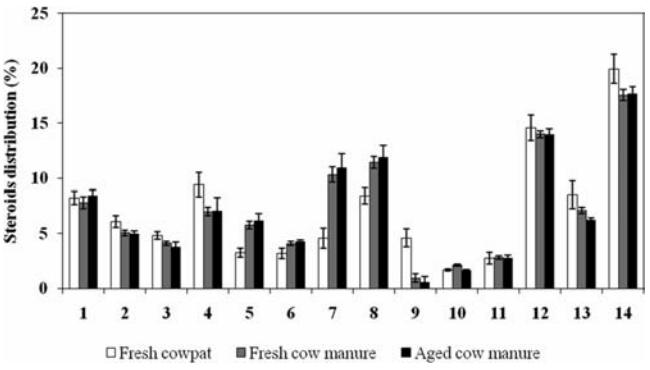

Figure 2. Average steroid profiles (in weight percentage ± standard deviation) in samples of cowpat, fresh cow manure, and aged cow manure.

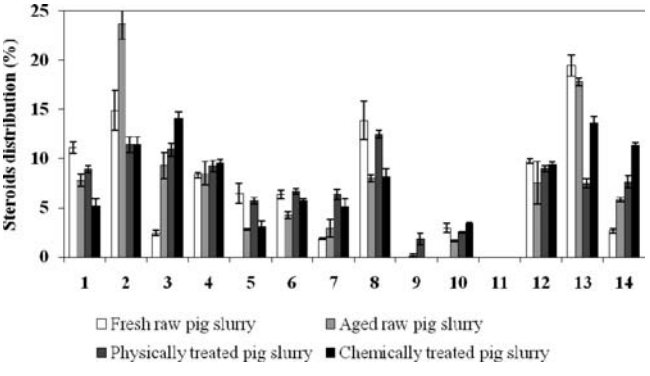

Figure 3. Average steroid profiles (in weight percentage ± standard deviation) in samples of fresh raw pig slurry, aged raw pig slurry, physically treated pig slurry, and chemically treated pig slurry.

The steroid profiles of the fresh cowpat and fresh pig slurry samples should be entirely controlled by the animal's diet and metabolism.[11−21] In fact, the C_{29} sterols [notably stigmasterol (no. 7) and sitosterol (no. 8)], which are common compounds of plants, dominated the sterol profiles of the cow feces samples, in agreement with the mainly herbivorous diet of cows. 5β-C_{29} stanols were also abundant in cow feces samples and are known to originate from the biohydrogenation of sitosterol (no. 8). These compounds reflect the cow's metabolism and the presence of anaerobic bacteria in the intestinal tract. By contrast, C_{27} and C_{29} steroids were approximately evenly distributed in the steroid profiles of the fresh pig slurry samples. As is usually observed, the steroids profiles of fresh pig slurry samples were characterized by high concentrations of coprostanol (no. 2) and 24-ethylcoprostanol (no. 13),[11,14] which reflect metabolic effects.[11,17] Note that 5β-epistigmastanol (no. 11) was not present in the pig slurry samples, whereas this compound represented ∼3% of the quantified steroids in cow feces (Figures 2 and 3).

Effects of Straw Addition, Storage, and Treatment Processes on Steroid Profiles. Figures 2 and 3 illustrate the effects of straw addition, storage, and physicochemical treatment on the steroid profiles of cow feces and pig slurries. The main effects of straw addition in cow feces were found to be increases in the relative proportions of stigmasterol (no. 7, $p < 0.05$) and sitosterol (no. 8, $p < 0.05$) (Figure 2). These compounds are the main constituents of plant lipid membranes and waxes,[30,31] and their relative proportions were increased in the manure samples. However, storage had no visible effect on the steroid profiles of cow manures, as the profiles of the fresh and aged cow manure samples were statistically indistinguishable ($p > 0.05$) from each other (Figure 2).

On the contrary, both storage and treatment appeared to have major effects on the steroid profiles of pig slurries, leading to a marked decrease in the proportions of coprostanol (no. 2) and 24-ethylcoprostanol (no. 13), associated with a concomitant increase in the epicoprostanol (no. 3) and 24-ethylepicoprostanol (no. 14) proportions (Figure 3). Studies on the fate of steroids in wastewater treatment plants have established that coprostanol (no. 2) is degraded by bacteria during the microbial

digestion process,[17,22−25] and it is likely that the decrease in coprostanol (no. 2) content observed in the treated slurries was caused by the same microbial degradation process. The observed concomitant decrease in the proportion of 24-ethylcoprostanol (no. 13) might have the same origin. This "microbial hypothesis" is consistent with the observed increase in relative abundances of epicoprostanol (no. 3) and 24-ethylepicoprostanol (no. 14) that also characterized the transition from fresh to stored/treated pig slurries. A previous study[32] did indeed demonstrate that anaerobic digestion could produce epicoprostanol, the 3α-epimer of coprostanol. This might be due to the survival during storage and treatment processes of bacterial communities that can reduce the sterols present in the feces into their 3α,5β-stanol epimers. This process might also possibly account for the increase in the proportion of 24-ethylepicoprostanol (no. 14) that was seen to accompany the increase in epicoprostanol in the aged and treated pig slurry samples.

Evidence for a Loss of Specificity of the Steroid Ratios Used So Far to Distinguish Cow from Pig Feces. As highlighted in the Introduction, fecal steroids have been identified as compounds that could help distinguish between fecal contaminations of cow or pig origin. The validity of using the absolute abundance of a single steroid for source tracking is called into question by the low to very low concentrations of steroids in waters and the propensity of some key steroid compounds such as coprostanol to degrade under aerobic conditions (see previous section). This explains why ratios of two or more steroids, instead of the absolute concentration of a single steroid, have been proposed as possible proxy indicators suitable for discriminating between cow and pig fecal pollution sources. Six such ratios have been proposed over the past 20 years, with reference values typical of each source.[11,14,33] Table 4 reports these six ratios for cow and pig feces based on the present data set, compared with the different reference values obtained from the literature. The results in this table clearly show a loss of specificity for five of the six ratios. Only the sitostanol/coprostanol ratio preserves its specificity.

Table 4. Comparison of Published and Measured Diagnostic Values (i.e. maximum and minimum) for the Six Steroid Ratios Cited in the Literature [11,14,32] as Being Capable of Discriminating between Pig and Cow Feces

	diagnostic value	
ratio	literature	this study
$5\beta C_{27+29}/C_{27+29}$[a]	$R \approx 1$ (cows)[11]	$1.3 < R < 3.4$ (cows)
	$R > 2$ (pigs)[11]	$1.2 < R < 4.8$ (pigs)
$5\beta C_{27+29}/C_{27+29}$[b]	$R \approx 1$ (cows)[11]	$0.6 < R < 1.3$ (cows)
	$R > 2$ (pigs)[11]	$1.1 < R < 4.0$ (pigs)
$5\beta C_{27+29}/C_{27+29}$[c]	$R \approx 1$ (cows)[11]	$0.5 < R < 1.1$ (cows)
	$R > 2$ (pigs)[11]	$0.8 < R < 3$ (pigs)
5β-stanols/C_{27}[d]	$R < 0.7$ (cows)[14]	$0.9 < R < 2.2$ (cows)
	$R > 3.7$ (pigs)[14]	$1.5 < R < 6.8$ (pigs)
[cop/(cop + 24-ethylcop)] × 100[e]	$38 < R < 47$ (cows)[32]	$33 < R < 47$ (cows)
	$51 < R < 61$ (pigs)[32]	$44 < R < 67$ (pigs)
sitostanol/coprostanol	$2.3 < R < 3.3$ (cows)[32]	$1.5 < R < 3.3$ (cows)
	$0.2 < R < 0.7$ (pigs)[32]	$0.2 < R < 1.0$ (pigs)

[a] Coprostanol + epicoprostanol + 24-ethylcoprostanol + 24-ethylepicoprostanol/(cholesterol + sitosterol). [b] Coprostanol + epicoprostanol + 24-ethylcoprostanol/(cholesterol + sitosterol). [c] Coprostanol + 24-ethylcoprostanol/(cholesterol + sitosterol). [d] Coprostanol + epicoprostanol/cholesterol. [e] [Coprostanol/(coprostanol + 24-ethylcoprostanol)] × 100.

Figure 4 compares all of the available data, allowing for the estimation of the values of 5β-stanols/C_{27} ratio in pig and cow feces. The data presented here include (i) the 35 cow and pig manure/slurry samples investigated in this study, (ii) the five pig slurry and two cow manure samples investigated earlier by Jardé et al.,[14] (iii) the six pig and cow feces samples investigated by Leeming et al.,[11] and (iv) the two cow and pig feces samples analyzed by Shah et al.[18] and Rogge et al.[34] What is the cause of the loss of specificity observed in Figure 4? Is it a side effect of the storage and treatment of slurries and manures before their land application? Alternatively, could it be a consequence of variations in animal diet and/or animal metabolism?

Figure 4 clearly shows that the loss of specificity of the 5β-stanols/C_{27} ratio is due mainly to the addition of the four fresh pig slurry samples collected in a pig fattening farm. This result is particularly important because these samples are representative of the most applied swine manure. These new data shift the entire pig feces sample population toward lower values of the 5β-stanols/C_{27} ratio, thereby leading to an over-lapping of the pig (0.9−2.2) and cow (1.5−6.8) feces fields. From this observation, we infer that the extreme variation of the 5β-stanols/C_{27} ratio now displayed by the pig feces end member might be more likely to be caused by a primary factor, rather than a secondary process that might develop during storage or treatment of the pig feces. However, the respective roles of diet and metabolism are difficult to establish precisely because the necessary data are not available in the literature. Therefore, we are left to consider the present data set, which, although containing more analyses than the entire published data set, remained nevertheless rather limited in extent. If we consider just the four fresh pig slurry samples, we observe that two come from animals fed with exactly the same proportions of wheat (90%) and soybean (10%) (Table 2). Yet, the 5β-stanols/C_{27} ratios of these two samples exhibit a dispersion (1.4 versus 2.1). The addition of rapeseed to the diet could be regarded as a factor that reduces the 5β-stanols/C_{27} ratio in pig feces, as the sample representative of this type (Table 2) of diet yielded the lowest 5β-stanols/C_{27} ratio (0.9) observed in the fresh raw slurry subpopulation. However, the fact that different 5β-stanols/C_{27} ratios were obtained for animals fed with exactly the same diet casts doubt on the hypothesis that the observed effects could be linked to diet variations.

The role of metabolism is also quite difficult to evaluate in the absence of accurate data on important indicators included in the samples taken as representative of the different pig populations. In fact, in the case of human steroid profiles, the conversion of cholesterol to coprostanol (and thus the relative abundance of coprostanol) depends strongly on the age of the individual from which the feces are produced. According to Midtvedt et al.,[35] mammal intestines at birth are devoid of intestinal flora, which gradually develop later, during the growth of individuals. Because pigs are mammals like humans, it is possible that the metabolism of pigs also might evolve with age in terms of the capacity to convert sterols into stanols, with piglets producing much lower amounts of coprostanol (and thus having much lower 5β-stanols/C_{27} ratios) than adult pigs. To test this hypothesis, further studies based on fresh pig feces samples coming from pig populations of different known ages are clearly required.

Implication for the Use of Steroids to Specify Pollution Sources in Waters. Inclusion of the new series of samples

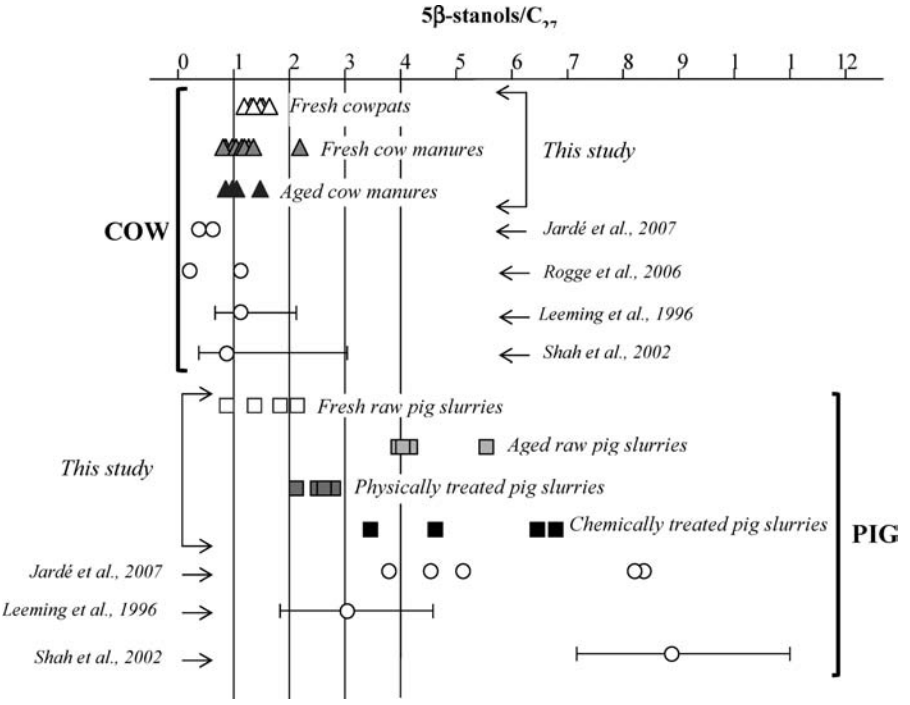

Figure 4. Variation of the 5β-stanol/C_{27} ratio in cow and pig feces: Comparison of literature data[11,14,18,33] with the present data set.

produces an extreme variability in ratios such as 5β-stanols/C_{27} that evidently compromises the use of steroid ratios to discriminate between cow and pig fecal contamination sources. The question that arises is whether steroids, taken together, can nonetheless serve as indicators to differentiate between cow and pig pollution sources. This question was addressed in this work by treating all samples by the PCA method. PCA methods are commonly used in chemometric data analysis of pollutants.[36−39] Figure 5 shows a plot of the PCA based on 35 fecal samples using the six most significant stanol compounds. The first two components of the PCA explained 79% of the total variance, with the first (F1) and second (F2) components of the PCA accounting for 61% and 18%, respectively. The figure illustrates a clear separation of the pig and cow feces samples. The main contributive variables on the F1 axis (Table 5), in decreasing order of importance, are 24-ethylepicoprostanol (26.0%), coprostanol (23.3%), sitostanol (21.1%), and 24-ethylcoprostanol (18.0%), whereas epicoprostanol (68.7%) is the main contributing variable on the F2 axis (68.7%). The PCA reveals that, in cow feces (group 1 in Figure 5), the stanol subseries is dominated by 24-ethylepicoprostanol and sitostanol whereas, in pig slurries (group 2 in Figure 5), this subseries is dominated by coprostanol, epicoprostanol, and 24-ethylcoprostanol. It also indicates that the distinction between cow and pig feces depends mainly on four stanol compounds, namely, coprostanol, 24-ethylcoprostanol, 24-ethylepicoprostanol, and sitostanol. Hence, we propose that these stanols, taken together, represent a valuable tool for differentiating pig from cow feces.

In summary, this study investigated the effects of storage and treatment processes on the steroid profiles of a large number of cow feces and pig slurry samples collected at a regional level. The pig feces data considerably increased the range of values obtained for steroid ratios previously considered as having the potential to discriminate between pig and cow feces. The range of values increased to such an extent that the two types are now combined,

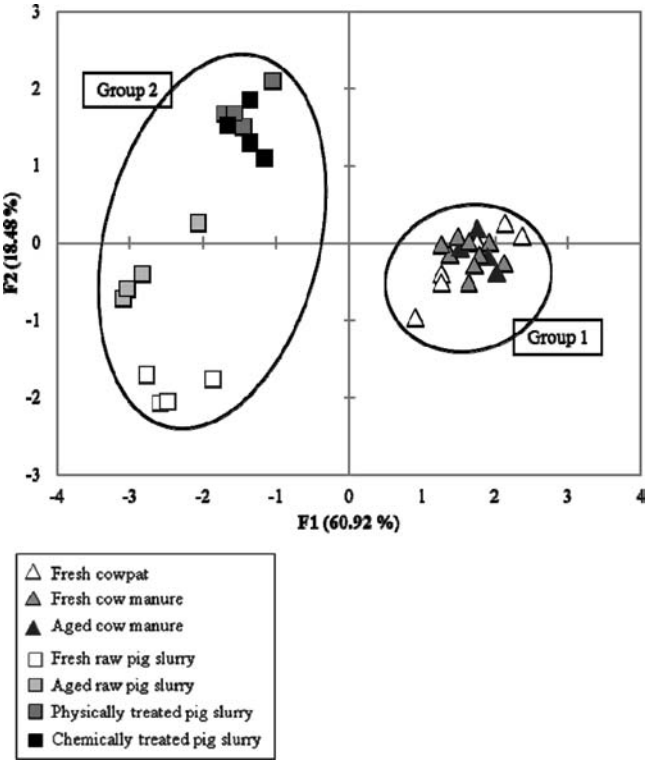

Figure 5. Plot of principal component analysis of the 35 analyzed samples using the six most discriminant stanol compounds. F1 axis, principal component 1; F2 axis, principal component 2.

making it impossible to use these ratios for purposes of discrimination. The cause of this increase is not known, although it seems likely to be linked to differences in the metabolism of animals in relation to their age and/or variations in the animal

Table 5. Factorial Coordinates of Variables and Relative Contributions (%) of Variables to Principal Components 1 (F1) and 2 (F2)

	factorial coordinates of variables[a]		contributions of variables (%)	
	F1	F2	F1	F2
coprostanol	−0.92	−0.16	23.3	2.2
epicoprostanol	−0.44	0.87	5.4	68.7
24-ethylcoprostanol	−0.81	−0.51	18.0	23.6
24-ethylepicoprostanol	0.97	0.06	26.0	0.3
campestanol	−0.48	0.16	6.2	2.4
sitostanol	0.88	−0.17	21.1	2.7

[a] Percentage of each stanol.

diet, rather than secondary mechanisms of steroid degradation during storage or/and treatment of the pig slurry. However, the specificity of steroids to serve as a tool for differentiating cow and pig feces can be restored by considering not just a few steroid compounds, but rather the entire stanol family, notably the six most diagnostic stanol compounds: coprostanol, epicoprostanol, 24-ethylcoprostanol, 24-ethylepicoprostanol, campestanol, and sitostanol. Chemometric analysis of the fingerprint of these six stanol compounds using PCA allows pig feces to be distinguished from cow feces, despite the strong variability and lack of specificity of classical steroid ratios. The PCA of stanol profiles developed in this study could appear as the first step of a promising approach to identify further fecal contamination sources in waters.

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