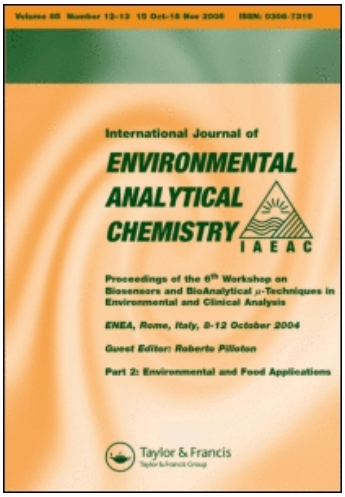

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Determination of fecal sterols in the sediments of different wastewater outputs by GC-MS

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DETERMINATION OF FECAL STEROLS IN THE SEDIMENTS OF DIFFERENT WASTEWATER OUTPUTS BY GC-MS

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An improved method for the analysis of fecal sterols in sediments was applied to distinguish livestock wastewater, domestic sewage, and industrial wastewater pollution in the receiving waters of Taiwan's rivers. The method included direct saponification, solvent phase extraction, derivatization with *N*-methyl-*N*-trimethyltrifluoroacetamide and catalyst, and separation by gas chromatography with an HP-50^+ capillary column, followed by qualitative and quantitative analysis by mass spectrometry. Recoveries of nine sterols by this method were 78–89%. The indicators of biopollution markers ((coprostanone × coprostanol)/epicoprostanol) in different sources of wastewater effluents were calculated as human 0.913 ± 0.251, pig 0.224 ± 0.135, cow 0.023 ± 0.001, duck 0.007 ± 0.001; such indicators are feasible for distinguishing between different animal sources of fecal pollution in water.

Keywords: Coprostanol; Fecal pollution; Sewage; Sterol profile

INTRODUCTION

The assessment of sewage pollution is of considerable importance for public health, esthetic, and ecological concerns. Sewage usually contains a variety of pathogenic micro-organisms together with many undesirable chemical pollutants, and hence, contamination of potable water supplies is an obvious hazard to public health. Determination of the content and source of sewage pollution is important in monitoring this type of pollution. While bacterial determinations, such as the FC/FS value (the ratio of fecal coliforms to fecal streptococci) [1] or flora of fecal streptococci [2,3], have been used to determine the contributions of the different sources of fecal pollution entering our aquatic systems, their use has been questioned mainly due to highly variable survival rates under environmental stress such as temperature, salinity, and sunlight [4–6]. For this reason, the use of chemical indicators of sewage contamination provides a useful alternative.

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Sterols have been used to detect fecal pollution, with most studies having focused on coprostanol (5β-cholestan-3β-ol) [7]. Coprostanol is produced primarily in the intestines of higher animals by enteric microbial reduction of cholesterol, the main steroid found in tissues of vertebrates [8]. This process is the important known source of coprostanol, and thus the presence of this compound in natural environments is considered a specific indicator of fecal contamination [9]. Coprostanol has a low solubility in water and tends to associate with particulate matter. Once coprostanol is incorporated into the sediments, its degradation is very limited [10]. Coprostanol concentration is barely influenced by various treatments such as chlorination or aeration of overlying water, and it persists in anoxic sediments. For the above reasons, coprostanol can be a useful tracer of fecal pollution [11].

The fecal sterol profile is dependent on the balance of dietary intake, metabolic production of sterols, and the biota resident in the digestive tract. The relative amounts of cholesterol and its metabolites found in human feces are as follows: cholesterol makes up about 20% of the neutral sterol concentration; coprostanol, 65%; coprostanone, 10%; and cholestanol, cholestanone, and epicoprostanol collectively about 5% [12]. Some marine mammals contain a high percentage of epicoprostanol (5β-cholestan-3α-ol) in addition to coprostanol [11]. Grimalt *et al*. proposed the ratio of 5α-stanols : 5β-stanols to be a more reliable criterion for assessing fecal input [13]. The sterol profiles of herbivores were dominated by C29 sterols, and only sheep feces presented the unusual stereoisomer, 24-ethylepicoprostanol. Cats and pigs had similar fecal sterol profiles to humans. However, the concentration of coprostanol was roughly 10 times more abundant in the feces of humans than in those of cats and pigs [14,15]. Both 5α- and 5β-stanols were in very low abundance in bird and dog feces due to the absence of low activity or the necessary anaerobic biota to reduce Δ5- or Δ5, 22-sterols to stanols. The 'sterol fingerprints' of humans and animals are sufficient to distinguish wastewater from different animals.

Fecal sterols in sediment are frequently analyzed in several steps: extraction of total lipids, removal of solvent, saponification of lipids, extraction of nonsaponifiable materials with an organic solvent, and derivatization prior to analysis. These steps are time-consuming and laborious. Furthermore, small amounts of sterols can be lost during the cleanup and extraction processes, as well as by absorption on Soxhlet filter cartridges [16]. The objectives of this study thus were: (1) to develop a feasible gas chromatography-mass spectrometry (GC-MS) method involving direct saponification to replace the conventional multi-step protocol for sterol extraction; (2) to apply the modified method to the identification and determination of nine sterols in outflow sediments from wastewater treatment plants of humans and different livestock including pigs, ducks, and cows; and (3) to examine the source specificity of fecal sterols and detail the distinguishing human- and livestock-sourced wastewater pollution.

EXPERIMENTAL

Chemicals

All chemicals and solvents for GC-MS analyses were of analytical-reagent grade. Sterol standards, campesterol, cholestanol, cholestanone, cholesterol, coprostanol, coprostanone, epicoprostanol, sitosterol, and stigmasterol were purchased from

Sigma (St. Louis, MO). 5α-Cholestane as an internal standard (I.S.), and *N*-methyl-*N*-trimethyltrifluoroacetamide (MSTFA) and trimethyliodosilane (TMSI) as silylating reagents were obtained from Sigma. Triethylamine was from Chem Servic (West Chester, PA).

Sample Collection

Samples were collected from the following: (1) outflow surface sediment (about 4 cm) from different livestock farms, including pigs, cows, and ducks; (2) surface sediment of effluent from municipal wastewater treatment plants in the Taipei area; (3) bed sediments in the upper (Yuan-Fuh) and lower (Wann-Dan) stream of the Kao-Ping river (area of high livestock feeding activities); (4) surface sediment in the drainage of the Ju-Nan and San-Shia industrial parks (areas less polluted by raw sewage). Samples of sediments and sludge after collection were centrifuged at 1500 *g* for 10 min. The supernatant was discarded, and the residue was freeze-dried, ground, and then stored in amber glass containers for further extraction.

Sample Preparation and Derivatization

Sample (0.5 g, dry weight) was thoroughly mixed with 0.5 g of anhydrous sodium sulfate in a 60-mL screw-capped vial, and 10 mL of 0.5 N methanolic KOH was added. The mixer was then directly saponified by incubation in a water bath at 60°C for 60 min with periodic shaking. After saponification, samples were cooled to room temperature, adding 10 mL of deionized water and adjusting to pH 7, and then extracted with dichloromethane (3×20 mL). The combined dichloromethane extracts were reduced in volume by rotary evaporation. The rotary evaporator flask was rinsed three times with 1 mL of hexane, and each rinsate was used for silica gel extraction (J.T. Backer). The less polar substances were removed by elution with 5 mL of hexane, and the sterols-containing fraction was collected using acetone (4×1.5 mL). The combined extracts were concentrated with a stream of nitrogen. We used a one-step derivatization for the keto- and hydroxy-groups of sterols. The sterols were converted to their trimethylsilyl (TMS) ethers by adding 80 µL of MSTFA and 1 µL of catalyst (TMSI/dichloromethae/triethylamine: 142/858/2, v/v), closed, and allowed to stand at 60°C for 60 min.

Instrumentation

Analyses were carried out on a Hewlett-Packard (Avondiele, PA) GC-MS system consisting of a Model 6890 gas chromatograph, a Model 5972 mass-selective detector, an HP G1701BA MS ChemStation, and a Model 7683 autosampler. For separation of sterols, a HP-50+ (30 m × 0.25 mm i.d., cross-linked 50% diphenyl–50% dimethylsiloxane, 0.25-µm film thickness) fused-silica capillary column was used. The carrier gas was helium with high purity with a flow rate of 2.0 mL/min. The injection volume was 1.0 µL; a splitless injection mode was used. The initial column temperature of 180°C was held for 1 min, programmed at 20°C/min to 270°C, then at 30°C/min to 295°C, and held for 8 min. The transfer line was constant at 280°C. The ionization voltage was 70 eV. In the scanning mode, the mass range was 50–550 amu at a rate of 1.53 scans/s. In the selected ion monitoring (SIM) mode, two characteristic ions for each sterol were used for peak identification. Each eluted peak was identified by

its relative retention time and matching the area ratios of two ions with those of sterol standards.

Quantification

The detection limit for each sterol was calculated based on the weight giving a signal three times the peak-to-peak noise of the background signal. A regression analysis was performed on the measured peak area ratios against the increasing weight ratios of sterols to I.S. to obtain the linearity of SIM responses and to plot calibration curves for the quantitative measurement of sterols. The used I.S. for sterol analysis is 5α-cholestane, a non-polar, saturated compound of similar chemical structure that elutes well before sterols in GC. The data were analyzed using Statistical Analysis Systems (SAS) software.

RESULTS AND DISCUSSION

Derivatization

In the capillary gas chromatographic analysis of sterols, the derivatization process has the advantages of providing sharp peaks, reducing retention time, and improving sensitivity. The formation of trimethylsilyl (TMS) ethers by BSTFA is mainly used in the GC of environmental sterols. However, derivatization of coprostanone and cholestanone, the carbonyl-containing compound, was incomplete and also unstable. Thus, the derivatization method for the keto- and hydroxy-groups of hormones was selected [17]. Briefly, the reagent was a 100/1 (v/v) mixture of MSTFA/catalyst, where the catalyst was TMSI/dichloromethane/triethylamine (142/858/2, v/v). The reaction conditions were 60 min at 60°C. We submitted the mixture of standards for derivatization, respectively, with only MSTFA and MSTFA/catalyst. Figure 1(a) shows the chromatogram obtained after derivatization with MSTFA only. The stanones were not derivatized completely, and the derivatized stanones degraded quickly with time. Coprostanone and cholestanone were split into three and two peaks, respectively, and underivatized coprostanone overlapped with derivatized stigmasterol. In contrast, when a MSTFA/catalyst was applied for derivatization, trimethylsilation was completed for all nine standards (Fig. 1b). The underivatized peaks of coprostanone and cholestanone were no longer eluted, and the resolution was satisfied. However, the chromatogram obtained after complete derivatization of coprostanone gave two peaks (approximately a 1 : 10 ratio in abundance) with a similar spectrum: the m/z 458 for molecular ion, the m/z 143 for base peak ion, and with 96% similarity (Fig. 2). We infer that different donors of electrons in derivatization of coprostanone produce cholest-2-en-5β-ol TMS ether and its $\Delta 3$ isomer. Derivatization with MSTFA/catalyst was completed for all the standards and without any noticeable degradation after 4 days ($P > 0.05$). A good stability of derivatized compounds was found to be comparable with or better than literature values [17,18].

Gas Chromatography-Mass Spectrometry

The choice of a capillary column was based on the resolution between nine fecal sterols and the ability to be operated securely at high temperature. Chromatograms

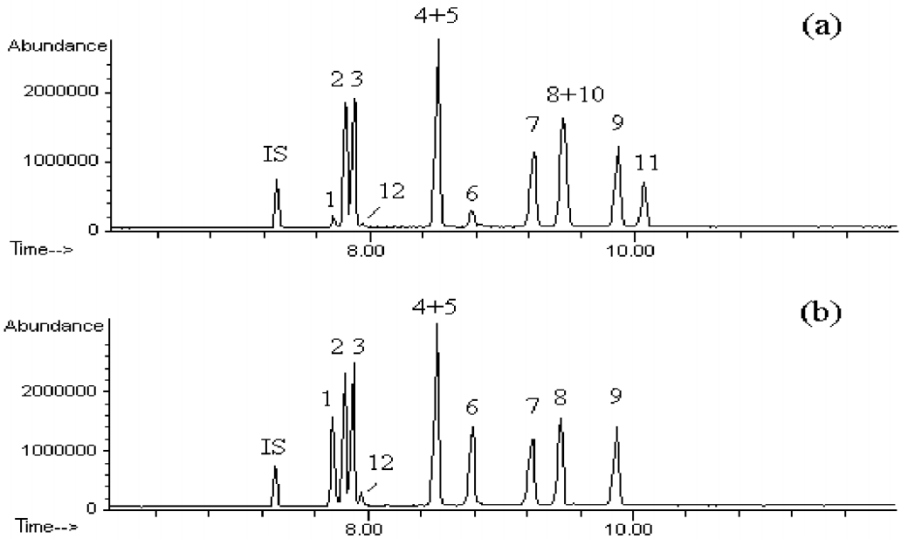

FIGURE 1 Chromatogram of standard sterols derivatized (a) with MSTFA and (b) with MSTFA/catalyst (TMSI/dichloromethane/triethylamine: 142/858/2, v/v). IS: internal standard (5α-cholestane); 1: coprostanone; 2: coprostanol; 3: epicoprostanol; 4: cholestanol; 5: cholesterol; 6: cholestanone; 7: campesterol; 8: stigmasterol; 9: sitosterol; 10: underivatized coprostanone; 11: underivatized cholestanone; 12: coprostanone isomer. Separation with 30 m × 0.25 mm × 0.25 µm HP-50+ capillary column and oven programmed at 180°C for 1 min, 20°C/min to 270°C, and then 30°C/min to 295°C and final hold-up for 5 min.

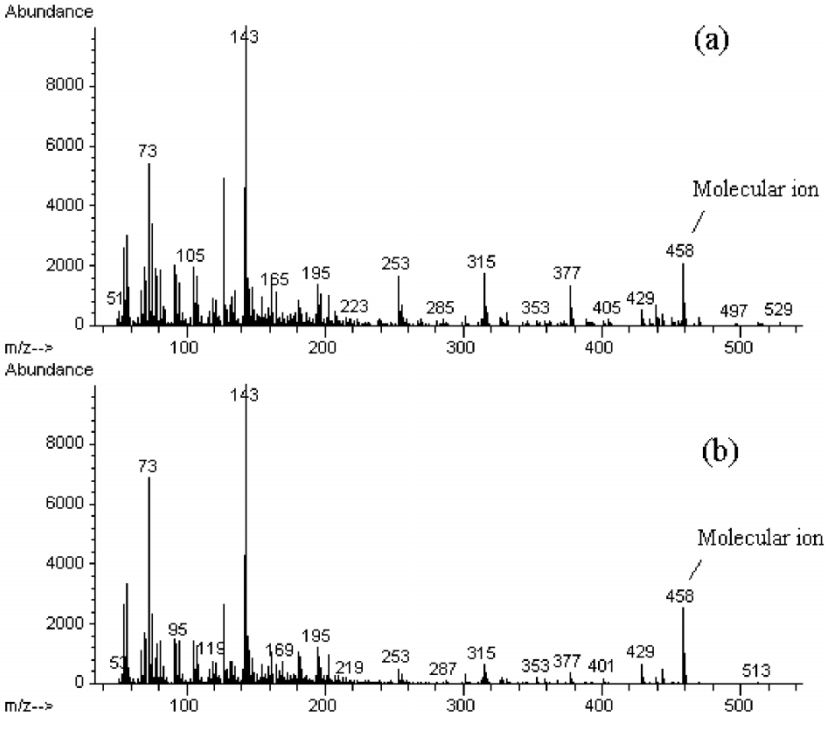

FIGURE 2 Mass spectra of (a) derivatized coprostanone and (b) isomer of derivatized coprostanone.

of a mixture of standards such as trimethylsilyl (TMS) ethers were obtained using a HP-5MS non-polar column and a more polar HP-50+ column. On the non-polar system, epicoprostanol was produced through the degradation of cholesterol, coeluted with coprostanol. Coprostanol and epicoprostanol have the same molecular weight and similar mass spectra. Although the ratio of m/z 215 to m/z 233 could be used to distinguish the epimers [19], good separation of both would be better for their co-existence in nature samples. With the polar system, adequate separation of coprostanol and epicoprostanol was achieved, though cholesterol (MW 386) eluted in the cholestanol (MW 388) region. GC-MS equipped with an HP-50+ column was chosen for the analyses to ensure the separation of nine fecal sterols. Under the GC conditions employed, sterols eluted between approximately 9 and 12 min.

Quantitative determinations by SIM were carried out, and the ions were selected to limit interferences by possible coeluting compounds. The molecular ion (M^+) was sufficiently abundant to be used for coprostanone (m/z 458), cholesterol (m/z 458), cholestanol (m/z 460), and cholestanone (m/z 458). For the other sterol TMS derivatives, the $[M-90]^+$ fragment ion was selected corresponding to the neutral loss of a TMS (OH) group from the molecular ion. This was the case for coprostanol and epicoprostanol (both at m/z 370), campesterol (m/z 382), stigmasterol (m/z 394), and sitosterol (m/z 396; Table I). Under the present SIM conditions, the detection limits were in the range of 9–30 pg. These limits show that the method has a good sensitivity. The GC-SIM-MS responses were linear with correlation coefficients varying from 0.997 to 0.999 in the concentration range of 0.2–20 ng/μL for the sterols studied.

The standard addition method was used to evaluate the recovery of the analysis. Recoveries ranged from 78 to 89%, and the CVs ranged from 5 to 11% (Table I). The method involving direct saponification is less efficient in the recovery of fecal stanones, including coprostanone and cholestanone, than fecal stanols but has a higher recovery and lower CVs for stanones than the preceding study [18]. Better results are attributed to the elimination of the initial extraction step thus simplifying the procedure and reducing it to only 4 h in total.

Quantification of Interests in Sediments

To evaluate the feasibility of using the developed method for routine analysis, the method was applied to the analysis of the various animals' sterol profiles in outfall

TABLE I GC-SIM-MS data of sterols as their trimethylsilyl derivatives

Sterols and stanones	*MW*	*Retention time* (min)	*Ions selected* (m/z)	*Detection limit* (pg)	*Linearity*[a]	*Recovery* (%)[b]
Campesterol	472	8.90	129 382	9	0.999	89 ± 11
Cholestanol	460	8.20	215 460	22	0.997	88 ± 8
Cholestanone	458	8.50	143 458	13	0.999	84 ± 8
Cholesterol	458	8.20	329 458	30	0.997	88 ± 8
Coprostanol	460	7.60	370 215	9	0.999	83 ± 6
Coprostanone	458	7.50	143 458	13	0.999	78 ± 9
Epicoprostanol	460	7.70	370 215	13	0.999	81 ± 5
Sitosterol	486	9.60	357 396	14	0.998	83 ± 9
Stigmasterol	484	9.20	129 384	21	0.999	89 ± 5

[a]Linearity was described with linear correlation coefficients for calibration curves.
[b]Data are expressed as average ± standard deviation.

sediments. The baseline is stable, and there are no major interfering peaks from the sample matrix (Fig. 3). The concentrations of sterols were within the overall ranges of 0.1–405 μg/g. The sterol profile of sewage effluents (human) was dominated by coprostanol (36%) and cholesterol (38%) (Table II). C_{27} sterols were present in sewage at higher levels than C_{29} sterols, whereas the opposite was observed for livestock wastewater. Only a trace amount of epicoprostanol was found in sewage. The sterol profile of pigs was similar to that of humans, but a difference was that the levels of epicoprostanol and sitosterol were greater in pigs. For ducks and cows, major fecal sterols were cholestanol, cholesterol, and sitosterol. The total proportion of phytosterols, including campesterol, stigmasterol, and sitosterol, was 54% in ducks, and the rate was significantly higher than that of humans (9%), pigs (18%), and cows (28%). Coprostanol was present at very low levels, and the low ratio of 5β-stanol

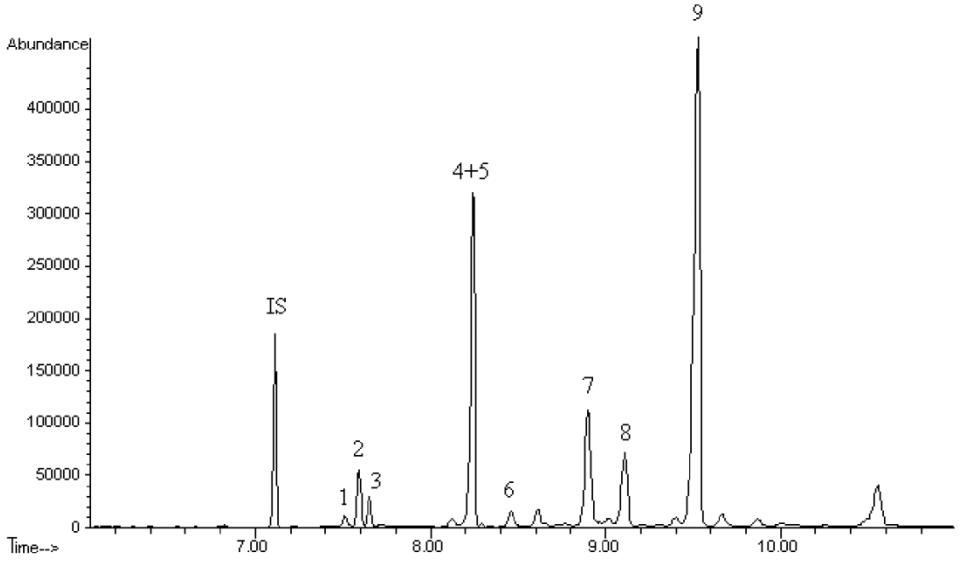

FIGURE 3 Total ion current chromatogram of sterols in the outfall sediments of ducks. 1: coprostanone; 2: coprostanol; 3: epicoprostanol; 4: cholestanol; 5: cholesterol; 6: cholestanone; 7: campesterol; 8: stigmasterol; 9: sitosterol; IS: 5α-cholestane.

TABLE II Concentration in percentage (average ± standard deviation) of sterols in outfall sediments of different animal sources

	Human ($n^a = 4$)	*Pig* ($n = 9$)	*Cow* ($n = 1$)	*Duck* ($n = 1$)
Campesterol	4.7 ± 2.3	4.8 ± 0.9	4.4 ± 0.5	5.9 ± 0.2
Cholestanol	14.1 ± 2.5	11.3 ± 2.9	17.7 ± 0.7	23.0 ± 1.3
Cholestanone	0.7 ± 0.6	1.8 ± 0.3	0.7 ± 0.1	0.1 ± 0.004
Cholesterol	37.6 ± 6.9	21.4 ± 8.9	37.2 ± 1.8	19.9 ± 2.2
Coprostanol	35.8 ± 4.1	29.5 ± 6.5	11.5 ± 0.8	2.3 ± 0.1
Coprostanone	2.4 ± 2.6	5.3 ± 1.6	0.8 ± 0.04	0.2 ± 0.02
Epicoprostanol	0.9 ± 1.0	9.1 ± 2.3	3.9 ± 0.3	0.7 ± 0.04
Sitosterol	3.0 ± 2.0	14.1 ± 1.5	19.0 ± 0.7	43.9 ± 3.8
Stigmasterol	0.8 ± 0.5	2.9 ± 0.8	4.8 ± 0.3	3.9 ± 0.2

[a]Number of animal sources.

relative to their sterols precursors for ducks indicates that anaerobes capable of reducing sterols to 5β-stanols were present in low numbers. The finding is similar to a study of sterol profiles in fecal samples [14].

Using Sterol Fingerprints to Determine Fecal Pollution from Humans and Livestock

An important feature of using fecal sterols as pollution indicators stems from the association of specific components and their distributions with the feces of different animals, which may allow different sources of fecal pollution to be distinguished through recognition of mixed inputs. We proposed ((coprostanone $\times$ coprostanol)/epicoprostanol) as an indicator for assessing fecal input. The selection of fecal sterol biomarkers for this study was based on their prevalence in feces and importance in biotransformation pathway of cholesterol. The formation of the three sterols is considered to occur as follows: 4-cholesten-3-one is formed by the oxidation of the 3β-hydroxy group of cholesterol to a ketone and isomerization of the 5–6 double bond to the 4–5 position; coprostanone is formed by the reduction of the 4–5 double bond; subsequently, coprostanol and epicoprostanol are formed by the reduction of the 3-keto group to the 3β-hdroxy and 3α-hydroxy groups, respectively. To our knowledge, the above formations have been produced predominantly through the mediation of anaerobic bacteria in the digestive tract of higher animals and not reported to occur naturally in fresh or marine waters. The source specificity of the three fecal biomarkers is due to a combination of diet, biosynthesis, and biotransformation. The levels of ((coprostanone $\times$ coprostanol)/epicoprostanol) in different sources of wastewater effluents are as follows: human 0.913 ± 0.251, pig 0.224 ± 0.135, cow 0.023 ± 0.001, duck 0.007 ± 0.001. The ratio of human : pig : cow : duck is 130 : 32 : 3.4 : 1. The value of the indicator of human effluent was significantly different from that of swine, cattle, and duck effluent.

The abundance of sterols detected in the sediments of different sampling streams is given in Table III. Yuan-Fun had the highest concentration, followed by Wann-Dan, Taipei sewage, San-Shia, and Ju-Nan. Yuan-Fun and Wann-Dan are both heavily polluted by piggery wastewater, and Taipei sewage mainly receives municipal wastewater of Taipei city. It is easy to recognize the stream being polluted by bioactivity through fecal sterols as sediments from both industrial parks have lower concentrations in total. The sterols in Taipei sewage had a similar magnitude of concentration as in the previous study by Jeng and Han [20]. The decreasing order of recommended indicator values was calculated to be Taipei sewage, Yan-Fuh, Wann-Dan, San-Shia, and Ju-Nan. As previously mentioned, human activity contributes a higher indicator value, followed by pig. Thus, Yuan-Fuh and Wann-Dann would have smaller indicator values to Taipei. The industrial effluent without human or animal fecal discharge from Ju-Nan has not only the lowest concentration but also the smallest indicator value. Given the demonstrated differences in sterol profiles between the animals tested and provided with more information regarding the physical setting, the biomarker can be an indicator used to distinguish sources of fecal pollution.

CONCLUSION

A major advantage of the present GC-MS method is the selective and rapid recovery of sterols from sediment by direct saponification. Moreover, the subsequent

TABLE III Concentration and indicator value (average ± standard deviation) of sterols in the sediments of different sampling streams

	Taipei sewage	*Yuan-Fuh*	*Wann-Dan*	*Ju-Nan*	*San-Shia*
Campesterol	4194 ± 5479[a]	14 749 ± 458	5749 ± 4197	26 ± 9	247 ± 18
	(4.7 ± 2.3)[b]	(5.1 ± 0.2)	(5.7 ± 0.03)	(1.4 ± 0.5)	(7.2 ± 0.5)
Cholestanol	10 942 ± 9910	15 784 ± 507	8562 ± 4328	296 ± 14	334 ± 48
	(14.1 ± 2.5)	(5.5 ± 0.2)	(9.4 ± 2.5)	(15.4 ± 0.7)	(9.7 ± 1.4)
Cholestanone	294 ± 165	2334 ± 49	1189 ± 357	7 ± 6	36 ± 1
	(0.7 ± 0.6)	(0.8 ± 0.02)	(1.4 ± 0.7)	(0.4 ± 0.3)	(1.1 ± 0.02)
Cholesterol	27 455 ± 22 895	44 555 ± 1791	14 435 ± 11 041	831 ± 40	1507 ± 105
	(37.6 ± 6.9)	(15.4 ± 0.6)	(14.0 ± 0.8)	(43.1 ± 2.1)	(43.9 ± 3.1)
Coprostanol	26 542 ± 24 824	134 918 ± 3933	38 374 ± 32 119	302 ± 23	342 ± 38
	(35.8 ± 4.1)	(46.7 ± 1.4)	(35.9 ± 5.8)	(15.6 ± 1.2)	(10.0 ± 1.1)
Coprostanone	794 ± 378	14 609 ± 347	3475 ± 1883	24 ± 15	107 ± 13
	(2.4 ± 2.6)	(5.1 ± 0.1)	(3.8 ± 0.9)	(1.2 ± 0.8)	(3.1 ± 0.4)
Epicoprostanol	345 ± 248	19 711 ± 401	7497 ± 3850	388 ± 20	81 ± 9
	(0.9 ± 1.0)	(6.8 ± 0.1)	(8.2 ± 2.1)	(20.1 ± 1.0)	(2.4 ± 0.3)
Sitosterol	1378 ± 955	36 142 ± 1113	17 660 ± 13 010	54 ± 22	555 ± 41
	(3.0 ± 2.0)	(12.5 ± 0.4)	(17.4 ± 0.3)	(2.8 ± 1.1)	(16.2 ± 1.2)
Stigmasterol	383 ± 244	6179 ± 125	4006 ± 2452	–[c]	223 ± 22
	(0.8 ± 0.5)	(2.1 ± 0.04)	(4.2 ± 0.6)		(6.5 ± 0.6)
Indicator[d]	0.913 ± 0.251	0.346 ± 0.008	0.166 ± 0.025	0.009 ± 0.006	0.131 ± 0.006

[a]Unit: ng/g. [b]Unit: %. [c]Below detection limit. [d]Indicator value: ((coprostanone × coprostanol)/epicoprostanol).

MSTFA/catalyst produced a complete trimethylsilyation reaction for nine of the standards and enhanced the GC/SIM/MS properties. The results presented herein show that sterols are readily detectable in raw sewage and estuarine sediment and that they survive microbiological degradation during treatment. Furthermore, the development of appropriate component ratios of ((coprostanone × coprostanol)/epicoprostanol) could possibly be used to distinguish between fecal pollution of human and animal origin.

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