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Analysis of Faecal sterols in Freshwater Sediments and Source Samples.

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MANAGEMENT PERSPECTIVE

This report is primarily a description of methods used by NWRI laboratories for the analysis of faecal sterols as source tracers. This work was conducted under Great Lakes priorities and is important to agencies involved in Remedial Action Plans. It contributes information towards Environment Canada initiatives on the assessment of human activities on Great Lakes ecosystems (Great Lakes 2020). The manuscript complements other publications by the same research team that describe the potential use of faecal sterols to assess impacts of sources such as sewage treatment facilities and livestock operations on embayments, harbours and nearshore lake areas. These tracer applications aid in the source identification and investigation of pollutant transport hence, they provide important information for the development of effective strategies to mitigate potentially adverse health effects attributable to contaminants or pathogens that may be associated with human or animal waste effluents.

Analyse des stérols fécaux dans les sédiments d'eau douce
et dans des échantillons de diverses sources

C.H. Marvin, J.P. Coakley, T. Mayer, M. Brown et P.A. Thiessen

SOMMAIRE À L'INTENTION DE LA DIRECTION

Ce rapport porte principalement sur la description des méthodes appliquées par les laboratoires de l'INRE pour les analyses de stérols fécaux utilisés comme traceurs de sources. On a effectué ces travaux dans le cadre des priorités d'assainissement des Grands Lacs; de plus, ils sont aussi importants pour les organismes qui collaborent aux plans d'assainissement, car ils fournissent des informations utiles pour les initiatives d'Environnement Canada concernant l'évaluation des activités humaines dans les écosystèmes des Grands Lacs (Grands Lacs 2020). Ce manuscrit complète d'autres publications de la même équipe de recherche, qui décrivent l'utilisation possible de stérols fécaux pour évaluer l'impact de sources comme les stations d'épuration des eaux d'égout et les activités d'élevage de bétail sur les enfoncements, les ports et les zones riveraines des lacs. Les traceurs, qui facilitent l'identification des sources et l'étude du transport des polluants, fournissent des informations utiles pour le développement de stratégies efficaces destinées à atténuer les effets potentiellement nocifs pour la santé des contaminants ou organismes pathogènes pouvant être associés aux effluents d'eaux résiduaires d'origine humaine ou animale.

ABSTRACT

Coprostanol (5β-cholestan-3β-ol), a mammalian metabolite of cholesterol and its primary isomer epicoprostanol (5β cholestan-3α-ol) have been used in characterization and tracing of sewage-contaminated sediments. Related compounds, some of which are also used as tracer accessories include cholesterol (cholestan-5-en-3β-ol), coprostanone (5β-cholestan-3-one), dihydrocholesterol (or cholestanol, 5α-cholestan-3β-ol) and cholestanone (5α-cholestan-3β-one). These compounds have the necessary characteristics of tracers in that their primary source is mammalian faecal matter. Furthermore, they are persistent in the sedimentary environment and readily quantifiable at low concentrations. This report documents the analytical methods applied at the National Water Research Institute to determine coprostanol and related compounds in sediments and source samples in support of research into the dispersal of contaminated sediments in freshwater systems. We have also calculated ratios of these compounds as a means of assessing and discriminating contamination arising from different sources including sewage treatment plants and agricultural-based activities.

RÉSUMÉ

On a utilisé le coprostanol (5β-cholestan-3β-ol), un métabolite du cholestérol chez les mammifères et son principal isomère, l'épicoprostanol (5β-cholestan-3α-ol) pour la caractérisation et le dépistage des sédiments contaminés par les eaux d'égout. Ces composés apparentés, dont certains sont aussi utilisés comme traceurs, sont notamment le cholestérol (cholestan-5-en-3β-ol), la coprostanone (5β-cholestan-3-one), le dihydrocholestérol (ou cholestanol, ou 5α-cholestan-3β-ol) et la cholestanone (5α -cholestan-3β-one). Ces composés sont utiles comme traceurs parce que les matières fécales des mammifères sont leur principale source. De plus, parce qu'ils sont persistants dans le milieu sédimentaire, on peut les doser facilement, même à de faibles concentrations. Ce rapport documente les méthodes analytiques utilisées par l'Institut national de recherche sur les eaux pour doser le coprostanol et ses composés connexes dans les sédiments et dans les échantillons de diverses sources pour le soutien des recherches qui portent sur la dispersion des sédiments contaminés dans les réseaux d'eau douce. Nous avons également calculé les rapports de ces composés pour l'évaluation et la détermination de différentes sources de contamination, notamment les stations d'épuration des eaux d'égout et les activités agricoles.

INTRODUCTION

Coprostanol (5β-cholestan-3β-ol) belongs to a large family sterols characterized by carbon numbers of 22 to 29 (Figure 1). It is almost uniquely produced by microbial reduction of cholesterol in the gut of humans (Kitchmer, 1971, Midtvedt and Midtvedt, 1993). For example, samples of bird guano from seagull rookeries in Hamilton Harbour (J.P. Coakley, unpubl. data) contained minute values of coprostanol (27 $\eta g \cdot g^{-1}$ or less), which are approximately 1 millionth the levels in human waste (Murtaugh and Bunch, 1967). Faeces of marine mammals, including some whales and seals (Venkatesan and Santiago, 1989) and pigs and cats (Leeming et al. 1997) also contain significant, but relatively minor, amounts of coprostanol. Because of this specificity of origin, coprostanol (C27) has been used extensively as a tracer for sewage-related pollution, both in water and in sediment. However, its low solubility in water and its affinity for sediment particles, make it more useful as a tracer of sediments contaminated by discharges of mammalian waste usually occurring through sewage treatment systems.

Despite its wide use, some researchers urge caution in the use of coprostanol as a tracer, especially in marine areas characterized by very low concentrations in sediments. This concern is primarily due to possible presence of coprostanol from the faeces of marine mammals, and from the breakdown of algal sterols such as stigmasterol. Grimalt et al. (1990) used the ratio of coprostanol to cholestanol to distinguish coprostanol derived from diagenesis of algal sterols from that of human origin. Studies of marine sedimentary environments offshore of the southern California coast (Venkatesan and Santiago (1989), and of freshwater and marine sedimentary areas of Australia (Leeming et al. (1997) indicate that unambiguous confirmation of contamination originating from human waste could be obtained by calculating the ratio of some sterol epimers (e.g. coprostanol to epicoprostanol) in the sediments. For this reason any determination of sedimentary coprostanol should include the epimers mentioned above.

In this paper, we describe the methodology currently used at the National Water Research Institute for the determination of sterols in a variety of matrices including freshwater sediments and source samples. Source samples were used to determine the specificity of faecal sterols in source identification. This specificity is important for

discerning the sources of sanitary and livestock-related pollution to aquatic systems and subsequently for effective implementation of remedial measures.

METHODS

Sample collection and preparation

Sediment samples were collected from Lake Ontario (E-30 and WB) and from the inlet area of a stormwater detention pond in Toronto (HRIN5). Sediment samples were collected using a Shipek sampler or mini box corer (Mudroch and MacKnight, 1994). Sediments making up the top 2 cm layer (comprising the previous 5-10 years deposition depending on the local sedimentation rate) were carefully collected and refrigerated at 4° C while onboard the survey vessel until storage in a cold room at NWRI. Care was taken to avoid loss of the soft surface layers. Before analysis, samples were freeze-dried, homogenized, and passed through a 62 μm sieve to remove the sand fraction. Source samples included raw sewage treatment plant (STP) effluent, and pig manure from holding tanks at two Ontario livestock farms.

Analytical Standards

Sterol compounds were all obtained from Sigma (Canada). They were as follows; epicoprostanol (95%), cholesterol (99+%), stigmasterol (95%), dihydrocholesterol (95%), stigmastanol (97.2%) and coprostanol (98%). The derivatizing agent N-methyl-N-trimethylsilyl-trifluoroacetimide (MSTFA) was obtained from Pierce (Rockford Ill). All solvents were of analytical grade. Water was prepared using a Milli-Q purification system. A sediment sample from a reference site in Lake Ontario, previously determined not to contain any detectable sterols, was used for spike recovery studies.

Extraction

Previously, the most utilized technique for extraction of sterols was Soxhlet extraction. This technique consumes large volumes of solvent and is generally time-consuming (24 hours). Extractions performed in this study were in dichloromethane using an ASE 200 accelerated solvent extractor (Dionex Corp.). Extraction cells were lined with Dionex D28 cellulose filters. Dry sub-samples (0.5 g - 1.0 g) were weighed and mixed with Ottawa sand (Fisher Scientific) to fill the entire volume of the extraction cell.

A 2,000 psi fluid pressure was maintained and cells were heated to 100°C. Optimum operating parameters are shown in Table 1.

Table 1. Optimal ASE-200 operating parameters.

Oven temperature	100°C
preheat	5 min
heat	5 min
static time	15 min
flush%	70% (dicholoromethane)
purge time	60 sec
pressure	2,000 psi

Cleanup

Sample extracts requiring cleanup were subjected to an open-column alumina procedure. A glass pipette was packed with 1.0 g of alumina and the extract was added to the top of the column. Sterols were eluted with 12 mL of dichloromethane.

Heavily contaminated samples (e.g., pig manure) were also subjected to a gel permeation chromatography procedure using an Autoprep 1002 GPC column packed with SX-3 Bio-Beads (Bio-Rad, Richmond CA) with a dichloromethane-hexane (50:50, v/v) mobile phase at 5 mL/min. Sterol elution times on the gel column were determined from spiked extracts.

Derivatization

Extracts were gently evaporated to dryness under nitrogen and derivatized with 100µl N-methyl-N-trimethylsilyl-trifluoroacetimide (MSTFA) for 20 minutes at 130°C. After cooling, extracts were reconstituted in 1 mL of hexane for GC-MS analysis.

Chromatography

Extracts were analyzed using a Hewlett Packard Model 5890 Series II gas chromatograph equipped with a Model 5971A mass selective detector (Hewlett-Packard, Mississauga, Ont). The instrument was operated in full scan or selective ion monitoring (SIM) mode depending on the data quality objectives. GC-MS operating parameters are shown in Table 2.

Table 2. GC-MSD operating parameters.

injection port	on-column
injection volume	1µl
column	0.25 mm i.d. 30 m DB-5, 0.25 µm phase
mass transfer line temp	300°C
full scan acquisition	50-500 amu
SIM ions (m/z)	388, 355, 231, 386, 316, 215, 370, 233, 264
Temperature program:	
initial temperature	100°C
ramp rates	100°C to 170°C @ 20°C/min
	170°C to 300°C @ 3°C/min
final temperature	300°C
carrier gas	helium @ 40cm/sec

Quality Assurance and Quality Control

Sediment samples were spiked with a laboratory standard containing four polycyclic aromatic hydrocarbons including naphthalene-d_8, fluorene-d_{10}, pyrene-d_{10} and benzo[a]pyrene-d_{12}. Spike recoveries were typically greater than 75%. Method blanks were carried through the complete sample preparation and analysis procedures. Between-run reproducibilities were typically 10%. The analysis of suspended sediments provided data complementary to that obtained from bottom sediments, however, sample sizes were frequently only on the order of several hundred milligrams. These small sample sizes imposed a method detection limit of approximately 60 ng g^{-1} (3:1 S/N) for individual sterols in a 1 g dry weight sample when analyzed in full scan mode. Method detection limits ten-fold lower could readily be achieved when selected ion monitoring (SIM) was employed.

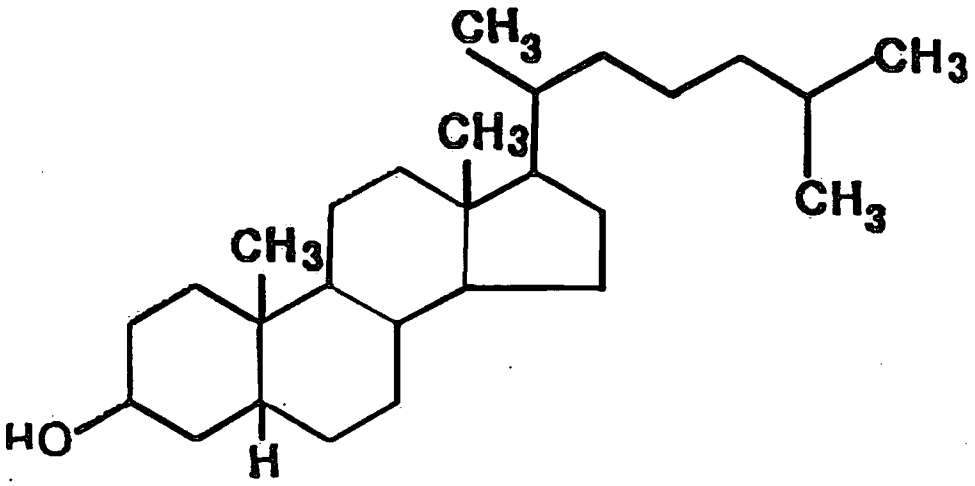

Figure 1. Structure of coprostanol, 5β-cholestan-3β-ol.

RESULTS AND DISCUSSION

The hydrophobicity of coprostanol and related sterols results in their being associated primarily with particulate matter in aquatic systems; our methodology is designed for processing of solid samples. Initial methods development focused on optimization of the extraction and analysis procedures. Examples of analytical procedures used previously at NWRI and other laboratories are reported in Coakley et al. (1992) Bachtiar et al. (1996), DeLuca and Fox (1995) and Jones et al. (1994). The accelerated solvent extraction (ASE) method was found to have distinct advantages over conventional Soxhlet extraction including lower solvent volumes and shorter extraction times of roughly 30 minutes per sample. The instrument has the capability to perform a series of 24 completely automated extractions. Under the described conditions, sterol compounds were recovered by ASE from all matrices studied with satisfactory recoveries. The ranges of recoveries of sterol compounds from spiked Lake Ontario reference sediment are shown in Table 3.

Table 3. Recovery of sterol compounds from spiked reference sediment.

Compound	Recovery
Cholesterol	90 - 95%
Dihydrocholesterol	90 - 95%
Stigmasterol	90 - 95%
Stigmastanol	90 - 95%
Coprostanol	90 - 95%
Epicoprostanol	90 - 95%

Methods previously used at NWRI were based on analysis using gas chromatography with flame ionization detection (FID). These methods, although robust, did not have the benefit of mass spectrometric confirmation of the analytes. Attempts were also made to develop methods that precluded the use of a derivatization procedure. We found that analysis of sterols in their native form without derivatization resulted in rapid decreases in column performance after roughly thirty samples. In addition, these methods did not readily result in the separation of two key sterol isomers, coprostanol and

epicoprostanol. Our analysis of a wide variety of sample matrices and reports from other analysts (Venkatesan and Santiago, 1989) have shown that epicoprostanol is frequently present at significant levels in some environmental samples compared with levels of coprostanol. Based on these observations, the levels of coprostanol in some reports in the literature may actually be the sum total concentrations of both coprostanol and epicoprostanol. Our sample preparation methodology combined with the described temperature program on a DB-5 column results in baseline resolution of these isomers. Figure 2 shows a GC-MS chromatogram of an extract of Lake Ontario reference sediment spiked with six sterols at levels ranging from 50 to 100 µg/g (parts per million).

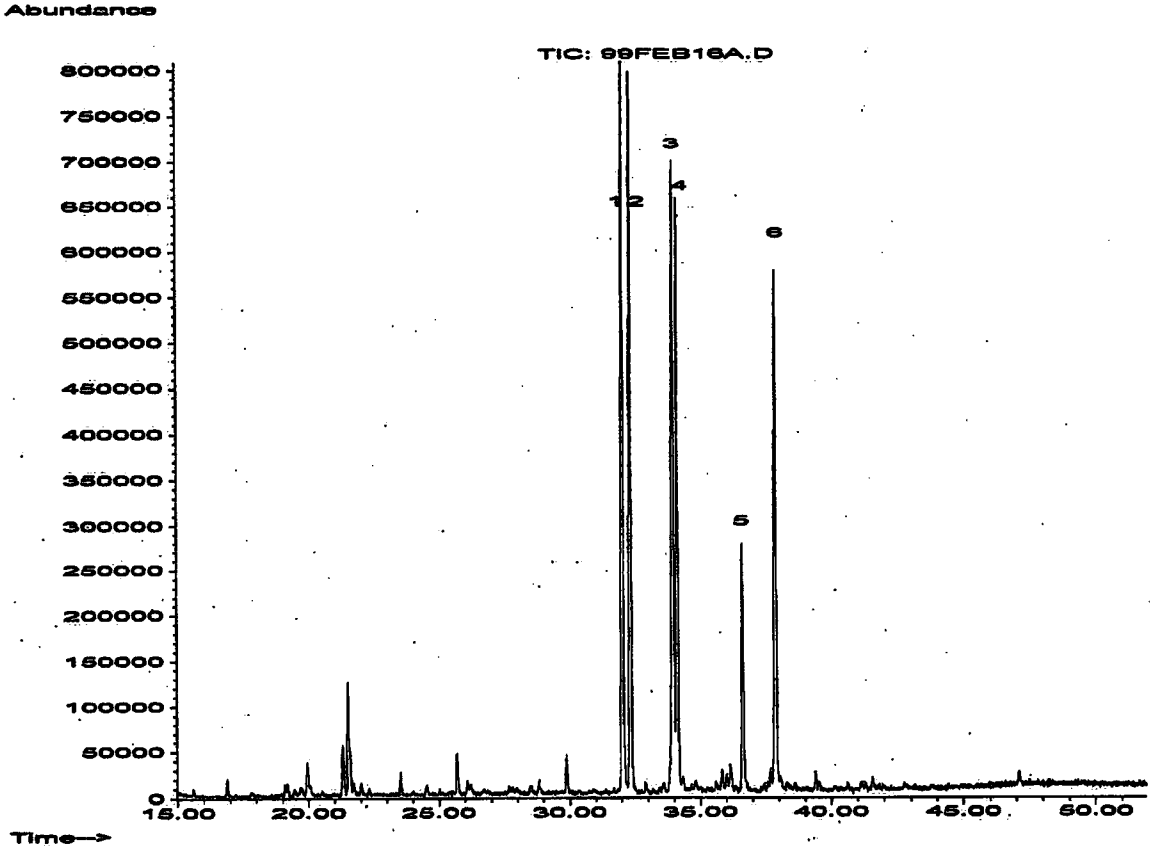

Figure 2. Gas chromatography - mass spectrometry total ion chromatogram showing the separation of six selected sterols in an extract of spiked Lake Ontario reference sediment. Peak numbers correspond to: 1. Coprostanol, 2. Epicoprostanol, 3. Cholesterol, 4. Dihydrocholesterol, 5. Stigmasterol, and 6. Stigmastanol.

Our primary interest in the analysis of sterols stems from their utility as tracers of contamination originating from municipal sewage treatment plants (STPs). We have

applied our methodology to the analysis of Great Lakes sediments from numerous nearshore areas including Hamilton Harbour, an embayment of western Lake Ontario. The harbour receives discharges from four STPs. Most of our work has focused on the northeast area of the harbour near the Burlington Skyway STP outfall. Figure 3 shows a typical chromatogram from the analysis of a sediment sample from Hamilton Harbour collected near the STP outfall. The compounds of interest are well resolved and there are minimal interferences which has resulted in our ability to accurately quantitate the analytes. These data, taken in context with current velocities and meteorological data, have allowed us to determine the temporal transport pattern of the STP effluent in this area of the harbour (Coakley et al., 2000).

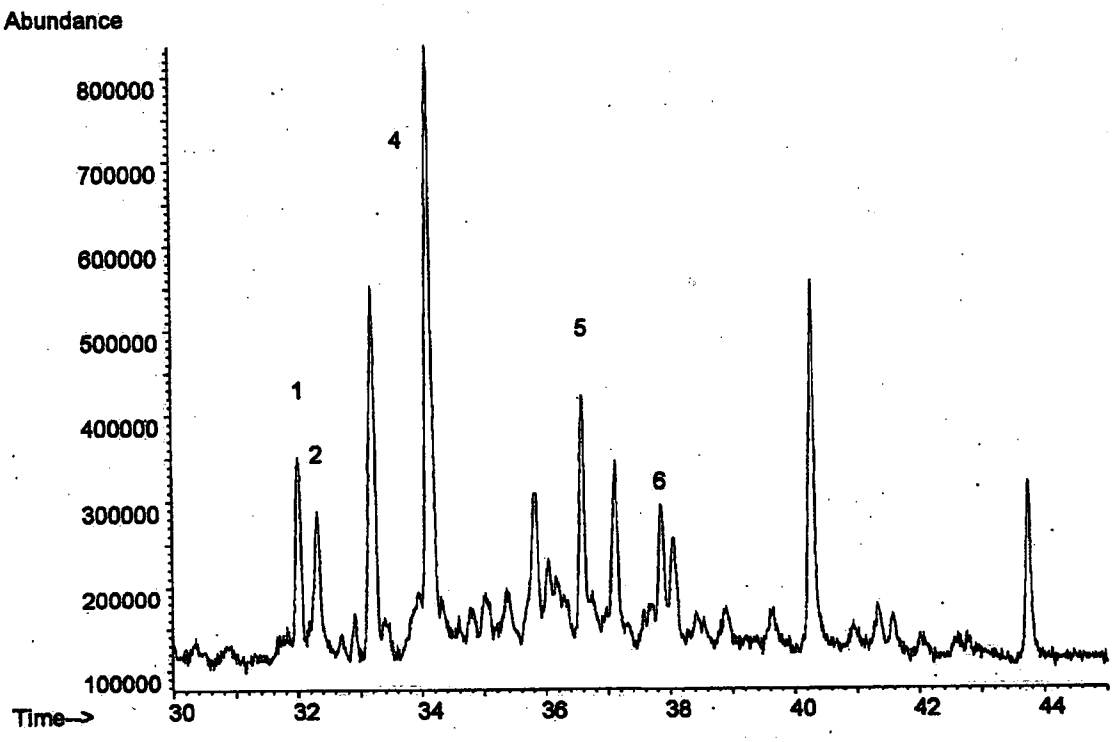

Figure 3. Full scan gas chromatography - mass spectrometry total ion chromatogram showing sterols determined in an extract of Hamilton Harbour sediment. Peak numbers correspond to: 1. Coprostanol, 2. Epicoprostanol, 4. Dihydrocholesterol, 5. Stigmasterol, 6. Stigmastanol.

We applied our methodology to the analysis of a variety of source samples and generated sterol profiles for the purpose of investigating the potential utility of these

compounds as indicators of impacts of shore based activities such as agriculture on aquatic systems. These matrices included pig manure, raw STP effluents and effluents from combined sewer overflows (CSOs). Table 4 lists the sterol concentrations determined in these source and reference samples. Figures 4, 5 and 6 show GC-MS chromatograms from the analysis of extracts of these source samples that were prepared using the method described above. Each of these profiles appear to be distinct, particularly in the differences in the ratios between coprostanol:epicoprostanol and cholesterol:dihydrocholesterol. The differences in these profiles will be further investigated through analyses of source samples collected from different geographical areas.

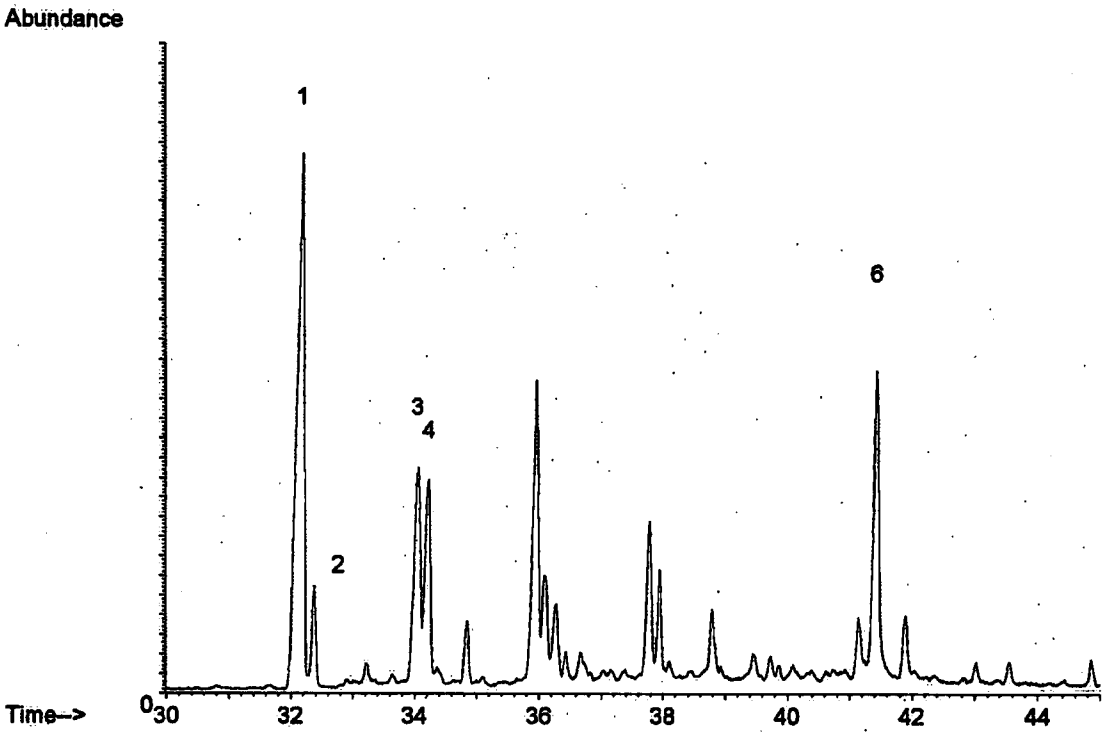

Figure 4. Full scan gas chromatography - mass spectrometry total ion chromatogram showing sterols determined in an extract of pig manure. Peak numbers correspond to: 1. Coprostanol, 2. Epicoprostanol, 3. Cholesterol, 4. Dihydrocholesterol, 6. Stigmastanol.

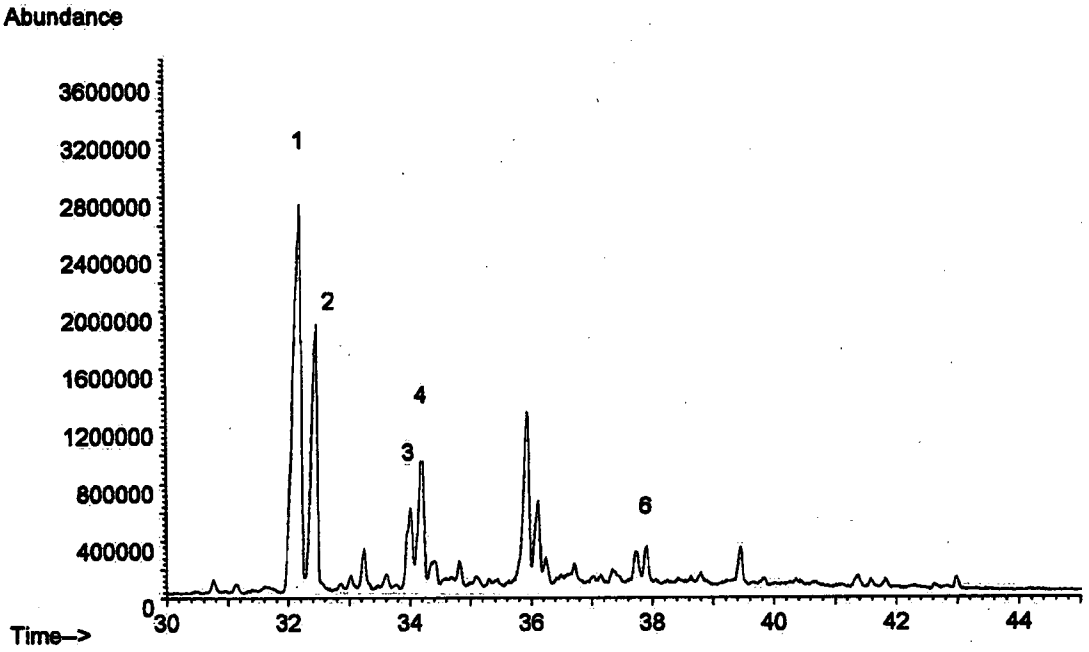

Figure 5. Full scan gas chromatography - mass spectrometry total ion chromatogram showing sterols determined in an extract of STP effluent. Peak numbers correspond to: 1. Coprostanol, 2. Epicoprostanol, 3. Cholesterol, 4. Dihydrocholesterol, 6. Stigmastanol.

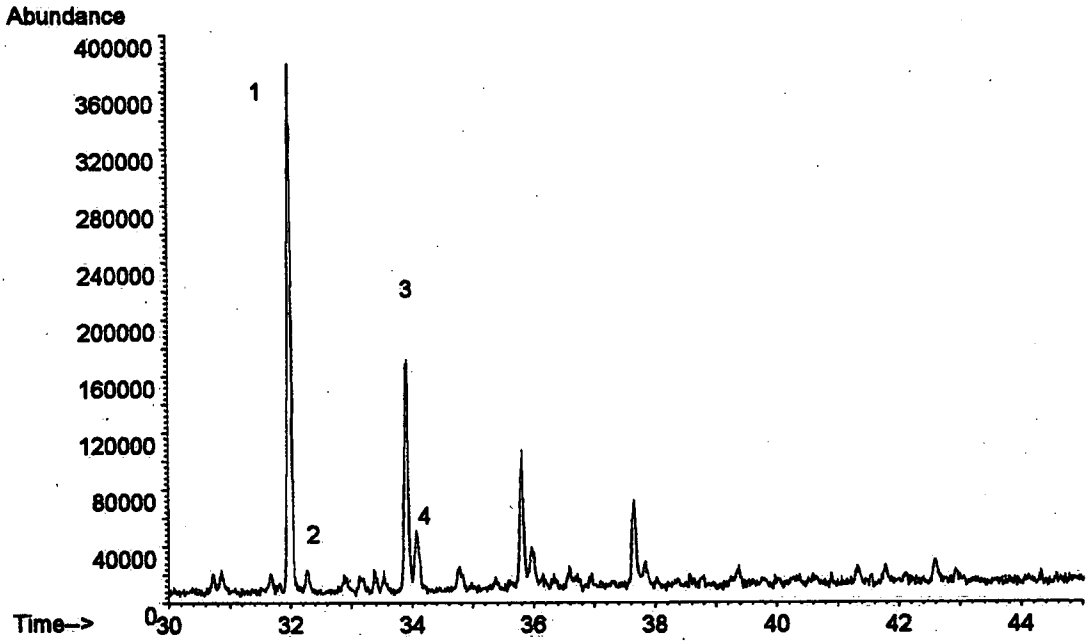

Figure 6. Full scan gas chromatography - mass spectrometry total ion chromatogram showing sterols determined in an extract of CSO effluent. Peak numbers correspond to: 1. Coprostanol, 2. Epicoprostanol, 3. Cholesterol, 4. Dihydrocholesterol.

Table 4. Concentration of sterols in source and reference samples. All values are in percent dry weight.

Sample	Coprostanol	Epicoprostanol	Cholesterol	Dihydrocholesterol	Stigmasterol	Stigmastanol
70-tank[a]	9.72 ± 0.97	1.28 ± 0.03	2.43 ± 0.29	3.30 ± 0.13	<0.8	0.84 ± 0.03
Site 3[a]	12.9 ± 1.19	1.40 ± 0.12	1.52 ± 1.03	3.60 ± 0.02	<0.8	1.45 ± 0.19
HRIN-5[b]	<0.01	<0.01	<0.01	<0.01	<0.8	<0.01
S-tank[a]	6.95 ± 0.14	0.78 ± 0.01	1.95 ± 0.64	2.19 ± 0.03	<0.8	0.70 ± 0.02
STP[c]	1.06 ± 1.77	5.81 ± 1.40	0.93 ± 0.42	2.95 ± 0.60	<0.8	0.32 ± 0.14
HCSO-10[d]	0.71 ± 0.01	<0.01	0.20 ± 0.0	<0.01	<0.8	<0.01
WB[b]	<0.01	<0.01	<0.01	<0.01	<0.8	<0.01
E-30[b]	<0.01	<0.01	<0.01	<0.01	<0.8	<0.01

[a]pig manure.
[b]reference sites, HRIN-5, pond sediment; WB, western basin of Lake Ontario; E-30, eastern basin of Lake Ontario.
[c]sewage treatment plant effluent.
[d]combined sewer overflow.

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