Method Validation for the Simultaneous Determination of Fecal Sterols in Surface Waters by Gas Chromatography–Mass Spectrometry

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Abstract

Besides microbiological methods, fecal pollution of surface waters is estimated by gas chromatographic (GC) determination of sterols present in human and animal sewage effluents. The most frequently used biomarkers for the evaluation of contamination levels include coprostanol, cholesterol, dihydrocholesterol, stigmasterol, β-sitosterol, and stigmastanol. Although several GC techniques are used to measure these compounds in aquatic systems, the analytical performance of GC–mass spectrometric (MS) determination of these sterols has not been systematically characterized. Therefore, the aim of this work is to validate a simple and rapid GC–MS method for the simultaneous analysis of six sterols, considering all parameters and requirements defined by Good Laboratory Practice. Following liquid–liquid extraction of spiked surface water samples, the extracts are silylated and analyzed by GC–MS. The method is evaluated for linearity and limits of detection and quantitation, as well as for precision, extraction efficiency, and stability. The assay is linear up to 160 ng; the limits of detection and quantitation are 5–10 ng and 20 ng, respectively. The within- and between-day precision ranged from 1% to 9% and 1% to 16%, respectively. The extraction efficiency was 65–80%. The stability studies indicate that the sterols in surface water samples begin to degrade after 24 h of refrigerated storage. However, three freeze/thaw cycles could be performed without their decomposition. The method is applied to the analysis of surface water and wastewater samples. The technical advantages make this GC–MS analysis suitable for routine environmental monitoring of fecal pollution in aquatic systems.

Introduction

Fecal pollution of surface waters by human and animal sewage effluents, which can contain a variety of pathogenic micro-organisms, has been considered as a risk not only to human health, but also to the integrity of aquatic ecosystems (1,2). Therefore, monitoring of fecal contamination plays an important role in the prevention of waterborne infectious diseases and the protection and improvement of the quality of the aquatic environment (3,4). Fecal pollution has traditionally been examined by microbiological methods based on the isolation, cultivation, and enumeration of indicator bacteria, including fecal coliforms, streptococci, and clostridia (5,6). However, the reliability of these measurements has been questioned because the culture media, conditions of incubation, and time delay between sample collection and beginning of analysis can significantly influence the species isolated and colony count (7–9). In addition, humans and nearly all warm-blooded animals host bacterial groups used to evaluate fecal contamination. Consequently, microbiological methods do not provide information for the identification and distinction of pollution sources (10,11). To overcome these limitations, in the past few decades, several biologically derived low-molecular-weight organic substances present in human and animal wastes have been proposed as biological indicators (biomarkers) of fecal contamination (12,13). Because of their high amounts in feces and untreated sewage effluents, sterols from the gastrointestinal absorption of dietary steroids as well as endogenous cholesterol (CHL) synthesis and metabolism have been proven to be the most suitable biomarkers among the potential compounds (14–16). When wastewater containing fecal matter is discharged into aquatic systems, sterols can be detected directly in water samples (17,18). Although sterols can be degraded in surface waters under aerobic conditions, they are rapidly bound to waterborne microparticulates, subsequently deposited at the water–sediment boundary, and finally incorporated into the anaerobic sediments where their decomposition is very limited (18,19). Thus, the concentrations of fecal sterols in surface waters and their sediments have been reported to correlate with current and previous contamination levels, respectively (17–20). Because significant differences have been recorded in the individual sterol composition of human and animal feces because of alterations in dietary intake of steroids and endogenous CHL production and metabolism, the sterol patterns of water and sediment samples can provide information required to distinguish and determine the sources of fecal

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pollution (21–23). For example, the sterol coprostanol (COP), which is produced in the gastrointestinal systems of humans and higher animals by the microbial reduction of CHL, has been extensively used as an indicator of human fecal pollution (24,25). It can also be metabolized to cholestanol, which is the corresponding 5α epimer of COP (22,25). High ratios of COP/COP–CHL have been reported to be associated with anthropogenic fecal contamination in seawater and sediment samples, and the differences in the relative amount of COP epimers have been used to discriminate fecal input from human and marine mammal sources in the vicinity of a sewage outfall (25). An abundance of CHL and β-sitosterol (β-SIT) with minimal level of COP in surface waters has been suggested as an indicator of fecal pollution from dogs and birds (24). In addition, the distribution of COP and other fecal sterols in the sediment cores of urban estuaries has been shown to correlate with historical trends in wastewater contamination (26). The ratios of plant-derived sterols and those of animal origin in overland flow samples from grazing-land have also been used to trace pollutant sources (27).

Fecal sterols in environmental and biological samples are usually quantitated by gas chromatography (GC) with flame ionization detection (FID) (28–30) and GC–electron capture detection (31), and their identification is verified by GC–mass spectrometry (MS) (28,30,32–38). Although these GC techniques have been widely used to measure the sterol concentrations in fresh, marine, storm, and sewage waters (28,32–38), the analytical performance of GC–MS determination has not been completely characterized. Because cost and time effectiveness are of high concern in environmental monitoring, the identification and quantitation of sterols in a single chromatographic run are also desirable. Therefore, the objective of this study was to validate a simple and rapid GC–MS method for the simultaneous identification and quantitation of the most frequently measured fecal sterols, including COP, CHL, dihydrocholesterol (DCHL), stigmasterol (SROL), β-SIT, and stigmastanol (SNOL). This GC–MS analysis was evaluated for linearity, precision, extraction efficiency, limit of detection (LOD), limit of quantitation (LOQ), and stability according to the guidelines and regulations of Good Laboratory Practice specified for chromatographic techniques (39). Finally, the method was applied to quantitative analysis of fecal sterols in Hungarian surface waters and wastewater samples from urban sewage treatment plants. The results presented in this paper show that because of the time-effectiveness and validity parameters, this GC–MS method is suitable for routine monitoring of fecal pollution in aquatic systems.

Experimental

Materials

Dichloromethane [high-performance liquid chromatography (HPLC) grade], acetonitrile (HPLC grade), sodium hydroxide, and standards of fecal sterols, including COP (5β-cholestan-3β-ol), CHL (5-cholesten-3β-ol), DCHL (3β-hxdroxy-5α-cholestane), SROL (3β-hydroxy-24-ethyl-5,22-cholestadiene), β-SIT (24β-ethylcholesterol), and SNOL (24α-ethyl-5α-cholestan-3β-ol) were provided by Sigma-Aldrich Chemical Company (Stenheim, Germany). Perylene-d_{12} and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were obtained from Supelco (Bellefonte, PA). Perylene was purchased from Fluka Chemie AG (Buchs, Switzerland).

Preparation of standard solutions

For the optimization of GC–MS conditions, a standard mixture of COP, CHL, DCHL, SROL, β-SIT, SNOL, and perylene (surrogate standard) was prepared in dichloromethane at a concentration of 1.0 mg/mL for each of component. For quantitative determination, perylene-d_{12} was used as an internal standard (IS) and dissolved in dichloromethane to obtain a stock solution of 1.0 mg/mL. To check the extraction efficiency (recovery) of the fecal sterols in unknown water samples, a 0.125-mg/mL perylene surrogate standard solution was prepared in acetonitrile.

Derivatization procedure

The GC–MS analysis of target sterols was carried out after derivatization with BSTFA–TMCS. A 25-µL aliquot of the standard mixture and 20 µL of IS solution were pipetted into a 1.5-mL autosampler vial (Supelco) and dried completely under a nitrogen flow. The residue was reconstituted in 125 µL of dichloromethane and mixed with 125 µL of BSTFA–TMCS. The vial was closed with a Teflon-lined screw cap, and the sample was silylated at 70°C for 30 min. Following derivatization, 1 µL of the mixture was injected into the GC–MS system.

Instrumentation and GC–MS conditions

The samples were analyzed on a Hewlett-Packard (Palo Alto, CA) GC–MS system consisting of an HP 5890 GC with an HP 5973 mass selective detector (MSD) and an Agilent 7683 automatic liquid sampler (Agilent Technologies, Palo Alto, CA). Separations were accomplished using an HP-5MS fused silica capillary column (Hewlett-Packard) coated with phenylmethylsiloxane (30-m × 0.25-mm i.d., 0.25-µm film thickness) using the Environmental Protection Agency (EPA) Method 8270 (40) with minor modifications. Briefly, the GC–MS parameters were as follow: carrier gas, helium, 1.4 mL/min, constant flow; injection, splitless, 1 µL sample; inlet temperature, 280°C; oven ramps, 150°C for 0.5 min, 150–300°C at 20°C/min, and 300–310°C at 25°C/min; GC–MS interface temperature, 280°C; MSD ion source temperature, 230°C; MSD quadrupole temperature, 150°C; ionization energy, 70 eV; and solvent delay, 7.0 min. System control, data acquisition, and analysis were performed with the HP G1701AA MSD Productivity ChemStation software, Rev. A.03.01 (Agilent) on a HP Vectra XA 5 computer (Hewlett-Packard). Data were acquired in the full scan mode between ions of *m/z* 50 and 550.

Preparation of calibrators and calibration curves

The target sterols and surrogate standard were dissolved in dichloromethane to prepare the standards as 1.0 mg/mL stock solution. The calibrators were prepared by measuring 20 µL of IS and 5-, 10-, 15-, 20-, 25-, 30-, 35-, and 40-µL aliquots of the stock solution into autosampler vials and dried under a nitrogen flow. The residues were reconstituted in 125 µL of dichloromethane, mixed with 125 µL of BSTFA–TMCS, and derivatized as described previously. The final mass concentrations of calibration standards were 20, 40, 60, 80, 100, 120, 140, and 160 ng for each of the com-

Table I. Target (T) and Qualifier (Q_1 and Q_2) Ions of *m/z* Applied for the GC–MS Quantitation of Fecal Sterols

Target compound	T	Q1	Q2
Perylene	252	253	250
COP	370	215	355
CHL	329	368	353
DCHL	215	445	355
SROL	255	394	484
β-SIT	357	396	381
SNOL	215	473	383
Perylene-d_{12} (IS)	264	260	132

ponents and that of IS was 80 ng in 1-µL injected volume. The calibration curves were obtained by plotting the target compound–IS response ratios of characteristic ions selected from the mass spectra as a function of the respective concentrations of the individual components. The target (T) and qualifier ions of *m/z* (Q_1 and Q_2) applied for the GC–MS quantitation are shown in Table I. The identity of the target sterols was examined by comparing the obtained mass spectra with those of the corresponding reference substances using the HP 5973 MSD reference collection, NIST MS search program, and spectrald database collection, Version 1.5 (1996) (Hewlett-Packard).

Precision studies

The within- and between-day precisions were determined at low, medium, and high concentrations that spanned the linearity range using 20 replicate chromatograms (39). Standards of the sterols and surrogate were dissolved in dichloromethane to obtain solutions at concentrations of 80, 160, and 320 µg/mL. At each concentration, 20- × 125-µL aliquots of these solutions and 20 µL of IS were pipetted into autosampler vials and dried under a nitrogen flow. The residues were dissolved in 125 µL of dichloromethane and mixed with 125 µL of BSTFA–TMCS, and they were then prepared for GC–MS analysis as detailed in the derivatization procedure. Eight vials were inserted into the autosampler, and the remaining were stored at –20°C until GC–MS analysis. The within- and between-day precision were expressed in percent relative standard deviation [coefficient of variation (CV)].

Recovery studies

The extraction efficiency (recovery) was determined at three different concentrations, adjusted by adding known amounts of COP, CHL, DCHL, SROL, β-SIT, STOL, and perylene (surrogate standard) to a surface water sample. The standards were dissolved in dichloromethane to obtain a 160-µg/mL stock solution, and 62.5-, 125.0-, and 250.0-µL aliquots of this were mixed with 250 mL of surface water and 10 g of NaOH (three replicates at each concentration). The spiked water samples were shaken in an incubator at 250 rpm/min at 60°C for 1 h. After transferring to a separatory funnel, the samples were vigorously shaken with 3 × 50 mL of dichloromethane. The dichloromethane extracts were completely dried in a Rotavapor (Büchi, Flawil, Switzerland), and the residues were washed into autosampler vials with 4 × 0.5 mL of dichloromethane and mixed with 20 µL of IS. The mixtures were then evaporated to dryness under a nitrogen flow and reconstituted in 125 µL of dichloromethane, mixed with 125 µL of BSTFA–TMCS, derivatized, and analyzed by GC–MS as described previously. The recovery was calculated by comparing the amounts of analytes obtained from the extracted water samples with those measured for the corresponding sterols dissolved in dichloromethane (39) and analyzed directly.

LOD and LOQ

The LOD and LOQ were determined by adding 4.2-, 8.3-, 16.6-, 31.3-, and 62.5-µL aliquots of a 160-µg/mL stock solution containing the target sterols and perylene to a mixture of 250 mL of surface water and 10 g of NaOH. The spiked water samples (two replicates at each concentration) were then shaken in an incubator at 250 rpm at 60°C for 1 h and extracted, derivatized, and analyzed by GC–MS as detailed in the Recovery studies section. The LOD and LOQ were defined as previously described (39).

Stability studies

To characterize the stability of the compounds under typical working conditions, the effect of storage at 4°C and freeze/thaw cycles on the stability of the analytes and surrogate was investigated by adding 62.5-, 125-, and 250-µL aliquots of a 160-µg/mL stock solution containing the target sterols and perylene to a mixture of 250 mL of surface water and 10 g of NaOH. The spiked water samples (two replicates at each concentration) were stored at 4°C and –20°C for 3 days, extracted, derivatized, and analyzed by GC–MS daily. The recovery of the compounds was calculated by comparing the concentrations measured on day 0 with those obtained on days 1, 2, and 3.

The system stability test was carried out by replicate injections performed every hour up to 24 h, using a derivatized mixture containing 80 ng of the target compounds (39). The system stability was calculated by comparing the CV values obtained in the test with the corresponding data of the within-day precision (CV_{rel}).

Preparation of water samples for GC–MS analysis

Surface water samples (n = 38) were collected in various regions of Hungary. Raw domestic wastewater samples (n = 10) were purchased from urban sewage treatment plants. The samples were processed for extraction within 24–36 h. Water samples (250 mL) were mixed with 200 µL of 0.125 mg/mL surrogate standard solution and 10 g NaOH. They were then saponified in a shaking incubator at 60°C for 1 h. Following saponification, the surface water samples were extracted, derivatized, and analyzed by GC–MS, as described in the previous sections. The wastewater samples were prepared for GC–MS in the same manner, but they were mixed with 50 mL of dichloromethane and ultrasonicated with a 10-µm amplitude for 10 min before extraction. The concentrations of target sterols and surrogate were calculated on the basis of calibration curves by the data analysis software.

Results

Representative total ion chromatograms of standard sterol mixture, spiked surface water, and sewage water samples are presented in Figure 1. The run time was 22.5 min, and the six sterols,

IS, and surrogate were completely separated. The following retention times were determined: 9.10 min for IS, 9.14 min for surrogate, 9.88 min for COP, 10.48 min for CHL, 10.57 min for DCHL, 11.53 min for SROL, 12.06 min for β-SIT, and 12.17 min for STOL. Spectral analysis of the target compounds showed higher than 90% identity with those of the reference substances in every case.

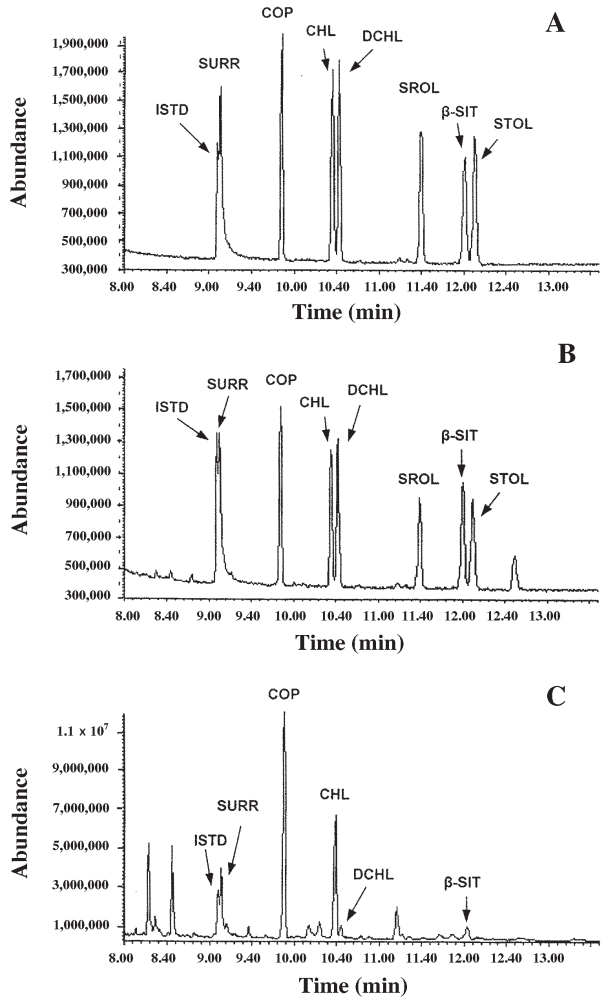
The calibration curves for surrogate, COP, CHL, DCHL, SROL, β-SIT, and SNOL were obtained from three independent series. The target compound–IS response ratios were linear up to 160 ng for each target molecule. The lines of best fit obtained by linear regression were described by the following equations. For COP:

$$y = 3.37x - 0.017 \quad \text{Eq. 1}$$

where $r^2 = 0.993$. For CHL:

$$y = 1.88x - 0.004 \quad \text{Eq. 2}$$

where $r^2 = 0.997$. For DCHL:



Figured 1. Representative chromatograms of the standard sterol mixture, spiked surface water, and domestic wastewater samples. Standard mixture of the target sterols, the internal standard (IS), and surrogate standard at 80 ng mass concentration (A); surface water sample spiked with target sterols (B); and raw wastewater sample from an urban sewage treatment plant (C).

$$y = 2.35x + 0.005 \quad \text{Eq. 3}$$

where $r^2 = 0.997$. For SROL:

$$y = 0.91x + 0.001 \quad \text{Eq. 4}$$

$r^2 = 0.998$. For β-SIT:

$$y = 1.19x - 0.001 \quad \text{Eq. 5}$$

where $r^2 = 0.998$. for SNOL:

$$y = 1.18x + 0.008 \quad \text{Eq. 6}$$

where $r^2 = 0.998$. For the surrogate:

$$y = 20.26x - 0.068 \quad \text{Eq. 7}$$

where $r^2 = 0.986$.

The within- and between-day precision data of repeated GC–MS analyses ($n = 20$) at three different mass concentrations of the target compounds are summarized in Table II. The extraction efficiencies of the six sterols and surrogate are presented in Table III. The recoveries did not change significantly with the concentration, and they were approximately 65% for COP; 75% for CHL, DCHL, and SNOL; 80% for SROL and β-SIT; and 75% for the surrogate. The LOD and LOQ of the six sterols following

Table II. The Within- and Between-Day Precision of the GC–MS Method at Three Different Mass Concentrations of the Target Sterols

	Within-day precision CV (%)			Between-day precision CV (%)		
Target compound	40 ng	80 ng	160 ng	40 ng	80 ng	160 ng
Perylene	2.29	0.68	1.32	6.76	2.18	1.42
COP	6.52	5.38	8.23	8.84	4.96	5.23
CHL	5.05	3.65	6.39	11.12	6.17	3.53
DCHL	2.67	2.69	7.04	7.35	5.37	2.20
SROL	6.39	3.06	5.67	11.35	10.14	5.70
β-SIT	8.89	4.99	7.78	15.55	11.75	8.94
SNOL	3.55	2.20	4.70	7.85	5.84	2.74

Table III. Recovery of the Target Sterols Following Liquid–Liquid Extraction from Native Matrix at Three Different Concentrations*

	Recovery (%)		
Target compound	40 µg/mL	80 µg/mL	160 µg/mL
Perylene	78.0 ± 4.3	75.4 ± 4.2	78.0 ± 3.8
COP	64.3 ± 6.0	64.7 ± 5.9	67.2 ± 10.5
CHL	81.5 ± 9.1	75.4 ± 2.3	75.9 ± 4.4
DCHL	80.0 ± 9.1	76.6 ± 3.8	75.8 ± 4.5
SROL	88.6 ± 6.5	81.5 ± 3.6	78.8 ± 4.2
β-SIT	80.1 ± 7.6	72.0 ± 6.9	79.6 ± 1.6
SNOL	82.7 ± 6.1	75.7 ± 2.9	75.4 ± 4.2

* Each value represents the mean (± standard deviation) obtained from three parallel extractions.

liquid–liquid extraction from native matrix are shown in Table IV. The LOD (at a signal-to-noise ratio of 3:1) were as follows: 5 ng for COP and 10 ng for the remaining five sterols and surrogate, and the LOQ (at a signal-to-noise ratio of 10:1) was 20 ng for each target compound in 1 µL injected volume.

The results of stability studies are demonstrated in Table V. The recovery data show that 20–30% and 30–40% of the original amounts of sterols degraded at each concentrations in the native matrix at 4°C on day 2 and day 3, respectively. In contrast, no decomposition of sterols occurred in spiked surface water samples after three freeze/thaw cycles performed on consecutive days. Our data also indicated that the GC–MS system stability, expressed in CV_{rel}, did not exceed the recommended value of 120% within a 24-h period (39).

Table IV. LOD and LOQ of the Target Sterols Measured by GC–MS Following Liquid–Liquid Extraction from Native Matrix*

Target compound	LOD (ng)	LOQ (ng)
Perylene	10	20
COP	5	20
CHL	10	20
DCHL	10	20
SROL	10	20
β-SIT	10	20
SNOL	10	20

* Each data point shows the mean of two replicate experiments.

Table V. Stability of the Target Compounds in Native Matrix at 4°C at the Three Standard Concentrations Tested*

Target compound	Concentration (µg/mL)	Recovery (%)† Day 1	Day 2	Day 3
Perylene	40	107.6	87.4	60.7
	80	98.4	75.9	73.3
	160	102.5	72.9	71.6
COP	40	108.3	88.0	67.9
	80	103.2	70.5	76.2
	160	83.6	63.7	62.8
CHL	40	104.8	79.3	64.0
	80	103.1	71.9	82.4
	160	93.3	67.0	61.1
DCHL	40	104.8	73.1	60.2
	80	100.2	71.0	79.6
	160	103.5	74.8	69.9
SROL	40	102.3	76.9	55.5
	80	100.7	72.7	81.7
	160	99.8	71.6	66.8
β-SIT	40	104.2	74.8	65.6
	80	97.2	71.8	78.7
	160	106.9	81.0	71.0
SNOL	40	104.7	78.3	65.3
	80	101.2	79.2	84.1
	160	103.8	78.2	71.3

* The results were obtained from two replicate analyses.
† Data are expressed in the percent of the original amounts measured on day 0.

The fecal sterol concentration was measured in 38 surface water and 10 sewage water samples. Their chemical identity was confirmed by spectral analysis, and the mass spectra of the target compounds showed higher than 90% identity with those of reference substances. Fecal sterols were detected in two surface water samples; their amounts were below the LOQ in one case, and the second sample contained 0.032 mg/L DCHL. Figure 2 depicts the total sterol concentration and sterol pattern of the 10 raw domestic wastewater samples analyzed. As shown, the total sterol concentration varied widely, and the following average concentrations of individual sterols were measured: 3.01 ± 1.69 mg/L (0.93–5.36 mg/L) COP; 1.29 ± 0.85 mg/L (0.26–2.93 mg/L) CHL; 0.28 ± 0.24 mg/L (0.1–0.80 mg/L) DCHL; 0.01 ± 0.03 mg/L (0–0.07 mg/L) SROL; 0.23 ± 0.16 mg/L (0.07–0.56 mg/L) β-SIT; and 0.05 ± 0.07 mg/L (0.02–0.18 mg/L) SNOL. The mean total sterol concentration was 4.88 ± 2.77 mg/L, and the samples contained 61.7% ± 9.0% COP, 26.4% ± 10.2% CHL, 6.0% ± 2.4% DCHL, 0.2% ± 0.3% SROL, 4.7% ± 1.6% β-SIT, and 1.0% ± 0.9% SNOL.

Discussion

Besides traditional microbiological methods, fecal pollution has been estimated by GC determination of human and animal sterols in various surface water samples (12–16). A time- and cost-effective approach for the evaluation of contamination levels involves the simultaneous identification and quantitation of COP, CHL, DCHL, SROL, β-SIT, and SNOL, which are the most frequently used biomarkers of fecal pollution (14–16). In several previous studies, these sterols have been quantitated by GC–FID and

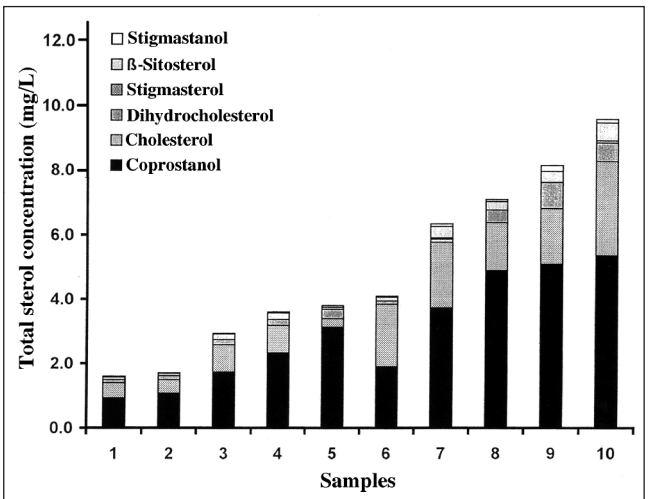


Figure 2. Total sterol concentration and sterol pattern of raw wastewater samples from various urban sewage treatment plants. The samples were prepared for GC–MS analysis as described in the text.

identified separately by GC–MS (32–38). Because the complete validation of the GC–MS method has not been performed, the evaluation and validation of a simple, rapid, and reliable GC–MS analysis that allows the simultaneous identification and quantitation of the previously mentioned sterols and permits the routine environmental monitoring of fecal contamination in aquatic systems is described in the present study.

Fecal sterols were determined both in spiked and native aqueous environmental samples using a modified version of EPA method 8270 that was originally developed for the GC determination of semivolatile compounds. The GC–MS system conditions were optimized with a standard mixture of the six sterols and one surrogate. An HP-5MS capillary column possesses a suitable stationary phase for the separation of highly hydrophobic sterol molecules. In order to improve the volatility, stability, and separation performance, sterols are usually silylated by BSTFA–TMCS, a generally used derivatizing agent (32–38). Besides providing high resolution for the target compounds, reducing the time consumption of the analysis was also an important requirement in the method optimization. After preliminary investigations, the incubation time for derivatization was shortened to 30 min.

The preparation of surface water samples for the GC–MS analysis was carried out by liquid–liquid extraction using dichloromethane as solvent. The accuracy of the assay strongly depends on the exact determination of extraction efficiency (recovery). Therefore, its correct determination had high priority for proper calculation of sterol concentrations. To check the recovery precisely for each sample, a surrogate standard with similar chemical structure and hydrophobic character to that of target sterols was used. Perylene proved to be a suitable substance for this purpose. It was not detected in the surface water and untreated wastewater samples. For quantitative analysis, deuterated perylene (perylene-d_{12}) was used as the internal standard. With it, the calibration curves of the target sterols and surrogate showed good linearity in a wide concentration range with regression coefficients higher than 0.990. Following liquid–liquid extraction, the recoveries were approximately 80% for all of the sterols, with the exception of COP, which had a recovery of 64.3–67.2%. A possible explanation for this difference is the more apolar character of the COP molecule that slightly reduced its solubility in dichloromethane and, therefore, worsened the recovery. The recoveries of the six sterols and surrogate were similar at the three different concentrations tested (see Table III), which allowed the determination of extraction efficiency for each unknown sample by adding a known amount of surrogate solution to the aqueous environmental samples before their preparation for analysis. This quality control feature was incorporated into the assay, and in this manner the quantitative data obtained by GC–MS could be corrected by a proportional factor to calculate the original concentration of the sterols in surface and sewage waters. The IS was added in the last step of sample preparation (prior to derivatization). In this way, concentration/dilution procedures could be carried out before the quantitative determination, and, consequently, the analytical range could also be extended, improving both the cost- and time-effectiveness of the GC–MS method.

The stability studies indicated that the sterols in surface water samples begin to degrade after 24 h of refrigerated storage, but three freeze/thaw cycles could be performed without their decomposition. This seems to be a significant concern to other laboratories because environmental samples are usually collected over a 1- or 2-day sampling trip, spend at least a day in transit, and may wait another day or more before analysis. In this case, it is recommended to freeze the samples after collection because the results show that sterols are stable to freeze/thaw cycles. The high stability of the GC–MS system and the simple and fast sample preparation allowed the GC–MS analysis of a large number of samples, as usually preferred or needed in routine methods for environmental monitoring.

Finally, the method was applied to quantitative determination of six fecal sterols in surface water and untreated domestic wastewater samples. The chemical identity of the sterols could be confirmed by spectral analysis, and the mass spectra showed higher than 90% identity with those of the reference compounds. Quantitative determination was also specific because chemically characteristic target and qualifier ions were selected from the mass spectra of the individual sterols and surrogate. Although 38 surface water samples have been analyzed, sterols were detected only in two cases, indicating that fresh fecal contamination did not occur in the majority of Hungarian surface waters examined. The total sterol concentration of raw domestic wastewater samples varied in a wide range, and the average distribution of individual sterol components showed a very similar pattern to that of in human feces (22).

In conclusion, the cost- and time-effectiveness, technical advantages, and good precision and recovery make this GC–MS analysis suitable for routine environmental monitoring of fecal pollution in aquatic systems.

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