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Short communication

Faecal sterols determination in wastewater and surface water

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Abstract

A simplified method to detect faecal sterols, as an alternative assessment of environmental faecal pollution is proposed. The aim of this study is the development of a method to determine sterols in water samples avoiding sample filtration through glass fibre filter. The method is based on a liquid–liquid extraction and a final GC–FID determination. The quantified sterols are coprostanol and 24-ethylcoprostanol, while 5α-cholestane is used as internal standard. The recovery of coprostanol and 24-ethylcoprostanol in wastewater ranges from 90 to 100% and the detection limit is 1–2 $\mu g\,l^{-1}$. Moreover the method proved to be useful for the sterols determination in surface water too.
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Keywords: Sterols; Faecal pollution; Coprostanol; 24-Ethylcoprostanol; Wastewater; Surface water

1. Introduction

Municipal wastes are often discharged into aquatic environments so that faecal pollution, caused by human and animal wastes, can lead to the deterioration of these environments compromising their employments: drinking water supply, recreational contact, shellfish or fish culturing and irrigation [1]. Faecal contamination has been traditionally measured using thermo-tolerant coliform bacteria, enterococci and *Clostridium perfringens* spores [2], but this approach has several shortcomings mostly related to the utilized analytical methods [3] and to the inability of these microrganisms to distinguish the origin of the faecal pollution [4]. Faecal sterols determination has been proposed as an alternative assessment of environmental faecal pollution indeed sterols are widely used as biomarkers for faecal contamination in sediments, surface waters, wastewaters and urine [5–9]. Coprostanol is produced by the microbial reduction of cholesterol in the digestive systems of higher animals [9], it is the major human faecal sterol and it constitutes about 60% of the total sterols found in human faeces while the principal faecal biomarker in herbivores is 24-ethylcoprostanol [4]. The most utilized detection method is based on a samples filtration through a glass fibre filter which retains the suspended particulate matter in water samples [4]; Nichols et al. [5] have applied this method for measuring faecal-derived sterols in storm water and sea surface micro layer, and they pre-concentrated 100 l of the sample before analyses. Also Mudge and Duce [10] have utilized this method in order to identify the source, transport path and sinks of sewage derived organic matter in the Ria Formosa Lagoon and they sampled 4 l of seawater for sterol determination. Jayasinghe et al. [11] have utilized a different method in order to extract and to detect sterols in environmental water samples: in this study the particle-associated sterols were extracted onto glass fibre filters and then the filters were supercritical fluid extracted and derivatized for gas chromatographic electron capture detection. Isobe et al. [12] filtered the water samples through a prebaked glass fibre filter and then it was ultrasonically extracted by 30 ml each of methanol, methanol/dichloromethane (1:1, v/v) and dichloromethane, consecutively. Suprihatin et al. [13] have filtered 5 l of water samples through glass fibre filters. The extraction was conducted using a modification of the method of Bligh and Dyer [14] and than the steroid contents of the silylated samples was determined using a gas chromatograph with mass spectrometer. Shah et al. [15] proposed an efficient diethyl ether-based soxhlet protocol to quantify faecal sterols from catchment waters after sample filtration through glass fibre filters. Ottoson and Stenström [7] have used the filtration method in order to evaluate the faecal contamination of grey water (5 l sample) in a local treatment system in Sweden. In all these studies the methods implies glass fibre filters and large water volumes. The sterols are retained by this kind of filter

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because they are bound to particulate matter but if particles are too fine, it is probable that, they could pass through the filters. Piocos and de la Cruz [16] developed a high performance liquid chromatography analysis with photodiode array detection and a SPE procedure to extract coprostanol, caffeine and urobiline in water. Cathum and Sabik [17] extracted coprostanol and steroids from surface water and effluent (1 l sample) by liquid–liquid extraction using dichloromethane. Also in the studies of Peng et al. [18] and of Szúcs et al. [19] the water sample from the Pearl River estuarine and the South China Sea, and from surface water and wastewater sample, respectively, were liquid–liquid extracted with dichloromethane using a separate funnel. Börjesson et al. [6] have determined a number of sterols in urine (sample volume was 100 ml) using a liquid–liquid extraction method with non-chlorinated solvents and they found it useful for their purpose. Since urine is assimilable to a liquid with a low particulate concentration, the aim of this study was to develop a method for sterols detection in water samples avoiding the filtration procedure and than to quantify coprostanol and 24-ethylcoprostanol in samples from a wastewater treatment plant (WWTP) and also from a surface water (Po river) to investigate the method applicability on different water samples.

2. Experimental

2.1. Chemicals

The following standards were used for calibration: 5α-cholestane (internal standard, I.S.), 5β-cholestan-3β-ol 98% (coprostanol) and 24-ethyl-5β-cholestan-3β-ol (24-ethylcoprostanol). GC hexane, chloroform and methanol were used for extraction and clean up; for the sterols derivatization we utilised *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). All chemicals were purchased from Sigma, USA. High purity water was prepared by a MillyQ Academic water purification system (Millipore, USA).

2.2. Solutions

Three stocks solutions of 5α-cholestane, coprostanol and 24-ethylcoprostanol diluted in a mixture of hexane–chloroform (4:1, v/v) were prepared at the concentration of $1\,g\,l^{-1}$. From these stock solutions were prepared other two solutions in acetone (one for the internal standard and the other for the calibration standards) at the concentration of $100\,mg\,l^{-1}$. All these solutions were stored at 4 °C for at least 1 month.

2.3. Samples collection

The WWTP is situated in northern Italy, it receives domestic and industrial discharges and has a capacity of 2,100,000 inhabitants equivalent. The average daily volume of wastewater is $550{,}000\,m^3$. Flow proportional 24 h composite samples of influent were collected, divided into five 1 l aliquots and stored in brown glass flasks at 4 °C for the sterols analysis. Grab surface water samples were collected from the Po river (the longest in Italy), divided into five 1 l aliquots and stored in brown glass flasks at 4 °C for the sterols analysis.

2.4. Samples preparation

All the glassware was rinsed with chloroform prior to use and every time a laboratory blank and a calibration curve were analysed with the samples. A 250 ml volume of wastewater or surface water sample was transferred into a separation funnel together with 2.5 g of NaCl in order to improve the separation of the two phases, than 125 μl of I.S. were added. A 50 ml volume of methanol and a 25 ml of hexane–chloroform (4:1, v/v) were added and the mixture was shaken for 30 min on a wrist action shaker at 600 rpm, after 15 min the lower aqueous phase was saved in a glass bottle and the upper organic phase was collected in a flat-bottom boiling flask. The remaining sample was transferred into the separating funnel with another 25 ml volume of hexane–chloroform (4:1, v/v). The extraction procedure was repeated and the pooled hexane–chloroform phase was evaporated under vacuum. The sample was saponified with 25 ml of 1 M potassium hydroxide in 96% ethanol at 80 °C for 90 min. After cooling at room temperature, 15 ml of water was added and the sterols were extracted twice in a separating funnel with 20 ml hexane–chloroform (4:1, v/v). The pooled organic phase was evaporated under vacuum and then re-suspended in 2 ml hexane–chloroform (4:1, v/v) and transferred into a glass tube. The sample was evaporated under a gentle stream of nitrogen. Sterols were converted to their corresponding trimethylsilyl (TMS) ethers by treatment with 100 μl of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The silylation was carried out at 60 °C for 1 h. Finally samples were evaporated under a gentle stream of nitrogen to dryness and were re-suspended in 250 μl of hexane–chloroform (4:1, v/v) before analysis.

2.5. Instrumentation and GC–FID procedure

GC analyses were performed with a Carlo Erba HRGC 5300 mega series (Carlo Erba Instruments, Milano, Italy), equipped with a 50 m × 0.323 mm I.D. (0.17 μm film thickness) capillary column J&W Scientific Inc. HP-1 (Agilent Technologies, CA, USA), a flame ionisation detector (FID) and a split/splitless injector (Carlo Erba Instruments, Milano, Italy). One microlitre of the samples extract (250 μl) was injected in the splitless mode at 200 °C and after 10 min the oven temperature was raised to 250 °C at $10\,°C\,min^{-1}$ and then to 310 °C at $4\,°C\,min^{-1}$ for 3 min. Hydrogen ultra-pure (99.998%) was used as a carrier gas and the inlet pressure was 70 kPa. Peak areas were obtained from the chromatograms acquired by the data-handling program Chrom-Card for Windows 1.19 (Carlo Erba Instruments, Milano, Italy). Sterols identification in the samples was based on comparison of their retention times with the standards. The relative standard deviation for five replicate injections of a water blank sample was 4.5%.

2.6. Quality assurance and quality control (QA–QC)

The reproducibility of the sterol analyses was examined through five replicate assays of a wastewater sample spiked with a mixture of sterols containing 25 μg of each sterol. Recovery

was tested in the same way and was calculated by comparing the amounts of analytes obtained from the extracted samples with those measured for the corresponding sterols dissolved in hexane–chloroform (4:1, v/v). The standard curve consisted of deionised water spiked with 3.12, 6.25, 12.5, 25, 50, 100 μg of each sterol. The water blank sample, which consisted of deionised water, reagents and internal standard, and the standard curve were run parallel to the other samples. The detection limit was determined by five repeated analyses of sterols at low concentration.

2.7. Statistical analyses

Statistical analysis was performed using the statistical program SPSS for Windows (version 12.0).

3. Results and discussion

3.1. Chromatographic separation

Fig. 1 shows representative chromatograms of the evaluated sterols in this study (a blank sample, a laboratory standard, a surface water and a wastewater sample); the retention times of the corresponding TMS ethers are 19.14 min (RSD = 3.8%), 22.23 min (RSD = 4.4%) and 26.38 min (RSD = 5.3%) for 5α-cholestane, coprostanolo and 24-ethylcoprostanol respectively. The excellent separation of the coprostanol and 24-ethylcoprostanol peaks allowed for quantification by simply measuring the peak areas.

3.2. Method validation

The detection limit was determined by repeated analyses of sterols at low concentration and it was 1–2 $\mu g\, l^{-1}$ while the quantification limit was 6 $\mu g\, l^{-1}$.

A linearity test was made using standard sterols added to wastewater samples. The linearity was good at concentrations ranged from 2 to 100 $\mu g\, l^{-1}$ both for coprostanol ($y = 236{,}255x$, $r = 0.998$, $p < 0.01$) and 24-ethylcoprostanol ($y = 134{,}958x$, $r = 0.964$, $p < 0.05$). The three sterols were added to wastewater sample and extracted with the described method; the mean recovery was between 90 and 100% and the coefficient variation was 2.11, 3.47 and 4.11% for 5α-cholestane, coprostanol and 24-ethylcoprostanol, respectively. Recovery data, standard

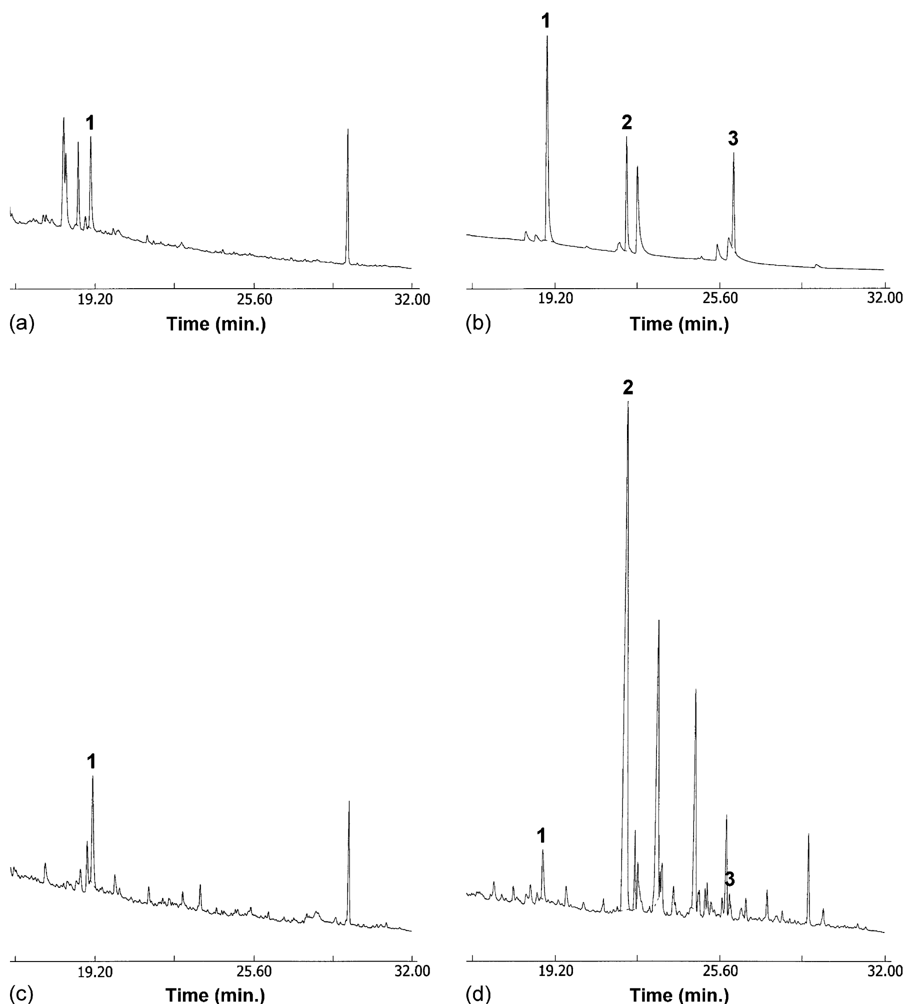

Fig. 1. Sterols chromatograms: (a) blank sample; (b) standard mix; (c) surface sample; and (d) wastewater sample. 1 = 5α-cholestane, 2 = coprostanol, 3 = 24-ethylcoprostanol.

Table 1
Percentage recovery data, standard deviations (SD) and coefficient variations (C.V.) of sterols added[a] to wastewater

Sample	5α-Cholestane	Coprostanol	24-Ethylcoprostanol
1	93	95	101
2	90	100	92
3	95	102	94
4	90	100	100
5	91	93	102
Mean	92	98	98
SD	1.94	3.41	4.02
C.V.	2.11	3.47	4.11

[a] 25 μg l^{-1} of each sterol was added to the samples.

deviation (SD) and coefficient variation (C.V.) for all sterols considered are shown in Table 1.

In order to check the method sensitivity, one wastewater sample was divided into ten 500 ml aliquots and stored in brown glass flasks at 4 °C for the sterol analyses. All the aliquots were analysed at the Department of Public Health and Microbiology, University of Torino, Italy but five aliquots (1–5) were extracted and analysed by two technicians and the other five aliquots (6–10) by other two different technicians. Table 2 shows the quantitative results obtained for the internal standard, coprostanol and 24-ethylcoprostanol.

Data reported in Tables 1 and 2 underline the good repeatability and reproducibility of this method moreover the differences between the sterols means concentration obtained by the two different groups of analysts were not statistically significant.

Cathum and Sabik [17] evaluated the concentration of steroids and coprostanol in effluent using a liquid–liquid extraction method and gas chromatography–mass spectrometry. They found only coprostanol at concentration of 14.67 μg l^{-1}. Isobe et al. [20] evaluated coprostanol concentration of influent and effluent sample taken from five sewage treatment plants located in the Tokyo metropolitan area. They applied a method based on water sample filtration onto pre-baked glass fibre filters and on sterols ultrasonic extraction with three different solvents. They found a mean coprostanol concentration of 327 μg l^{-1} in influent samples and of 1.5 μg l^{-1} in effluent samples.

Ottoson and Stenström [7] studied the faecal contamination of grey water for reuse. They used the method based on sample filtration through glass fibre filter and they found that coprostanol range between 3.1 and 14.9 μg l^{-1}. Szúcs et al. [19] validated a simple and rapid GC–MS method for the simultaneous identification and quantitation of the most frequently measured faecal sterols. The recovery was 65–80%. They analysed 10 raw domestic wastewater samples, the average coprostanol and 24-ethylcoprostanol concentrations was 3.01 ± 1.69 mg l^{-1} and 0.05 ± 0.07 mg l^{-1}, respectively. As shown in Table 2, we found a mean coprostanol concentration of 34.3 and 33.4 μg l^{-1} in a wastewater sample that are lower in comparison with Isobe et al. [20] and are similar in comparison with Cathum and Sabik [17]; the differences are probably due to different location, plant characteristics, considered areas and different methods applied.

Table 2
Comparison of the concentrations (μg l^{-1}) of the three sterols in a wastewater sample divided into ten aliquots and analysed by two different pair of technicians (group 1 and 2)

Sample	5α-Cholestane[a]	Coprostanol	24-Ethylcoprostanol
Analysed by group 1 (μg l^{-1})			
1	11.6	35.2	68.1
2	11.3	33.6	65.7
3	12.1	36.8	67.4
4	11.8	32.5	63.9
5	12.0	33.3	70.2
Mean	11.8	34.3	67.1
SD	0.3	1.7	2.4
C.V.	2.7	5.0	3.6
Analysed by group 2 (μg l^{-1})			
6	12.2	30.7	69.4
7	10.9	36.2	63.2
8	11.7	34.9	72.7
9	11.4	33.5	65.9
10	11.1	31.8	67.6
Mean	11.5	33.4	67.8
SD	0.5	2.2	3.6
C.V.	4.5	6.7	5.3

[a] 12.5 μg l^{-1} of I.S. added to the ten aliquots of the wastewater sample.

3.3. Method application on surface water

In order to check the applicability of the method on a sample with a low particle concentration, one surface water sample (Po river) was analysed. The five aliquots were analysed with the method described above and the results obtained were shown in Table 3. The mean coprostanol concentration was 8.7 μg l^{-1} while the 24-ethylcoprostanol concentration was below the instrumental detection limit. The coefficient variation was 3.94% for coprostanol. Leeming and Nichols [21] used the filtration method for coprostanol determination in water samples from Derwent estuary in Australia and its concentration ranged between 7 and 954 ng l^{-1}. Noblet et al. [8] evaluated coprostanol and other faecal sterol in water samples of Santa Ana River in California (USA). They filtered the surface water samples through pure glass filters and the sterols were extracted using supercritical fluid extraction. In this river the mean coprostanol concentration was 5 ng l^{-1}. Isobe et al. [20] studied the coprostanol concentration in water samples

Table 3
Sterols concentrations (μg l^{-1}) in a surface water sample (Po river) divided into five 1 l aliquots

Sample	5α-Cholestane[a]	Coprostanol	24-Ethylcoprostanol
1	12.3	9.0	<1
2	11.5	8.8	<1
3	12.1	9.2	<1
4	11.2	8.3	<1
5	12.0	8.4	<1
Mean	11.8	8.7	n.d.
SD	0.41	0.34	n.d.
C.V.	3.44	3.94	n.d.

n.d. = not determined.
[a] 12.5 μg l^{-1} of I.S. added to the five aliquots of the surface water sample.

from the Mekong delta in Vietnam. They applied a method based on sample filtration and on sterol ultrasonic extraction with three different solvents. They observed significant differences in coprostanol concentration in different seasons. The highest concentration of coprostanol were observed during dry season (0.001–97.1 $\mu g\,l^{-1}$) followed by the wet season (<0.001–13.5 $\mu g\,l^{-1}$). Shah et al. [15] conducted a study to evaluate a diethyl ether-based soxhlet extraction procedure for faecal sterols occurring from catchment waters. Moreover they compared this method with the Bligh and Dyer [14] chloroform extraction process. Their results suggested that the diethyl ether-based soxhlet extraction method was more efficient and reproducible than the Bligh and Dyer [14] one, but they filtered large volume of water (2.2–18.5 l). They found that coprostanol concentration ranged from 0 to 15.83 $\mu g\,l^{-1}$ and 24-ethylcoprostanol concentration ranged from 0.03 to 0.31 $\mu g\,l^{-1}$. Szúcs et al. [19] analysed 38 surface water samples, they detected faecal sterols in two samples, but coprostanol and 24-ethylcoprostanol were below the limit of quantification. The coprostanol concentrations in surface water are variable in the reported studies and our data are comparable with the concentration found by Isobe et al. [20] in wet season and by Shah et al. [15]. In conclusion our proposed method, based on liquid–liquid extraction of sterols from water samples has good extraction efficiency, repeatability and reproducibility that are similar to the other method ones [4,6,14]. This method, which requires low water volumes, is demonstrated to be useful for the sterol analyses in different water samples such as wastewater and surface water, but it is particularly indicated for samples with low concentration of suspended particle. On the bases of our positive results, in future we would suggest to plan monthly samplings of wastewater (influent and effluent), surface and also drinking water during all the year in order to asses the sterols concentration variability and their usefulness as faecal indicator.

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