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Application of pentafluorophenyldimethylsilyl derivatization for gas chromatography–electron-capture detection of supercritically extracted sterols

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Abstract

We report a convenient method of extracting and detecting sterols in environmental water samples. Particle-associated sterols were extracted onto glass-fibre filters then the filters were supercritical fluid extracted (at 33 MPa, 80°C for 30 min) followed by pentafluorophenyldimethylsilyl (flophemesyl) derivatization for gas chromatographic–electron-capture detection (GC–ECD). Optimal derivatization of epicoprostanol, coprostanol, cholesterol, dihydrocholesterol, stigmasterol, β-sitosterol and stigmastanol with neat flophemesyl chloride was achieved under mild conditions (i.e., room temperature, 15 min) without the need for added solvent. These sterols gave linear GC–ECD calibrations ($r^2 \geq 0.99$) while detection limits in the final solutions were 0.1–0.6 μg/ml. Overall recoveries for the supercritical extraction and derivatization steps were 80–110% (mean 92%). The procedure was applied to raw sewage and to river water samples and 0–55%-higher results were obtained over those achieved by modified Bligh and Dyer extraction. Flophemesyl derivatization of sterols allows selective ECD, improved separation from other compounds such as polychlorinatedbiphenyls that may be found in environmental samples, and reduced clean-up requirements (e.g., removing lipids by saponification which may be needed when flame ionization detection is used). Characteristic mass spectra of the flophemesyl derivatives are reported; these were obtained in order to distinguish between sterol classes and individual sterols. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Water analysis; Environmental analysis; Derivatization, GC; Sample preparation; Sterols; Flophemesyl chloride

1. Introduction

Coprostanol and other related sterols have been used to detect sewage contamination in environmental samples [1–5]. Sterols have also been used in geochemical studies as biogeochemical markers to identify the types of vegetation in ancient samples [6] and archaeological studies to identify latrines in historic sites [7]. This present analytical method may also provide an improved procedure for such studies.

Usually, sterols are solvent extracted, converted to their trimethylsilyl (TMS) ether derivatives and analyzed by gas chromatography (GC) with flame ionization detection (FID) for routine analysis and GC with mass spectrometric (MS) detection for structure elucidation [5]; on some occasions GC–MS has been used as a routine tool [7]. However, prior to GC–flame ionization detection (FID) analysis, clean-up of the extract is required in order to avoid any

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interfering peaks that are possible in the region of sterol peaks on the GC trace. This is usually achieved by saponification of the extract to remove lipids by conversion into their corresponding fatty acids, followed by extraction of sterols into hexane [8]. With trace analysis, especially when the analyte solution is concentrated in order to enhance the limit of detection, it is laborious to obtain GC–FID traces which are background peak free since FID is not selective and will respond to all co-extracted hydrocarbons.

Pentafluorophenyldimethylsilyl (flophemesyl) derivatization has previously been demonstrated for cholesterol and some related steroids [9,10]. This permits the use of GC–electron-capture detection (ECD) analysis, which also offers improved sensitivity, allowing smaller sample sizes to be analyzed and/or reduced extent of preconcentration to be used.

In order to avoid the saponification step and subsequent extractions required in sample work-up for FID, we have studied the use of supercritical fluid extraction (SFE) as an alternative preparation procedure. Nguyen et al. [11] demonstrated extraction of sterols from sewage sludges by in-situ TMS derivatization with static extraction followed by dynamic carbon dioxide flow. Rayner et al. [12] used carbon dioxide and a co-solvent of 7.5% ethanol–water (20:1) to extract coprostanol from sewage effluent.

This present study was initiated to investigate analysis of sterols by using flophemesyl derivatization and so exploit the above advantages arising from selective, sensitive detection. This approach, combined with SFE of sterols from sewage-impacted matrices has not previously been reported.

2. Experimental

2.1. Materials

Pesticide grade solvents (Merck) were used throughout and all glassware was detergent-washed, rinsed with water followed by distilled water and rinsed with solvent prior to use. Internal standard (I.S.), 1-chloro-9,10-diphenylanthracene, and sterol standards epicoprostanol (EPI), cholesterol (CHL), dihydrocholesterol (DHC), stigmasterol (SROL), β-sitosterol (β-SIT) and stigmastanol (SNOL) were purchased from Aldrich (Sigma–Aldrich, Castle Hill, Australia). Coprostanol (COP) was purchased from Matreya (Matreya, Pleasent Gap, USA). Pentafluorophenyldimethylsilylchloride was purchased from Acros (Acros Organics, Janssen Pharmaceuticals, Belgium) or Aldrich. Hydromatrix was purchased from Varian (Harbor City, CA, USA).

2.2. Standard solutions

Stock solutions (100 μg/ml) of individual sterols or mixtures were prepared in hexane–chloroform (4:1). These stocks were diluted to 0.5 to 5 μg/ml in hexane–chloroform or in anhydrous hexane. The I.S. was prepared in anhydrous hexane.

2.3. Sample filtration and spikes

Aliquots of sample (20 ml to 2 l) were vacuum filtered through the glass fibre filters (Advantec Toyo GF 75–47 mm) (Bonnet Equipment, Jannali, Australia). Where appropriate filters were then spiked with an aliquot of individual or mixed sterol standard and the solvent allowed to evaporate. Each filter was then cut into ca. 10 strips and subjected to supercritical extraction.

2.4. Supercritical fluid extraction

A Dionex Model 703 (Dionex, USA) SFE extractor with 10 ml stainless steel extraction cells was used. This instrument has eight horizontal extraction cells and allows only dynamic flow at a pre-set fluid flow-rate of ~1 ml/min. SFC-grade carbon dioxide (BOC Gases Australia) was used for extraction. Restrictors used had a typical flow-rate of ~500 ml/min supercritical fluid and were heated to 150°C during extraction. Oven temperature was maintained at 80°C and the pump pressure was at 33 MPa. Collection was into empty vials at temperatures from −5°C to 0°C. No co-solvent was added since nearly 100% recovery was obtained under these conditions (optimization studies will be published elsewhere).

Typically 30 min extractions of sterols from glass fibre filters were achieved as follows. The downstream end of each extraction cell was fitted with a

glass fibre filter disc and then packed with ca. 1 ml of glass-wool. The cell was then 1/3 filled with Celite, ca. 1 g of hydromatrix was added followed by the sample (consisting of glass fibre filter pieces mixed with Celite) and a further 1 g of hydromatrix. The cell was then filled with Celite, capped with a plug of glass wool and fitted with a filter disc.

2.5. Derivatization

Standard solutions (800 μl) were added into GC vials and gently evaporated to dryness with nitrogen. To collection vials containing supercritical fluid extracted sterols was added 1000 μl hexane–chloroform (4:1), 800 μl was taken out into a GC vial and evaporated to dryness to remove any moisture.

Five μl of neat flophemesyl chloride was then added into each vial and left for 15 min at room temperature with thorough mixing followed by quantitative addition of 20 μl of I.S. in anhydrous hexane. The solution was then evaporated to dryness under a stream of nitrogen, 800 μl of dry hexane added, mixed and analyzed by GC–ECD.

2.6. GC–ECD analysis

GC was carried out on a Shimadzu 14A instrument with Shimadzu AOC 17A auto-injector and CBM-101 Communications Bus Module (Shimadzu Scientific Instruments, Rydelmere, Australia). Data were acquired with Shimadzu Class LC10 software.

Carrier gas was helium at a pressure of 111 kPa, detector make up gas was methane–argon (5:95) at a pressure of 59 kPa. ECD temperature was 320°C and injector temperature was 300°C, except where otherwise stated. A BPX5 capillary column (25 m×0.22 mm I.D., film thickness 0.25 μm) (SGE International, Ringwood, Australia) was used for sterol separation with a column temperature program of 50°C held for 1 min then programmed at 30°C/min up to 300°C, with hold time of 40 min (total run time of ca. 49 min). Analysis was done under splitless (for 0.75 min) conditions. The range and current for the detector were both at 1. Injection volumes were 1 μl. The system was passified by chromatographing up to six injections of the blank. It took more than 8 h to saturate the injector liner with silanization agent, after which the response to a sterol standard became constant.

2.7. GC–MS analysis

GC–MS analysis was performed on a Hewlett-Packard 5890A gas chromatograph coupled with a Hewlett-Packard 5970 Series mass spectrometer equipped with the same type of column as for the GC–ECD study and the same temperature program was used except the split open time was 2 min. Helium column head pressure was 78 kPa. GC–MS source temperature, injector temperature and detector temperatures were 250°C, 290°C and 300°C, respectively and the mass-selective detector was operated in scanning mode with electron-impact ionization (70 eV). To obtain library spectra for the sterol derivatives in scanning mode, 1000 μg/ml solutions were prepared. One μl injections were made. Data analysis was by HP G1034C version C.3.0 software.

2.8. GC–ECD calibration curves

Two calibration curves were prepared: one for mixed sterol standards (0.5 to 5 μg/ml in hexane–chloroform, 4:1) which had been directly derivatized and analyzed by GC–ECD and another for mixed sterol standards that had been spiked onto blank filter papers, supercritical fluid extracted and then derivatized. Triplicate analyses were conducted. I.S., 1-chloro-9,10-diphenylanthracene was added to each solution at 1 μg/ml prior to analysis.

To check whether recovery of a particular sterol was affected by the presence of other sterols, the above procedures were repeated for: (i) coprostanol alone (i.e., a single sterol standard) and (ii) coprostanol in a mixture of the other six sterols in hexane.

2.9. Comparison of SFE with the Bligh and Dyer solvent extraction method

Triplicate aliquots of raw sewage (20 ml) from the inlet to the Werribee Treatment Plant (Melbourne, Victoria, Australia) were vacuum filtered. Each filter was cut into half and each then subjected to either SFE, or modified Bligh and Dyer (B&D) extraction according to Refs. [13,14]. Both types of extract were subjected to flophemesyl derivatization as this

gave an interference free ECD chromatogram in the region of sterol elution. Peak identity and purity were confirmed by GC–MS.

3. Results

3.1. GC–ECD chromatogram of flophemesyl derivatives of sterols

A partial gas chromatogram of the sterol flophemesyl derivatives is presented in Fig. 1. The I.S. (molecular mass 365) elutes before the region of derivatized sterols, which have greater nominal molecular masses (Table 1). The sterol flophemesyl derivatives had longer retention times than the respective TMS derivatives, and showed excellent resolution with baseline separation. The extra retention of these derivatives reduces the possibility of interfering impurities when analyzing environmental samples. Relative ECD mass response factors of the flophemesyl derivatives differ for many of the sterols (Table 1), EPI has the greatest response whilst COP is ~50% and CHL, β-SIT, SNOL are ~25% of that of EPI.

Table 2 shows the precision of the GC–ECD response for five consecutive injections of a 5 μg/ml solution of the sterols. A relative standard deviation (R.S.D.) of 3.1–3.6% was obtained for all sterols for area ratio of sterol/I.S. When data are normalised against coprostanol in each standard, the R.S.D. decreases to 0.5–1.5%. Note that the I.S. was only 0.1 μg/ml in these solutions, and so might have contributed to the higher R.S.D.s for the former data. An I.S. concentration of 1.0 μg/ml was used in subsequent work.

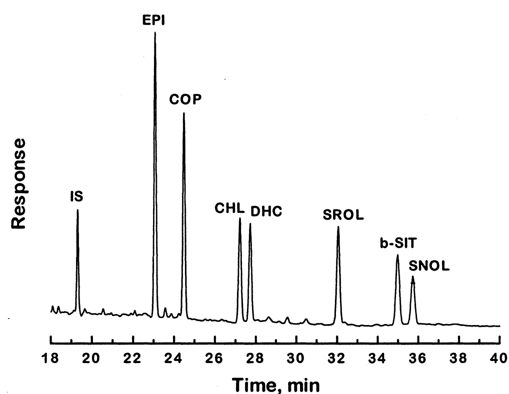

Fig. 1. Partial GC–ECD chromatogram of the internal standard (I.S.) and flophemesyl derivatives of standard sterols. EPI= Epicoprostanol, COP=coprostanol, CHL=cholesterol, DHC= dihydrocholesterol, SROL=stigmasterol, β-SIT=β-sitosterol, SNOL=stigmastanol, I.S.=1-chloro-9,10-diphenyl anthracene at 1 μg/ml. All sterols at 5 μg/ml.

Table 1
Molecular masses of I.S. and flophemesyl derivatives of sterols and relative mass response factors

Sterol	Nominal molecular mass of derivative	*f*
I.S.	365	1.0
EPI	612	5.7
COP	612	2.9
CHL	610	1.4
DHC	612	2.2
SROL	636	1.5
β-SIT	638	1.4
SNOL	640	1.4

f=Relative ECD mass response factor relative to the I.S. Other abbreviations as in Fig. 1.

It was found that the flophemesyl derivatives were stable for at least one week when stored under nitrogen in GC vials at room temperature in the light.

3.2. Optimization of derivatization of sterols with flophemesyl chloride

The sterol derivatization reaction with flophemesyl chloride was optimised for temperature, time, amount of derivatizing reagent and solvent volume (experiments were done in duplicate).

Derivatization was carried out at a number of times over the range 15–60 min and at both room temperature and elevated temperature (65°C), and no significant change in GC peak response was evident. Hence room temperature was adequate for observed maximum recovery of ECD response, and was used in all further studies.

It was found that as little as 5 μl of the derivatizing agent was sufficient for maximum derivative yield for a mixture of 5 μg of each of the tested sterols. From stoichiometry considerations 5 μl of the derivatization reagent should be able to react with ca. 10 mg of sterol assuming quantitative reaction, thus 5 μl of the derivatizing agent is more

Table 2
Peak area/I.S. peak area for seven sterols in five repetitive analyses by GC–ECD

	Area ratio sterol/I.S.			Area ratio sterol/COP		
	Mean	S.D.	R.S.D. (%)	Mean	S.D.	R.S.D. (%)
EPI	53.32	1.72	3.2	1.53	0.0081	0.5
COP	34.82	1.18	3.4	1.00	0.0000	0.0
CHL	29.62	0.97	3.3	0.85	0.0032	0.4
DHC	25.06	0.80	3.2	0.72	0.0094	1.3
SROL	24.63	0.86	3.5	0.71	0.0047	0.7
β-SIT	27.97	1.00	3.6	0.80	0.0091	1.1
SNOL	22.10	0.79	3.6	0.64	0.0090	1.4

S.D.=Standard deviation; R.S.D.=relative standard deviation; I.S.=internal standard (at 0.1 μg/ml).
Note: Area ratio of sterols to I.S. is high due to low concentration of I.S. used.

than adequate for most environmental samples since considerably less than 10 mg of total sterols would be extracted [5].

Increasing the injector temperature gives a small increase in the peak area for each sterol, with about a 20% increase observed from 300 to 320°C, suggesting that better transfer of derivatized sterol into the GC column occurs with higher injector temperature (Fig. 2). However, since a prior study on SFE with GC–ECD analysis used an injector temperature of 300°C to be consistent with this earlier work, it was decided to standardise on an injector temperature of 300°C throughout.

Poole et al. [9] have reported that ECD operation at 350°C is the optimum temperature for flophemesyl derivative detection. The maximum temperature of the ECD in our laboratory is 330°C, and approximately 4–18% increase in the detector response was observed for the sterols tested for a 10°C rise from 320 to 330°C. In this study, a detector temperature of 320°C was used due to system limitations.

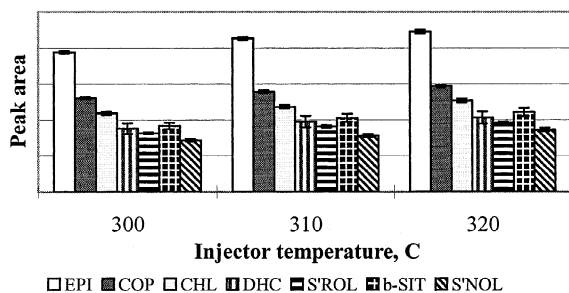

Fig. 2. Effect of injector temperature on ECD response.

3.3. Calibration curve of flophemesyl derivatives of sterols

Calibration curves for sterol standards extracted by SFE and then derivatized to their flophemesyl ethers showed good linearity ($r^2 \geq 0.99$) for the concentration range 0.5–5.0 μg/ml (Table 3). The minimum detection limit (with signal-to-noise ratio of 2:1) was 0.1 μg/ml for all sterols. This was similar to the statistically calculated LOD (limit of detection) as reported in Table 3, following the method of Colina et al. [15]. However, the calculated LOD for DHC, SROL and β-SIT is 0.2 μg/ml and CHL was higher at 0.9 μg/ml.

For standards prepared directly, calibration linearity was also good ($r^2 \geq 0.99$). Table 3 also gives results of the calibration slope ratios of the sterols in the two experiments, reported as (slope of SFE extracted and derivatized sterols)/(slope of directly derivatized sterols). Recoveries according to this ranged from 80–108%, with a mean of 92% (Table 3).

EPI, COP and CHL had significantly higher intercepts than the other sterols (Table 3). Normal blanks and sterol standards each without EPI, COP and CHL for both direct sterol standards and sterol standards through SFE showed there are no peaks at the respective retention times and hence the higher intercepts for these sterol curves are apparently not due to any impurities. This implies there may be curvature in the calibrations.

Recoveries of COP (calculated as described above) after SFE of standards containing COP alone and for COP in a mixture of six other sterols were 107 and

Table 3
Calibration data set ($R=a+bc$) for SFE extracted and derivatized sterol standards

Sterol	a	S_a	b	S_b	$S_{r(b)}$	$S_{R,c}$	Linearity		S	LOD (μg/ml), calculated	Recovery (%), calculated
							r^2	$1-S_{r(b)}$			
EPI	0.3536	0.0307	0.9669	0.0103	0.0107	0.0369	0.9998	0.9893	0.0382	0.1	96.3
COP	0.1624	0.0225	0.6192	0.0076	0.0123	0.0270	0.9997	0.9877	0.0436	0.1	80.2
CHL	0.3891	0.1596	0.5321	0.0538	0.1011	0.1915	0.9799	0.8989	0.3599	0.9	107.6
DHC	0.0641	0.0214	0.3908	0.0072	0.0184	0.0257	0.9993	0.9816	0.0658	0.2	93.0
SROL	−0.0569	0.0398	0.5228	0.0134	0.0256	0.0479	0.9987	0.9744	0.0916	0.2	92.2
β-SIT	−0.0076	0.0283	0.3809	0.0095	0.0249	0.0340	0.9987	0.9751	0.0892	0.2	91.7
SNOL	−0.0119	0.0131	0.2838	0.0044	0.0155	0.0157	0.9995	0.9845	0.0553	0.1	85.5

a=Intercept, S_a=intercept S.D., b=slope, S_b=slope S.D., c=concentration (μg/ml), $S_{R,c}$=regression S.D. of R to c, R=response, r^2=correlation coefficient, LOD=limit of detection=$3(S_{R,c}/b)[(n-2)/(n-1)]^{1/2}$, $S_{r(b)}$ (=S_b/b)=slope R.S.D., $S=S_{R,c}/b$, n=the total number of pairs of points (5), I.S. concentration used=1.0 μg/ml.
Recovery is determined on the basis of the slope difference of these data and that for directly derivatized sterol calibration data.

98%, respectively (recovery taken as the ratio of slope of SFE/COP curve to direct COP derivatized curve; Table 4). In both cases, good linearities were obtained ($r^2 \geq 0.99$). However, the two sets of standards were analyzed on two different days, and there was a variation in the slopes: ~0.4 for COP alone and ~0.6 for COP in a mixture of sterols (Table 4). Earlier work found that when the injector liner of the GC is completely deactivated with the silanization agent the flophemesyl derivative responses increase with time. The above results are consistent with incomplete deactivation when the COP alone samples were analyzed [16].

A comparison was made of GC–ECD results of a 5 μg/ml hexane solution of COP diluted 1:1 with either solvent or a sterol mixture not containing COP. These solutions should yield a response 50% of that of the original COP solution if no interference effect occurs in the presence of a suite of sterols. Responses of 47.2 and 50.2%, respectively were found for the two diluted solutions, showing that the presence of other sterols has little effect on the response of COP.

3.4. Comparison of SFE with B&D solvent extraction

Fig. 3 shows derivatized sterol chromatograms obtained from both SFE extraction and solvent extraction of a water sample. The sterol region is apparently free from interferences and there is no need to saponify or column separate the extracts prior to analysis. Table 5 shows recoveries from both extraction methods with respect to a number of sterols. Recovery is determined by comparing the difference between the spiked and unspiked results with the response obtained for the same concentration sterol in a standard solution. On average, SFE gave better recoveries for the sterols. For the limited data, reproducibility of the SFE method was slightly better. Note that spike recovery can be estimated where the sterols were not found in the unspiked

Table 4
Calibration data for COP standard (std) under different conditions

Sterol		a	S_a	b	S_b	Recovery (%)
COP	std	−0.0217	0.0153	0.3825	0.0063	
COP	std through SFE	0.1165	0.0419	0.4111	0.0173	107
COP in a mixture	std	−0.0019	0.0298	0.6365	0.0011	
COP in a mixture	std through SFE	0.1192	0.0582	0.6216	0.0219	98

Symbols as for Table 3.

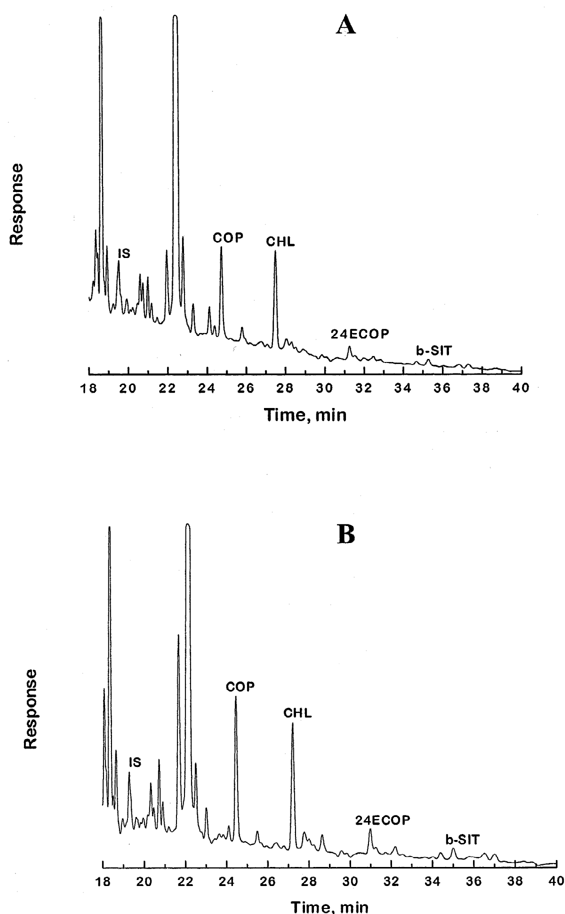

Fig. 3. Partial GC–ECD chromatogram of a field sample extraction. I.S. is at 1 μg/ml. (A) SFE; (B) modified Bligh and Dyer extraction.

sample, but no recovery can be estimated where the sterol was not available for the standard mixture. This comparison illustrates that the two extraction methods agree reasonably well, and therefore the SFE method can be considered to be an alternative procedure for this analysis.

3.5. GC–MS analysis of flophemesyl derivatives of sterols

The flophemesyl derivatives are characterised by ions *m/z* 58 and 77 [17]. Table 6 shows the principal ions detected in the present work. The base peak is the $[M-242]^+$ ion, but in 5α stanols, the base peak is the *m/z* 215 ion. The group, $[C_6F_5Si(CH_3)_2(OH)]$ has a molecular mass of 242 which is eliminated during electron impact. However, the $[M-242]^+$ peak is barely visible in 5α stanols and a peak of $[M-(SC+42)]^+$ ion (where SC=side chain) gives about 17% abundance in these sterols only. The characteristic $[M-(242+15)]^+$ ion is at 10–45% abundance in all sterols. Another distinguishing character of various sterol classes is the $[M-(242+SC)]^+$ ion. In 5α stanols, this is negligible and in 5β stanols it is about 12% abundant. However, in sterols with a double bond at C_5 (Fig. 5), this ion is about 30% of the base peak and in sterols where there are two double bonds at C_5 and C_{22}, this ion is around 55% of the base peak. Another significant ion is *m/z* 215 which is given by the elimination of the SC at C_{17} position (Fig. 5) and fragments of mass 242 and 42 from the molecular ion, [18] and usually 5α

Table 5
Recoveries of sterols from environmental water samples by using SFE and modified Bligh and Dyer (B&D) methods

Sterol	5 μg/ml Std	Unspiked sample Average (±1/2 range %)		Spiked sample Average (±1/2 range %)		Recovery (%)	
		SFE	B&D	SFE	B&D	SFE	B&D
EPI	5.89	ND	ND	6.71 (8.5)	5.97 (12.5)	114	101
COP	2.34	0.64 (2.8)	0.59 (1.8)	3.08 (6.4)	2.91 (7.7)	104	99
CHL	1.83	1.94 (5.6)	1.80 (9.3)	3.51 (7.6)	3.52 (14.4)	85.9	94.1
DHC	1.74	ND	ND	1.91 (7.0)	1.86 (15.1)	110	107
24ECOP[a]	–	0.33 (7.9)	0.30 (9.1)	0.36 (13.3)	0.32 (18.0)	–	–
SROL	1.81	ND	ND	1.85 (8.9)	2.02 (5.2)	102	112
β-SIT	1.57	0.14 (12)	0.15 (16)	1.61 (6.1)	1.56 (5)	94	90
SNOL	1.23	ND	ND	1.35 (13)	1.33 (13)	110	108

Symbols as for Table 3; *n*=2; both unspiked and spiked samples were analyzed.
[a] Note 24ECOP not present in standard, but present in samples. DHC not present in samples.
ND=Not detected. Values quoted are ratios sterol/I.S.

Table 6
Mass spectrometric fragmentation patterns of sterols

Sterol	$M^{\cdot+}$	SC	5α/5β	= Bond	$[M-242]^+$	$[M-(SC+42)]^+$	$[M-(242+15)]^+$	$[M-(242+SC)]^+$	$[M-(242+SC+42)]^+$	$[M-SC]^+$
EPI	612	113	5β		370 (100)	457 (–)	355 (21)	257 (12)	215 (80)	499 (–)
COP	612	113	5β		370 (100)	457 (8)	355 (44)	257 (12)	215 (65)	499 (–)
CHL	610	113		5-en-	368 (100)	455 (1)	353 (52)	255 (30)	213 (25)	497 (2)
DHC	612	113	5α		370 (6)	457 (17)	355 (28)	257 (4)	215 (100)	499 (–)
SROL	636	139	5β	5,22-dien-	394 (10)	455 (–)	379 (10)	255 (56)	213 (22)	497 (4)
β-SIT	638	141		5-en-	396 (100)	455 (1)	381 (44)	255 (30)	213 (25)	497 (3)
SNOL	640	141	5α		398 (6)	457 (16)	383 (23)	257 (5)	215 (100)	499 (–)

$M^{\cdot+}$=molecular ion, $[M-15]^+$=molecular ion−15 Da etc.
Figures in parentheses give size as percentage of base peak (100).

stanols have this as base peak whereas in 5β stanols its abundance is around 65–80%. This m/z 215 is absent CHL, β-SIT and SROL in which there is a double bond at position C_5 (Fig. 5) and the characteristic ion of these compounds is m/z 213 but with an abundance of 25%. A less significant peak of $[M-SC]^+$, at about 2–4% of the base peak, is only visible in sterols with double bonds. In all mass spectra of flophemesyl derivatives of sterols, there is a significant peak at m/z 81 which other authors have assigned to the ion $[Si(CH_3)F_2]^+$ [19]. Fig. 4 shows the MS spectra of the flophemesyl derivatives of sterols used in this study.

4. Discussion

Overall, there are many advantages arising from using SFE in place of conventional solvent extraction method [14] and GC–ECD in place of conventional GC–FID [5]. Conventional solvent extraction methods use organic solvents in large amounts and the extraction is lengthy and laborious. For example, the modified Bligh and Dyer extraction method uses 130 ml of chloroform–methanol mixture for a single extraction and takes two days [14]. In contrast, the SFE method used in this work is clean, convenient, rapid and uses no extracting solvents other than carbon dioxide which may be released directly to the atmosphere. Furthermore, this SFE method has the potential to be ‘‘class selective’’ (at least partially), and avoids steps in clean-up of environmental samples before analysis [20].

Usually, to avoid interfering peaks in the GC–FID chromatograms of environmental samples, a time-consuming saponification step is required to remove any oils and fats remaining in the extract: in some instances, a column separation step is required to remove other hydrocarbons [21]. ECD is selective (fats and oils are not derivatized) and hence there is no need to saponify the extract. All other low-molecular-mass halogenated compounds (e.g., polychlorinated biphenyls) and derivatizable compounds should elute earlier in the chromatogram. Flophemesyl chloride in pyridine derivatizes unhindered secondary hydroxyl groups (e.g., the 3β-OH group of cholesterol) when ketones are first protected [22]. However neat flophemesyl chloride, or flophemesyl chloride with ~10 μl of anhydrous hexane was used in this work. Given the fact that the major sterols found in sewage samples which may be useful in sewage analysis have no keto groups or hydroxyl groups hindered, derivatization with flophemesyl chloride poses no problems. With some environmental samples it was found to be desirable to add hexane to ensure that there was adequate mixing between the sterols and the derivatizing reagent. We found pyridine was not a suitable solvent for flophemesyl derivatization because when pyridine was added to flophemesyl chloride, it gave a white cloud formation and the solution became crystalline especially when evaporated down to low volume. However, Wannagat et al. [23] reported no evidence of *N*-flophemesylpyridinium salt complex formation. Five μl of reagent should give a sufficiently large molar excess to ensure that all the sterols in typical water extracts are derivatized.

TMS ether derivatives of sterols on a comparable capillary column gave coprostanol eluting before epicoprostanol [24] and flophemesyl derivatives give

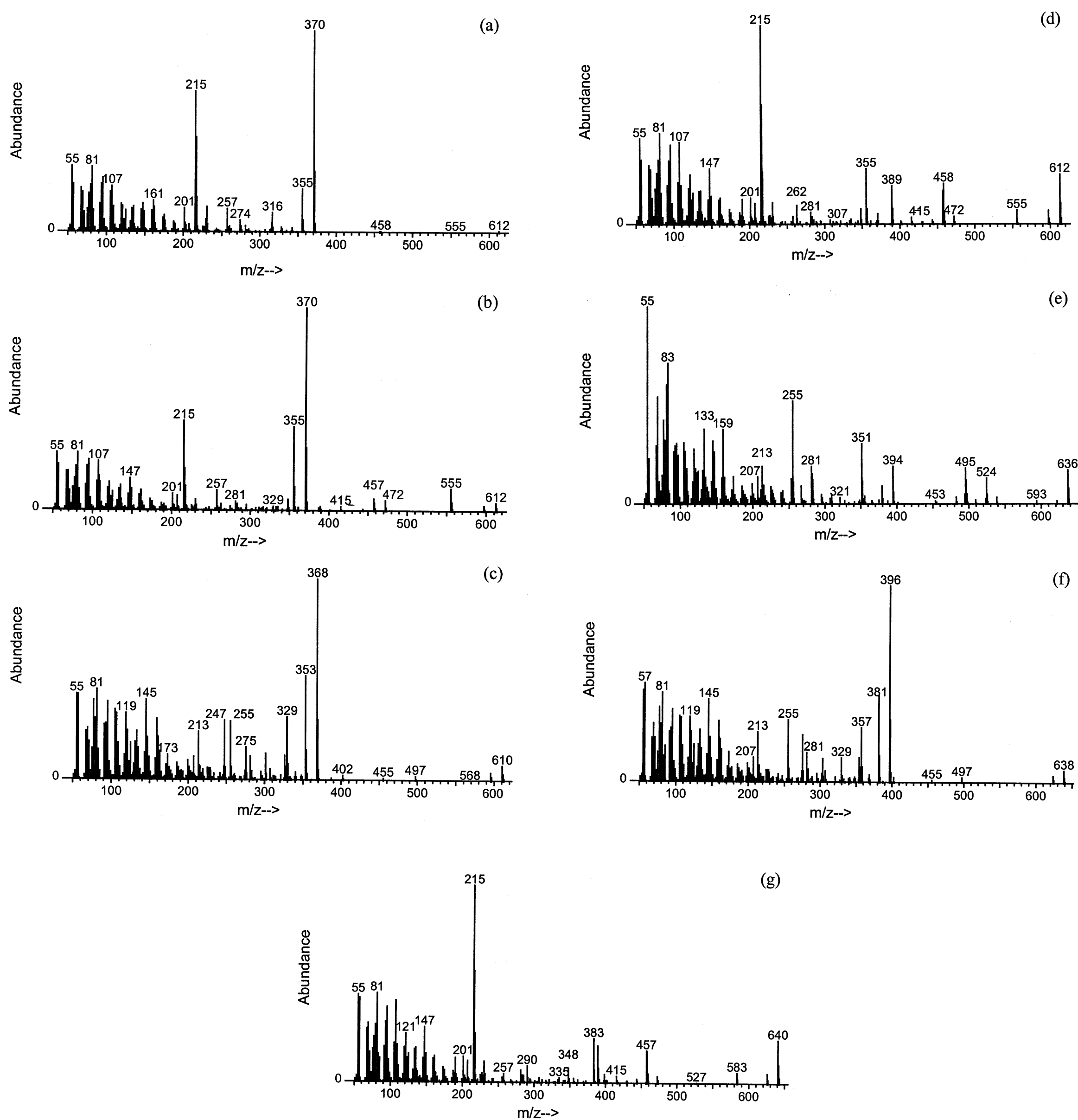

Fig. 4. Mass spectra of the flophemesyl sterol derivatives. (a) Epicoprostanol, (b) coprostanol, (c) cholesterol, (d) dihydrocholesterol, (e) stigmasterol, (f) β-sitosterol, (g) stigmastanol.

elution of these two compounds in reverse order. However, the net separations of the TMS and flophemesyl derivatives do not differ greatly. The LOD for flophemesyl ethers of sterols is of the order of 0.1 μg/ml for injected solutions (0.1 ng injected sterol), at least an order of magnitude less than that of TMS ethers of sterols by FID. This lower LOD is useful in environmental water analysis giving possibilities to collect and filter smaller volumes of water.

The injector liner of the GC should be treated with the flophemesyl reagent (i.e., should be silanized) prior to injection of any sterol samples. If not, due to

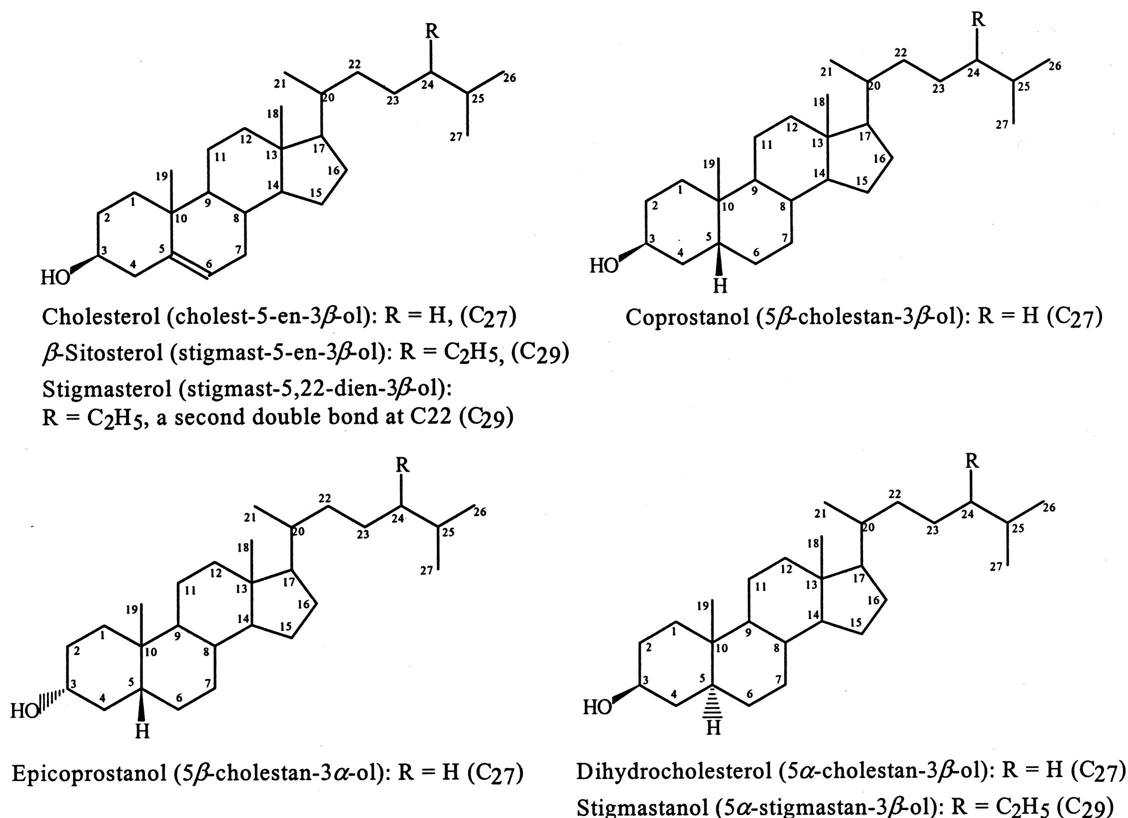

Fig. 5. Structure of sterols used in this study.

the ECD response enhancement over successive injections, it takes about 8–12 h for the ECD responses of sterols to reach plateau. Given this observation, we routinely re-analyze a sterol standard every three or four analyses for quantitative studies.

Mass response factors of the sterols tested vary widely (Table 1). EPI shows about twice the peak area of COP and four times the peak area of other sterols in a 5 μg/ml mixture analysis. Under the conditions tested, it is not evident that any inter-conversion of one sterol to another occurred.

After the derivatization procedure, the derivatization agent could not be evaporated off fully with nitrogen blow down. Additional vacuum has to be applied to fully remove the derivatization agent. However, due to the small amount (5 μl) of the derivatizing agent present in the mixture and its short elution time, it did not pose any problem to the analysis.

Ketones are not derivatized with flophemesyl chloride or *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The latter is the reagent of choice when analyzing sterols as TMS ether derivatives [21]. However, underivatized stanones can be chromatographed together with TMS ethers of sterols with usual GC–FID or GC–MS analysis. Benfenati et al. [25] used *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) to derivatize stanones into their corresponding trimethylsilyl ethers. When targeting analysis for sterols in a complex matrix, flophemesyl derivatization with GC–ECD is a useful approach. Simultaneous analysis for stanones would then require an additional GC–FID finish. Methods of determining sewage contamination in the environment based on the use of sterol to stanone ratios may be difficult with the present derivatization approach [26]. In most instances, stanones are not required to identify mammalian faecal waste (the main direction

of this work) and the presence of sterols at different relative concentrations can be diagnostic for this purpose [27].

The sterol fingerprinting method would appear to have benefits compared with the coliform bacteria counting method to determine sewage contamination, as bacteria are not always viable and can be multiplied under different conditions before the collection of samples and therefore information about the source and extent of contamination may be compromised [21]. However, the validation of the sterol approach still requires further work to gauge its limitations, and to perform parallel studies with bacterial studies on water samples.

Mass fragmentation patterns (Fig. 4) of flophemesyl derivatives of sterols show that their molecular ions are not very intense when compared to base ions. However, characteristic base peaks were identified (Table 6). There are characteristic differences between the mass fragmentation patterns of 5α and 5β stanols, saturated and unsaturated sterols and 5-en- and 5,22-dien- unsaturated sterols. Thus GC–MS under selective-ion monitoring (SIM) can be employed to identify sterols qualitatively and quantitatively based on their base peaks [7]. Limited flophemesyl derivative mass spectra were in the Wiley 6 spectral library (only CHL was found in spectral matching) and the analyzed mass spectra in this study were included in a custom library in the software.

The higher R.S.D.s for the two extraction methods compared could be attributed to the non-homogenised nature of the filtered filter halves, i.e., some algae which can contribute to CHL etc., would deposit inconsistently on the filter with vacuum filtration of water.

Compared with flophemesyl chloride, flophemesylamine or various combinations of flophemesyldiethylamine and flophemesyl chloride are more powerful derivatizing reagents, with the latter having catalytic properties [28]. Future studies will be based on this derivatizing mixture.

5. Conclusion

Extraction of sterols from environmental samples by SFE, direct derivatization with flophemesyl chloride and analyzing the derivatized sterols by GC–ECD and/or GC–MS are promising for routine determination of sterols which can be used as a molecular fingerprint for sewage contamination in the environment.

Pentafluorophenyldimethylsilyl (flophemesyl) derivatives of sterols were prepared with the supercritical fluid extract under the optimised conditions of 5 μl of flophemesyl chloride, 15 min at room temperature. These derivatives give cleaner, more sensitive and selective ECD gas chromatograms. The method does not use excessive amounts of hazardous organic solvents, is relatively inexpensive, simple, rapid and avoids clean-up steps.

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