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Influence of salinity and natural organic matter on the solid phase extraction of sterols and stanols: Application to the determination of the human sterol fingerprint in aqueous matrices

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ABSTRACT

Faecal sterols have been proposed as direct chemical markers for the determination of faecal contamination in inland and coastal waters. In this study, we assess the impact of (a) the concentration of dissolved organic carbon (DOC), (b) the nature of DOC, (c) the salinity and (d) the concentration of sterols and stanols on their solid phase extraction. When natural organic matter (NOM) is modelled by humic acid, increasing DOC concentration from 2.7 to 15.4 mg/L has no significant impact on the recovery of sterols and stanols. The modelling of NOM by a mixture of humic acid and succinoglycan induces a significant (24%) decrease in the recovery of sterols and stanols. For all concentrations of target compounds, no significant increase in recovery is associated with increasing the salinity. Moreover, an increase in the recovery of target compounds is induced by an increase in their concentration. The nine target compounds and the recovery standard (RS) exhibit the same behaviour during the extraction step. Thus, we propose that (a) the concentration of target compounds can be corrected by the RS to calculate more realistic concentrations without modifying their profile and (b) the sterol fingerprint can be investigated in the colloidal fraction of aqueous samples without altering the information it could provide about the source. The application of this analytical method to waste water treatment plant influent and effluents yields results in agreement with previous studies concerning the use of those compounds to differentiate between sources of faecal contamination. We conclude that this analytical method is fully applicable to the determination of sterol fingerprints in the dissolved phase (<0.7 μm) of natural aqueous samples.

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1. Introduction

The quality of inland and coastal waters is damaged by human and animal faecal contaminations. This leads to (a) risks for human health due to the occurrence of bacteria, protozoa and viruses and (b) economic losses due to bathing prohibition and shellfish farming closures. To improve water quality, Water Framework Directives related to waters used for bathing waters (2006/7/CE) and shellfish farming (2006/113/CE) require the establishment of bathing water profiles including identification and classification of the pollution sources. Since the faecal indicators, namely *Escherichia coli* and enterococci, used to monitor these contaminations do not allow the determination of their sources [1], several microbial source tracking (MST) methods have been developed using microbial and chemical markers [2,3].

Among chemical markers, sterols and stanols are considered as direct markers because they occur naturally in human and animal faecal matter. Sterols are involved in the composition of cell walls of animals (cholesterol, cholest-5-en-3β-ol) and plants (phytosterols), and are precursors of several biochemical pathways. Stanols in animal faeces mainly arise from the biohydrogenation of sterols in the intestinal tract. The profile of sterols and stanols in faecal matter is a function of three factors: (a) diet, (b) the metabolism of endogenous sterols and (c) the occurrence of anaerobic bacteria in the digestive tract of some animals, which biohydrogenate sterols into stanols [4]. This results in profiles, also known as "sterol fingerprints", characteristic of the species, which can be used to determine the particular sources of faecal contamination.

Due to the growing interest in determining the sources of faecal contamination, there has been a considerable research effort dedicated to the analysis of sterols and stanols. To investigate their profile in aqueous samples, these compounds need to be extracted from the aqueous matrix before they can be analysed by liquid or gas chromatography. The first method consists of freeze-drying the samples, followed by extraction of the solid by an organic solvent. This procedure is time consuming as 2 days are required for freez-

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ing and 3 days for freeze-drying [5]. A second method involves liquid–liquid extraction, based on the affinity of target compounds towards a liquid organic phase [6–14], but large volumes of organic solvents are necessary. The third method is solid phase extraction (SPE), which depends on the affinity between target compounds and solid media. This type of extraction has been successfully applied to the extraction and analysis of some sterols and stanols [15–17]. However, these studies have been only performed on a reduced number of compounds, whereas the identification of a faecal contamination source based on the sterol fingerprint would require a full determination of the profile.

In inland and coastal waters, sterols and stanols are hydrophobic compounds that are bound to natural organic matter (NOM). NOM appears to be composed of small molecular units (100–2000 g/mol) inherited from the degradation of biopolymers such as lignin, polysaccharides and proteins. Their interactions via hydrogen bonding, non-polar interactions and polyvalent cation interactions result in the formation of aggregates displaying macromolecular characteristics [18]. NOM negatively impacts SPE applied to pesticides [19,20], PAHs [21,22] and endocrine disruptors [23]. The negative impact of NOM on the SPE of sterols occurs at dissolved organic carbon concentrations higher than 20 mg/L [16]. However, in these investigations, NOM was modelled by commercial humic acid, which does not represent the natural heterogeneity of NOM.

The aim of this study is to test the SPE of nine sterols and stanols as a method to investigate their profile, in order to determine the sources of faecal contamination in freshwaters and seawaters. In this context, our study aims at investigating the impact of (a) the concentration of dissolved organic carbon (DOC), (b) the nature of DOC and (c) salinity on the recovery of the nine sterols and stanols by SPE using glass fibre disks containing C_{18}-bonded silica. The analytical procedure developed here is applied to the determination of the sterol fingerprint in two domestic waste water treatment plants (WWTP), as well as in effluents and one WWTP influent, to check whether their sterol fingerprints are concordant with previous results on the profiles of sterols and stanols in matrices characteristic of human faecal contamination.

2. Experimental

2.1. Reagents and chemicals

Organic solvents were of HPLC grade. Dichloromethane (DCM) was purchased from Carlo-Erba SDS (Val de Reuil, France), methanol (MeOH), isopropanol (iPr) and hydrochloric acid 37% (HCl) were purchased from VWR (West Chester, Pennsylvania, USA). The mixture of *N*,*O*-bis(trimethylsilyl)trifuoroacetamide and trimethylchlorosilane (99/1, v/v) (BSTFA + TMCS) was purchased from Supelco (St. Quentin Fallavier, France). Coprostanol (5β-cholestan-3β-ol), cholestanol (5α-cholestan-3β-ol), stigmasterol (24-ethylcholesta-5,22-dien-3β-ol), sitosterol (24-ethylcholest-5-en-3β-ol), α-cholestane and anhydrous magnesium sulfate ($MgSO_4$) were purchased from Sigma (St. Quentin Fallavier, France). Epicoprostanol (5β-cholestan-3α-ol), epicholestanol (5α-cholestan-3α-ol), campesterol (24-methylcholest-5-en-3β-ol) and sitostanol (24-ethyl-5α-cholestan-3β-ol) were purchased from Steraloids (Newport, Rhode Island, USA). Cholesterol and commercial humic acid (HA) were purchased from Aldrich (St. Quentin Fallavier, France). Succinoglycan (Rheosan®) was purchased from Rhodia (La Defense, Courbevoie, France). Cholesterol d_6 ([2,2,3,4,4,6-$^2H_6$]-cholest-5-en-3β-ol) was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). Sodium chloride (NaCl), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) and potassium chloride (KCl) were purchased from Fisher (Illkirch, France), Prolabo (Fontenay-sous-Bois, France), Fluka (Seelze, Germany) and Merck (Darmstadt, Germany), respectively.

2.2. Dissolved organic carbon and ultraviolet measurements

Dissolved organic carbon (DOC) was quantified on a Schimadzu TOC 5050A monitor (Duisburg, Germany) coupled with an automatic sampler ASI 5000A according to the standard method NF EN 1484 (1987). Ultraviolet measurements were performed at 254 nm with a quartz cell on a spectrophotometer Jenway 6405 (Dunmow, UK). The value of the specific UV absorbance at 254 nm ($SUVA_{254}$) is calculated as the ratio of absorbance to the DOC concentration in mg/L multiplied by one hundred [24].

2.3. Organic solutions of sterols and stanols

Solutions of individual compounds (coprostanol, cholestanol, epicoprostanol, epicholestanol, sitostanol, cholesterol, campesterol, stigmasterol, sitosterol and cholesterol d_6) were prepared by weighing 10 mg of pure compounds with a Sartorius R160D semimicro electronic balance (Goettingen, Germany) and dissolution in DCM using 10 mL volumetric flasks. Then individual compounds were dissolved in DCM at 1000 μg/mL. The solution of 5α-cholestane was prepared by taking 400 μL of a commercial solution at 10 mg/mL and diluting it in 10 mL of DCM, using a 500 μL GASTIGHT® syringe (Hamilton, Bonaduz, Switzerland) and a 10 mL volumetric flask. The final concentration of this solution was 400 μg/mL. A second solution of 5α-cholestane was prepared by diluting the first solution by 10, so that its final concentration was 40 μg/mL. Cholesterol d_6 was used as a recovery standard (RS) and 5α-cholestane was used as an internal standard (IS).

The limits of detection were determined using three solutions containing coprostanol, cholestanol, epicoprostanol, epicholestanol, sitostanol, cholesterol, campesterol, stigmasterol, sitosterol and cholesterol d_6 at 5, 10 and 15 ng/mL. A first solution was prepared by diluting 500 μL of each solution of individual compounds at 1000 μg/mL in 10 mL of DCM. This solution (S_1) containing sterols and stanols at 50 μg/mL was diluted by 100 to make up a solution (S_2) of sterols and stanols at 0.5 μg/mL. The solutions used for the determination of the limits of detection were prepared by diluting 100, 200 and 300 μL of S_2 in 10 mL of DCM. The solutions used for the calibration were prepared by diluting 20, 100, 200, 1000 and 2000 μL of S_1 and 100 μL of the 5α-cholestane solution at 400 μg/mL in 10 mL of DCM. Then, the calibration was performed by the internal standard method using five-point calibration curves (0.1, 0.5, 1, 5 and 10 μg/mL) with a constant IS concentration at 4 μg/mL. Two solutions were prepared in order to spike synthetic waters with sterols and stanols. The first solution was prepared according to the protocol described for S_1, while the second (S_3) was prepared by diluting S_1 in DCM by a factor 10. As a result, the concentration of S_3 was 5 μg/mL.

2.4. Synthetic waters

Three types of synthetic waters (SW) were prepared to investigate the effect of (1) the concentration of dissolved organic carbon (DOC), (2) the nature of DOC and (3) salinity on the recovery of target compounds by SPE. SW1 was used as synthetic freshwater with HA as a model of NOM. SW2 represented freshwater with a mixture of HA and succinoglycan to model NOM. SW3 represented seawater with HA as a model of NOM. The SWs were prepared on the day of the extraction with ultrapure water obtained from a Millipore reagent water system (Billerica, MA, USA). Their characteristics are reported in Table 1.

SW1 was prepared by dissolution of 2 g of NaCl (0.5 g/L) in 4 L of water and homogenization by magnetic stirring at room temper-

Table 1
Salinity, nature and amount of dissolved organic carbon (DOC) in synthetic freshwaters (SW1 and SW2) and in synthetic seawater (SW3).

N	Salinity[a] (‰)	DOC (mg/L)	Nature of DOC
SW1-1	0.5	2.6	Humic acid
SW1-2	0.5	4.4	Humic acid
SW1-3	0.5	15.4	Humic acid
SW2	0.5	4.5	Humic acid + succinoglycan
SW3	36.3	1.8	Humic acid

[a] The salinity corresponds to the sum of the concentrations of salts added in order to prepare SWs. It was not investigated in the dissolved phase after filtration at 0.7 μm.

ature. After homogenization of the solution, 20, 40 and 200 mg of solid HA were added in order to prepare SW1-1, SW1-2 and SW1-3, respectively. Then, those solutions were stirred for 1 h and filtered through a glass fibre filter (0.7 μm). The DOC concentrations in solutions SW1-1, SW1-2 and SW1-3 were 2.6, 4.4 and 15.4 mg/L, respectively.

SW2 was prepared by dissolving 2 g of NaCl (0.5 g/L) in 4 L of water and homogenization by magnetic stirring at room temperature. After homogenization of the solution, 20 mg of solid HA and 20 mg of solid succinoglycan were added. Then, the SW2 solution was stirred for 1 h at 50 °C and filtered through a glass fibre filter (0.7 μm). The DOC concentration of solution SW2 was 4.5 mg/L. The % of DOC derived from HA was investigated by preparing 1 L of a solution of SW1 (10 mg of solid HA) and 1 L of a solution of succinoglycan (10 mg) with a salinity of 5‰ (NaCl). These solutions were stirred for 1 h at 50 °C and filtered through a glass fibre filter (0.7 μm). Nine solutions were prepared in 50 mL volumetric flasks using calibrated pipettes using 45, 40, 35, 30, 25, 20, 15, 10 and 5 mL of the HA solution and 5, 10, 15, 20, 25, 30, 35, 40 and 45 mL of the solution of succinoglycan, respectively. DOC and UV were determined for the nine solutions as well as for the raw solutions of HA and succinoglycan in order to calculate $SUVA_{254}$, so this parameter could be plotted against the % of DOC inherited from HA.

SW3 was prepared by dissolution of NaCl (28.41 g/L), $MgSO_4$ (3.52 g/L), $CaCl_2·2H_2O$ (1.58 g/L), KCl (0.79 g/L) and $MgCl_2·2H_2O$ (5.18 g/L) in 4 L of water and homogenization by magnetic stirring at room temperature. After homogenization of the solution, 12 mg of solid HA were added. Then, SW3 was stirred for 1 h and filtered through a glass fibre filter (0.7 μm). The DOC concentration of the SW3 solution was 1.8 mg/L.

The recovery of sterols and stanols from SW1, SW2 and SW3 was investigated at four concentrations: 0.5, 1, 5 and 10 μg/L of the target compounds: coprostanol, cholestanol, epicoprostanol, epicholestanol, sitostanol, cholesterol, campesterol, stigmasterol, sitosterol and cholesterol d_6 (RS). Synthetic waters were spiked by adding 50 and 100 μL of S_1 and 50 and 100 μL of S_3 to 500 mL freshly prepared SW1, SW2 and SW3 under stirring. After 1 h, the pH was fixed at 1 with HCl (10%, m/m) and isopropanol was added in the proportion 10:1 (water:isopropanol) [21,25]. Then sterols and stanols were extracted by SPE. Each analysis was performed twice.

2.5. Environmental waters

The effluent from two domestic WWTPs and the influent from a rural WWTP in Brittany (North-West France) were sampled in February 2010. The first effluent (E1) was sampled at the urban WWTP of Rennes (France). This WWTP has a capacity of 360,000 equivalent-inhabitants and returns between 45,000 and 80,000 m^3/day of treated water to the River Vilaine. The incoming waste water is treated according to a six-step procedure: (1) debris removal, (2) sand removal, (3) oil removal, (4) biological treatments including aerobic, anoxic and anaerobic basins, (5) chemical treatment by addition of ferric chloride and (6) tertiary clarification by sand filtration. The second effluent (E2) was sampled at the urban WWTP of Noyal-sur-Vilaine (France). This WWTP has a capacity of 6,100 equivalent-inhabitants and returns between 800 and 2000 m^3/day of treated water to the River Vilaine. The incoming waste water is treated according to a five-step procedure: (1) debris removal, (2) sand removal, (3) oil removal, (4) activated sludge process (biological treatment) and (5) clarification by sedimentation. The influent (In) was sampled at the rural WWTP of La Meignanne (France). This WWTP has a capacity of 1,800 equivalent-inhabitants and returns 160 m^3/day (annual mean value) of treated water to the Brionneau brook. The incoming waste water is treated according to a five-step procedure: (1) debris removal, (2) sand removal, (3) oil removal, (4) biological treatments including anoxic and aerobic basins and (5) clarification by sedimentation.

After filtering exactly 1 L of E1 and E2 – and 100 mL in the case of the influent sample In – through a glass fibre filter (0.7 μm), cholesterol d_6 was added as a RS (50 μL of a solution at 50 μg/mL in DCM). After 1 h agitation, the pH was fixed at 1 with HCl (10% m/m) and isopropanol was added in the proportion 10:1 (water:isopropanol). Then, sterols and stanols were extracted by solid-phase extraction (SPE).

2.6. Solid-phase extraction

SPE experiments were performed with a filtration system supplied by Supelco. The vacuum was obtained with a N86KT pump from Laboport (Supelco supplier) and SPE disks used were Supelco ENVI-18 DSK (47 mm diameter). The extraction step was performed using a procedure previously described elsewhere [21]. The SPE disks were first washed with DCM and conditioned with MeOH and ultrapure water, and then the samples were added. After complete elution of the samples, the target compounds were recovered using DCM. Organic phases were dried over $MgSO_4$ and evaporated to dryness under a gentle stream of nitrogen. Organic phases were taken up with DCM and a solution of 5α-cholestane (40 μg/mL in DCM) as an internal standard (IS) for the quantification of sterols and stanols by gas chromatography–mass spectrometry (GC–MS). Final volumes were 500 μL for SWs with spiked concentrations of 0.5 and 1 μg/L and 1 mL for SWs with spiked concentrations of 5 and 10 μg/L for WWTP effluents and influent. The volume of the solution of 5α-cholestane was 50 and 100 μL, respectively, in order to reach a final concentration of IS of 4 μg/mL, which was the same concentration used for the calibration. Hence, the enrichment factor was 1000 for SWs spiked with 0.5 and 1 μg/L and for WWTP effluents, 500 for SWs spiked with 5 and 10 μg/L and 100 for the WWTP influent. Finally, the calibration range was 0.1 to 10, 0.2 to 20 and 1 to 100 μg/L for an enrichment factor of 1000, 500 and 100, respectively. Sterols and stanols were analysed as their trimethylsilyl derivatives. 50 μL of solution were dried under a gentle stream of nitrogen and then 50 μL of BSTFA + TMCS were added. The derivatization was performed at 70 °C for 30 min on the day of the analysis.

2.7. GC/MS analyses

Quantitative analyses were carried out by gas chromatography on a GC-2010 instrument (Shimadzu, Kyoto, Japan) equipped with a SLB 5MS capillary column (Supelco, 60 m × 0.25 mm ID, 0.25 μm film thickness) coupled to a QP2010 + mass spectrometer (Shimadzu) operating in selected ion monitoring (SIM) mode. Table 2 summarizes the selected *m*/*z* ratios used for the quantification of the compounds. The temperature of the transfer line was set at 250 °C, and molecules were ionized by electron impact using an energy of 70 eV. The temperature of the ionization source was set at 200 °C. Samples were injected in splitless mode at 310 °C. The oven temperature was programmed from an initial temperature

Table 2
Name, abbreviation, linearity analytical limit of detection (LD in ng/mL) and *m*/*z* fragments applied for the identification and the quantification of the nine target compounds and the recovery standard (Cholesterol d6). The analytical LD corresponds to the minimum concentration in the organic phase in order to have S/N ratio > 3.

Compounds	Abbreviation	Linearity	LD (ppb)	*m*/*z*	
				Quantification	Identification
Coprostanol	$3\beta5\beta$-C_{27}	0.996	5	215	230, 257, 306, 355, 370
Epicoprostanol	$3\alpha5\beta$-C_{27}	0.996	5	215	230, 257, 306, 355, 370
Epicholestanol	$3\alpha5\alpha$-C_{27}	0.995	5	215	230, 257, 306, 355, 370
Cholestanol	$3\beta5\alpha$-C_{27}	0.995	10	215	230, 257, 306, 355, 370
Sitostanol	$3\beta5\alpha$-C_{29}	0.989	10	215	230, 257, 306, 383, 398, 431, 473, 488
Cholesterol	$3\beta\Delta^5$-C_{27}	0.995	5	129	255, 329, 353, 368, 458
Campesterol	$3\beta\Delta^5$-C_{28}	0.997	5	129	255, 261, 343, 367, 382, 472
Stigmasterol	$3\beta\Delta^{5,22}$-C_{29}	0.997	5	129	255, 257, 355, 379, 394, 484
Sitosterol	$3\beta\Delta^5$-C_{29}	0.998	10	129	255, 357, 381, 396, 472, 486
Cholesterol d6	D_6-$3\beta\Delta^5$-C_{27}	0.997	5	131	261, 333, 343, 359, 374
α-Cholestane				217	357, 372

of 70 °C (held for 1 min) rising to 150 °C at 15 °C/min, then rising from 150 °C to 310 °C (held for 20 min) at 3 °C/min. Helium was used as the carrier gas, with a flow rate of 1.0 mL/min. The limit of detection (LD) of each compound was estimated by calculating the signal-to-noise ratio (S/N) for three solutions containing the target compounds at 5, 10 and 15 ng/mL. Each solution was analysed 10 times. LDs were defined as the concentration at which S/N > 3. The analytical limits of detection are presented in Table 2.

2.8. Statistical analyses

Statistical analyses were performed using XLSTAT 2010.2.03 from Addinsoft (Paris, France).

3. Results and discussion

3.1. Synthetic waters

3.1.1. Influence of DOC concentration

The influence of NOM on the SPE of organic compounds has long been recognized [19]. This so-called negative effect [22] may be caused by (a) saturation of sorption sites, (b) the interaction between NOM and target compounds that decreases their desorption during solvent extraction and/or (c) competition for the sorption of target compounds between the solid phase and NOM in solution [19]. In analytical development studies on SPE, HA has been used to model NOM. An increase in HA content leads to a decrease in the recovery of pesticides [19] and endocrine disruptors [23], as well as cholesterol, coprostanol, cholestanol, coprostanone (5β-cholestan-3-one) and cholestanone (5α-cholestan-3-one) [16]. In this last study, a decline in recovery was observed for the highest HA concentration of 20 mg/L, but not at low concentrations of HA ranging from 2 to 10 mg/L. Moreover, for the highest HA concentrations, the mean recovery was 31% for coprostanol, 28% for cholestanol and 57% for cholesterol. Due to these variations, the molecular ratios such as coprostanol/cholesterol [26] and coprostanol/(cholestanol + cholesterol) [27], which are used to determine the sources of faecal contamination, would not be representative of the original samples, thus leading to possible misinterpretations. To investigate the effect of NOM on the recovery of sterols and stanols, SW1 was prepared with three HA concentrations: 2.6, 4.4 and 15.4 mg/L. We chose this range of DOC as it represents the range of DOC concentrations observed in rivers of Europe [28,29]. Table 3 summarizes the results for the recovery of the target compounds.

For SW1-1 with 2.6 mg/L DOC, the recovery ranges from 53% (epicoprostanol) to 117% (sitosterol), with a mean value of 77%. The mean recovery, that is, for a given concentration, the average of the recoveries of each compound, increases from 61% to 76% over the range of concentrations of these compounds. A maximum recovery of 102% is obtained at a target molecule concentration of 5 μg/L (Fig. 1). The extractions are reproducible since the mean standard error ranges from 1% (1 μg/L) to 5% (5 μg/L). Moreover, the recovery of each target compound is similar, as highlighted by the standard deviations calculated for mean recovery at each concentration, which range from 4.9% (0.5 μg/L) to 9.3% (5 μg/L). For SW1-2 containing 4.4 mg/L DOC, the recovery ranges from 62% (coprostanol and epicoprostanol) to 118% (sitosterol), with a mean of 85%. The mean recovery varies from 77% to 93%, with a minimum of 68% for 1 μg/L and a maximum value of 102% for 5 μg/L (Fig. 1). Those extractions are reproducible since the mean of the standard error ranges from 1% (1 μg/L) to 4% (0.5 μg/L). Each compound is recovered in the same proportion, as shown by the standard deviations calculated for mean recoveries at each concentration, which range from 3.5% (1 μg/L) to 9.7% (5 μg/L). For SW1-3 containing 15.4 mg/L DOC, the recovery ranges from 54% (stigmasterol) to 102% (cholesterol), with a mean value of 81%. The reproducibility of these extractions is not as good as for SW1-1 and SW1-2, as highlighted by the mean standard error ranging from 2% (10 μg/L) to 15% (5 μg/L). The mean recovery increases from 62% to 95% along with the concentration of the compounds (Fig. 1). The recovery of each target compound is similar, as highlighted by the mean standard deviations of the recoveries, which range from 2.3% (1 μg/L) to 6.5% (0.5 μg/L).

The differences of recoveries obtained for a given concentration between the three types of SW1 (2.6, 4.4 and 15.4 mg/L of HA) were tested using the non-parametric test of Kruskal–Wallis for *k* samples. For a concentration of 0.5 μg/L of target compounds, the differences between SW1-1 and SW1-3 are non-significant. On the contrary, there are significant differences between SW1-2 and SW1-1, as well as between SW1-2 and SW1-3 (p-value < 0.0002). For 1 μg/L and 5 μg/L of target compounds, the differences between SW1-1 and SW1-2 are non-significant, while the differences between SW1-3 and SW1-1 as well as between SW1-3 and SW1-2

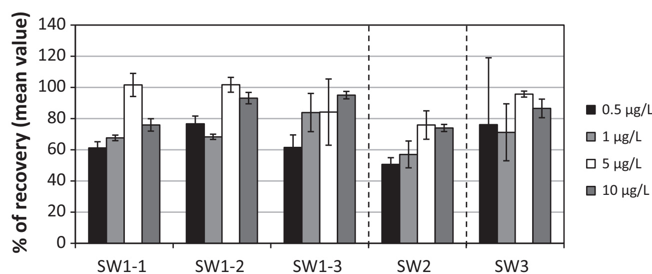

Fig. 1. Mean recovery of stanols, sterols and cholesterol d6 (RS) for their SPE from synthetic freshwaters (SW1 and SW2) and synthetic seawater (SW3). The uncertainties are reported as standard errors.

Table 3
Recovery of sterols, stanols and cholesterol d6 (RS) for synthetic freshwaters and seawater for 0.5, 1, 5 and 10 μg/L of target compounds. The reproducibility is illustrated by standard errors. The names of molecules are listed Table 2.

Compounds	SW1-1				SW1-2			
	0.5	1	5	10	0.5	1	5	10
$3\beta5\beta$-C_{27}	54 ± 6	58 ± 2	110 ± 8	64 ± 1	76 ± 1	62 ± 1	112 ± 4	92 ± 9
$3\alpha5\beta$-C_{27}	53 ± 9	59 ± 2	94 ± 7	58 ± 1	78 ± 3	62 ± 2	97 ± 5	85 ± 7
$3\alpha5\alpha$-C_{27}	65 ± 0	70 ± 1	91 ± 6	79 ± 7	85 ± 6	71 ± 3	89 ± 6	93 ± 4
$3\beta5\alpha$-C_{27}	67 ± 0	72 ± 4	93 ± 5	77 ± 4	85 ± 8	72 ± 0	91 ± 7	88 ± 1
$3\beta5\alpha$-C_{29}	68 ± 4	70 ± 5	109 ± 5	81 ± 3	84 ± 6	71 ± 1	107 ± 6	91 ± 3
$3\beta\Delta^{5}$-C_{27}	63 ± 3	72 ± 1	94 ± 9	78 ± 6	72 ± 6	69 ± 2	93 ± 4	97 ± 2
$3\beta\Delta^{5}$-C_{28}	60 ± 4	68 ± 2	108 ± 8	81 ± 4	71 ± 6	68 ± 1	109 ± 4	96 ± 2
$3\beta\Delta^{5,22}$-C_{29}	59 ± 4	67 ± 2	104 ± 8	79 ± 4	71 ± 5	68 ± 1	105 ± 4	96 ± 3
$3\beta\Delta^{5}$-C_{29}	60 ± 3	68 ± 1	117 ± 9	83 ± 4	72 ± 4	69 ± 2	118 ± 4	99 ± 3
D_6-$3\beta\Delta^{5}$-C_{27}	63 ± 6	72 ± 0	96 ± 8	78 ± 5	74 ± 6	70 ± 2	96 ± 4	95 ± 2

Compounds	SW1-3				SW2			
	0.5	1	5	10	0.5	1	5	10
$3\beta5\beta$-C_{27}	67 ± 16	85 ± 20	88 ± 24	97 ± 3	50 ± 4	53 ± 8	79 ± 6	70 ± 9
$3\alpha5\beta$-C_{27}	63 ± 15	86 ± 18	84 ± 24	95 ± 2	52 ± 3	54 ± 7	84 ± 6	72 ± 9
$3\alpha5\alpha$-C_{27}	63 ± 8	83 ± 12	87 ± 23	95 ± 6	57 ± 3	59 ± 8	73 ± 11	76 ± 0
$3\beta5\alpha$-C_{27}	58 ± 7	84 ± 9	80 ± 20	90 ± 4	57 ± 4	56 ± 9	74 ± 10	73 ± 1
$3\beta5\alpha$-C_{29}	58 ± 7	81 ± 10	81 ± 19	92 ± 1	52 ± 7	54 ± 9	74 ± 11	72 ± 1
$3\beta\Delta^{5}$-C_{27}	76 ± 5	88 ± 10	87 ± 23	102 ± 2	52 ± 4	57 ± 9	74 ± 9	77 ± 0
$3\beta\Delta^{5}$-C_{28}	55 ± 6	82 ± 10	83 ± 19	95 ± 2	44 ± 4	52 ± 10	77 ± 10	75 ± 1
$3\beta\Delta^{5,22}$-C_{29}	54 ± 4	81 ± 10	83 ± 19	95 ± 0	45 ± 6	57 ± 8	75 ± 10	75 ± 1
$3\beta\Delta^{5}$-C_{29}	60 ± 5	84 ± 12	82 ± 18	91 ± 1	50 ± 6	78 ± 9	73 ± 11	73 ± 1
D_6-$3\beta\Delta^{5}$-C_{27}	61 ± 7	85 ± 11	88 ± 23	99 ± 4	47 ± 1	50 ± 9	75 ± 9	76 ± 0

Compounds	SW3			
	0.5	1	5	10
$3\beta5\beta$-C_{27}	76 ± 42	69 ± 19	102 ± 5	73 ± 4
$3\alpha5\beta$-C_{27}	70 ± 37	67 ± 19	99 ± 5	72 ± 5
$3\alpha5\alpha$-C_{27}	72 ± 43	60 ± 14	82 ± 3	90 ± 4
$3\beta5\alpha$-C_{27}	87 ± 58	70 ± 18	94 ± 1	88 ± 6
$3\beta5\alpha$-C_{29}	95 ± 65	78 ± 22	110 ± 0	91 ± 5
$3\beta\Delta^{5}$-C_{27}	81 ± 39	73 ± 17	95 ± 0	92 ± 8
$3\beta\Delta^{5}$-C_{28}	71 ± 37	74 ± 19	96 ± 1	89 ± 8
$3\beta\Delta^{5,22}$-C_{29}	70 ± 35	74 ± 18	94 ± 1	89 ± 9
$3\beta\Delta^{5}$-C_{29}	70 ± 35	75 ± 18	92 ± 1	89 ± 5
D_6-$3\beta\Delta^{5}$-C_{27}	69 ± 36	71 ± 18	94 ± 1	92 ± 6

are significant (p-value < 0.0001). For 10 μg/L of target compounds, the differences between SW1-2 and SW1-3 are non-significant, while the differences between SW1-1 and SW1-2 as well as between SW1-1 and SW1-3 are significant (p-value < 0.0001). According to these results, in the range of the tested DOC concentrations, there is no clear trend for the impact of the increase in dissolved HA on the recovery of sterols and stanols.

3.1.2. *Influence of the nature of DOC*

In previous studies [16,19,21–23], the negative-effect of NOM on the recovery of target compounds by SPE was investigated using synthetic HA as a model of NOM. However, humic substances represent between 40 and 80% of NOM in freshwaters [30]. The other constituents of NOM, namely rigid biopolymers such as structural and fibrillar polysaccharides and flexible biopolymers including proteins and reserve polysaccharides [31], could have an influence on the extraction step. The proportion of polysaccharides in NOM is a function of the type of water, the period of sampling and the geographical localization. In the Yukon River (Alaska, USA), mono- and poly-saccharides appear to represent 24% of the DOC [32]. In another study, conducted by proton nuclear magnetic resonance (NMR) on the dissolved organic matter (DOM) in a suite of lakes, rivers, seawater and marine sediment interstitial water, DOC was found to be composed of approximately 9% polysaccharides in freshwaters and 6% in seawater [33]. However, in another NMR study, the DOM of Pacific Ocean water was mainly composed of polysaccharides [34], and approximately 50% of DOM of ocean surface waters worldwide would appear to be composed of polysaccharides [35].

To investigate the influence of the nature of DOC on the recovery of sterols and stanols by SPE, SW2 was prepared using a mixture of commercial HA and succinoglycan in order to model NOM. The commercial succinoglycan was obtained by pure fermentation of *Agrobacterium tumefaciens*. This compound is a microbial acidic exopolysaccharide composed of octasaccharide repeating units in which galactose and glucose monomers occur in a molar ratio of 1–7 [36]. The mass fraction of DOC inherited from HA (%C_{HA}) in SW2 was determined by measuring the $SUVA_{254}$, a parameter that is correlated with DOC aromaticity [24]. DOC analyses and UV measurements were performed on 11 solutions of known %C_{HA}. As a result, we found that %C_{HA} was linearly correlated with $SUVA_{254}$ (Fig. 2). This calibration allowed us to calculate %C_{HA} in SW2, yielding an estimate of 40%.

For SW2, the recovery ranges from 44 to 84%, with a mean value of 64% (Table 3). The mean recovery, that is the average of the recoveries of each compound at a given concentration, increases with the concentration of the compound from 51% to 74%, with a maximum of 76% for 5 μg/L of target molecules (Fig. 1). The extraction is reproducible since the mean standard error ranges from 2% (10 μg/L) to 9% (1 and 5 μg/L). Moreover, the recovery of each target compound is similar, as shown by the standard deviations of mean recoveries for each concentration, which range from 2.1% (10 μg/L) to 7.9% (1 μg/L). The differences of recoveries between SW1-2 and SW2 obtained for a given concentration were tested using the non-

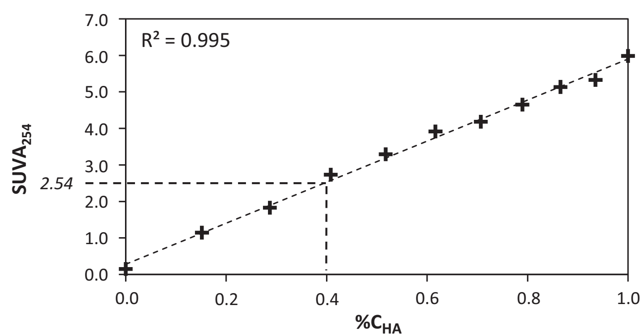

Fig. 2. Variation of specific UV absorbance at 254 nm as a function of the mass fraction of dissolved organic carbon inherited from humic acid in a binary mixture of humic acid and succinoglycan.

parametric test of Kruskal–Wallis for *k* samples. Compared with SW1-2, the modelling of NOM by a mixture of HA and succinoglycan (2:3) leads to a significant decrease (*p*-value < 0.0002 for 0.5, 5 and 10 μg/L and <0.0025 for 1 μg/L) in the recovery of target compounds. This decrease represents 34%, 16%, 25% and 21% of the mean recovery obtained for SW1-2 at target-compound concentrations of 0.5, 1, 5 and 10 μg/L, respectively.

3.1.3. Influence of the salinity

To test the SPE procedure for the recovery of sterols and stanols from coastal waters, a simplified seawater (SW3) was obtained by replacing bromide, fluoride and total alkalinity by chloride, and replacing strontium by calcium [37]. This recipe was applied to the determination of thermodynamic constants in seawater [38–40]. As a supplement to this recipe, HA was added in order to model NOM. After filtration at 0.7 μm, the final concentration of DOC in SW3 is 1.8 mg/L, which is in the range of values obtained in the Elorn estuary (Brittany, France) [41]. Table 3 reports the results for the recovery of the target compounds in SW3. The mean values range from 71% (1 μg/L) to 96% (5 μg/L), which are lower than the recoveries of coprostanol, cholesterol and cholestanol from natural seawater by SPE using Oasis HLB solid phase [16]. The recoveries are better at high concentrations than at low concentrations. Moreover, the reproducibility improves with the concentration, as highlighted by the standard error of the mean for each compound, which varies from 30% (0.5 μg/L) to 13% (1 μg/L) to 1% (5 μg/L) and 4% (10 μg/L). The target compounds are extracted in the same proportion, as highlighted by the mean standard deviations of the recovery, which are 8.8% (0.5 μg/L), 5.1% (1 μg/L), 7.1% (5 μg/L) and 7.6% (10 μg/L). The differences of recoveries obtained for a given concentration between SW1-1 and SW3 were tested using the non-parametric test of Kruskal–Wallis for *k* samples. The differences are significant for target-compound concentrations of 0.5 and 10 μg/L (*p*-value < 0.0002 and =0.0102, respectively).

3.1.4. Discussions on synthetic waters

Three hypotheses are proposed to explain the negative effect of NOM on the SPE of molecules: (a) saturation of sorption sites, (b) interaction between NOM and target compounds, which decreases their desorption during solvent extraction or (c) competition between the solid phase and NOM in solution for the sorption of target compounds [19]. Previous studies on the negative effect of NOM on the SPE of different types of target compounds [16,21,23] demonstrated that the decrease in recovery due to high DOC concentrations, modelled by HA, is positively correlated with the hydrophobicity of the molecules. Assuming that increasingly hydrophilic molecules will bind less and less well to HA in water systems, then our results contradict hypothesis (a), while they back up hypotheses (b) and (c). Recovery experiments carried out on two ^{14}C-labelled pesticides have indicated (i) that about 1% of the ^{14}C-labelled compounds remain on the solid phase after extraction by organic solvent and (ii) that about 38% of the ^{14}C-labelled compounds are present in the eluted water [19]. Moreover, the analysis of eluted water from the SPE of pesticides from lake water samples indicates a recovery of between 8% and 38% of the target compounds [20]. These results show that the target compounds can be eluted via their interaction with NOM, either as complexes or by adsorption, which seems to contradict hypothesis (b) and to validate hypothesis (c).

From the results of the present study, we can identify four trends. Firstly, an increase in the concentration of the target compounds leads to an increase in the recovery. This can be explained by losses of molecules during the analytical procedure. If we assume that the absolute amounts of these losses are constant, then they represent a higher percentage of the target compounds at low concentrations than at high concentrations. Secondly, the recovery remains stable when the concentration of HA increases from 2.6 to 15.4 mg/L, which seems to contradict hypothesis (a). Thirdly, we observe that an increase in the salinity from 0.5‰ to 36.3‰ induces a significant increase in the recovery of 25 and 14% at compound concentrations of 0.5 and 10 μg/L, respectively. The increase in salinity could lead to a decrease in the solubility of HA, and then produce an increase in the interaction between HA and the SPE phase. This trend seems to contradict hypothesis (b) and to validate hypothesis (c). The last observation concerns the nature of NOM. The modelling of NOM by a mixture of HA and a polysaccharide (succinoglycan) leads to a 25% decrease in the recovery. The interaction between HA and succinoglycan could induce an increase in the apparent solubility of HA via H-bonding between water and succinoglycan, which could lead to a decrease in the interaction between HA and the solid phase. Such a hypothesis would contradict hypothesis (b) and would support hypothesis (c). However, during the extraction step of SW2, two SPE disks were required to perform the extraction because of clogging of the SPE disk. This is probably due to the occurrence of polysaccharides that are known for their fouling behaviour in filtration processes [42]. The fouling of SPE disks by polysaccharides could have hindered the interaction between HA and the solid phase. Such a hypothesis would appear to support hypothesis (a). By combining the results of this study with previous data, we can conclude that the competition between the solid phase and dissolved NOM for the sorption of target compounds could be the main phenomenon explaining the negative effect of NOM on their SPE from natural samples.

For the three types of synthetic water, the mean standard deviations of recovery for each concentration range from 2.1% to 9.7%,

Table 4
Concentrations (μg/L) of stanols and sterols in two WWTP effluents (E1 and E2) and one influent (In).

Compounds	E1	E2	In
Coprostanol	0.73	0.79	98.7
Epicholestanol	nd	nd	2.2
Epicoprostanol	0.17	0.16	1.8
Cholestanol	0.28	1.14	13.0
24-Ethylcoprostanol[a]	0.37	0.36	23.9
Sitostanol	0.36	0.34	6.4
Cholesterol	0.66	1.06	122.4[b]
Campesterol	nd	0.23	3.2
Stigmasterol	0.26	0.17	3.8
Sitosterol	0.31	0.33	27.7

nd: non-detected.

[a] 24-Ethylcoprostanol: 24-ethyl-5β-cholestan-3β-ol. This molecule was not purchased as a pure standard. It was quantified using the calibration curve of coprostanol.

[b] This value was outside of the calibration range that was from 1 to 100 μg/L for the analysis of In.

Table 5
Signification of molecular ratios calculated for sterols and stanols and values obtained for the WWTP effluents (E1 and E2) and the WWTP influent (In).

Ratio	Ref	Range	Signification	E1	E2	In
R_1 = cop/(cop + cholestanol)	[44]	[0.7;1.0] [0.0;0.3]	Sewage contamination Uncontaminated sites	0.7	0.4	0.9
R_2 = cop/chol	[26]	[0.2;1.0] >1.0	Sewage contamination High levels of sewage contamination	1.1	0.7	0.8
R_3 = cop/(cholestanol + chol)	[27]	<0.06 >0.06	Small scale sewage effect Important sewage effect	0.78	0.36	0.73
R_4 = cop/(cop + 24etcop)	[45]	<0.38 >0.73	Herbivores feces Human feces	0.67	0.69	0.80
R_5 = sito/cop	[5]	<1.0 >1.0	Human or porcine feces Bovine manures	0.5	0.4	0.1

cop: coprostanol; chol: cholesterol; epicop: epicoprostanol; 24etcop: 24-ethylcoprostanol; sito: sitostanol.

with a mean value of 5.6 ± 0.5% (standard error). Moreover the difference between the recoveries of (1) the RS and (2) sterols and stanols divided by the recovery of the RS is 6.9 ± 0.5% (standard error) with a 95% confidence interval from 5.8 to 7.9%. For the extraction of sitosterol ($3\beta\Delta^5$-C_{29}) from SW2, this value is 55.1%, which suggests this value represents an outlier. However, with the exception of this value, both of these results highlight the fact that the nine target compounds and the RS exhibit similar behaviours during the extraction step. Thus, (a) the sterol fingerprint can be investigated in the colloidal fraction of aqueous samples without modifying its information content and (b) the concentrations of target compounds can be corrected by the RS to calculate more realistic concentrations without modifying their profile. The correction factor is calculated as the ratio of the theoretical concentration of the RS to its experimental value. The correction is performed by multiplying experimental concentrations of sterols and stanols by this factor. As a consequence, we apply this analytical procedure to the analysis of a WWTP influent and two WWTP effluents, to ascertain whether their sterol fingerprints, analysed by SPE, are concordant with previous results on sterol and stanol profiles in matrices characteristic of human faecal contamination.

3.2. *WWTP influent and effluents*

Faecal contamination of freshwaters and coastal waters by human faecal matter is associated with domestic waste water treatment plants (WWTP), being mainly due to the effluents and influents that are directly discharged into watercourses when their flow is higher than the WWTP capacity during episodes of heavy rain. To test the applicability of the SPE method to determine the origin of faecal contamination, we studied the profile of sterols and stanols in two domestic WWTP effluents from urban areas (E1 and E2) and a domestic WWTP influent from a rural area (In). According to the results obtained on synthetic waters, the concentrations of sterols and stanols are normalized here to the concentration of the RS (cholesterol d_6). The results are summarized in Table 4. Eight sterols and stanols are detected in the dissolved fraction (<0.7 μm) of E1. The main compounds present are coprostanol (0.73 μg/L) and cholesterol (0.66 μg/L). Nine sterols and stanols are detected in the dissolved fraction of E2, and the main compounds are cholestanol (1.14 μg/L), cholesterol (1.06 μg/L) and coprostanol (0.79 μg/L). These values are in the range of the concentrations of campesterol, stigmasterol, β-sitosterol, cholesterol and coprostanol in filtered (<0.1 μm) WWTP effluents from southern Germany [43]. In the present study, ten sterols and stanols are detected in the dissolved fraction of In, their summed concentrations being 96 and 66 times higher than the sum of detected compounds in E1 and E2, respectively. The main compounds present are cholesterol (122.4 μg/L) and coprostanol (98.7 μg/L).

Based on sterol fingerprints characteristic of animal species [4], several sterol ratios have been developed to track the input of human faecal matter into the environment (Table 5). These ratios are calculated here for E1, E2 and In (Table 5). The values obtained for the ratios R_1, R_2 and R_3 are characteristic of sewage-contaminated areas, with the exception of the value of E2 for R_1. The values of the ratios R_4 and R_5 are characteristic of human faecal matter. The extraction of sterols and stanols from the dissolved phase of two effluents and one influent yields results in agreement with previous studies on the use of these compounds to differentiate between sources of faecal contamination.

4. Conclusion

This investigation of the recovery of sterols and stanols by solid phase extraction from three types of synthetic waters has allowed us to assess the impact of (a) the concentration of dissolved organic carbon (DOC), (b) the nature of DOC, (c) the salinity and (d) the concentration of target compounds. When NOM is modelled by humic acid, increasing DOC from 2.6 (SW1-1) to 15.4 (SW1-3) mg/L has no significant impact on the recovery of sterols and stanols. The modelling of NOM by a mixture of humic acid and succinoglycan (2:3—SW2) leads to a significant (24%) decrease in the recovery of sterols and stanols. Increasing the salinity from 0.5 (SW1-1) to 36.3‰ (SW3) produces a 25% and 14% increase in the recovery at 0.5 and 10 μg/L of target compounds, respectively, while no significant differences are observed between SW1-1 and SW3 for concentrations of 1 and 5 μg/L of target compounds. For the three types of synthetic water, an increase in the concentration of target compounds produces an increase in their recovery. Moreover, the RS and the nine target compounds all exhibit similar behaviours during the extraction step. Thus, we propose that (a) the concentration of target compounds can be normalized to the RS to calculate more realistic concentrations without modifying their profile and (b) the sterol fingerprint can be analysed in the colloidal fraction of aqueous samples without altering the information content. The application of this analytical method to WWTP influent and effluents yields results in agreement with previous studies on the use of these compounds to differentiate between sources of faecal contamination. In conclusion, this analytical method is fully applicable to the determination of sterol fingerprints in the dissolved phase (<0.7 μm) of natural aqueous samples.

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