Development of the Analysis of Fecal Stanols in the Oyster Crassostrea gigas and Identification of Fecal Contamination in Shellfish Harvesting Areas

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Lipids:

Development of the analysis of fecal stanols in the oyster *Crassostrea gigas* and identification of fecal contamination in shellfish harvesting areas

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Abstract

The objective of this work was to study the effects of washing and purification steps on qualitative and quantitative analysis of fecal stanols in the oyster *Crassostrea gigas* using either single or combination of lipid purification steps on silica gel or aminopropyl bonded silica gel (NH_2) or a washing step. Among the three analytical pathways compared, the two including water extraction or NH_2 purification did not lead to higher recoveries and decreased repeatabilities of extractions compared to the single purification on silica gel. This latter led to similar recoveries (ca. 80%) and repeatabilities (ca. 10%) for both spiked standards (coprostanol and sitostanol). This analytical pathway has been applied to oysters collected in a harvesting area in Brittany (France) where fecal contaminations are important and allowed to quantify eight stanols in oysters. The relative proportions of fecal stanols of these oysters were combined with principal component analysis in order to investigate the usefulness of their stanol fingerprints to record a fecal contamination and to distinguish its source between human, porcine and bovine contaminations. Oysters non-fecally contaminated by *Escherichia coli* did not present specific stanol fingerprints while oysters fecally contaminated had a bovine fingerprint, suggesting a contamination of these samples by bovine sources. As a consequence, the method developed here allows the use of stanol fingerprints of oysters as a microbial source tracking tool that can be applied to shellfish harvesting areas subjected to fecal contaminations in order to identify the different sources of contamination and improve watershed management.

Abbreviations

BSTFA	N,O-bis- (trimethylsilyl)trifluoroacetamide
DCM	Dichloromethane
DW	Dry weight
GC-MS	Gas chromatography- mass spectrometry
MeOH	Methanol
NH_2	Aminopropyl-bonded silica gel
PCA	Principal component analysis
TMCS	Trimethylchlorosilane

1. Introduction

Coastal and shellfish harvesting areas are subjected to fecal contaminations from human and animal waste leading to sanitary risks due to the presence of source-specific microbial pathogens in contaminated waters and shellfish [1,2,3]. Among shellfish, several species of bivalves such as mussels and oysters have been used as biological models for research in ecotoxicology and biomonitoring since they are suspension filter-feeders which may bioaccumulate and record environmental contaminants into their tissues [4, 5].

Fecal contamination of shellfish is particularly acute in France which is the first European producer of oysters (mainly *Crassostrea gigas*) [6]. In Brittany, one of the main areas of production of *Crassostrea gigas*, shellfish can be subjected to fecal contamination leading to the closure of shellfish harvesting areas [7]. Therefore, to limit i) sanitary risks linked to the consumption of contaminated shellfish and especially *Crassostrea gigas* and ii) economic loss due to the closure of shellfish harvesting areas, it is crucial to improve watershed management by controlling and limiting the sources of fecal contamination within these environments.

For this purpose, the actual European Shellfish Directive on shellfish harvesting (854/2004/EC) imposes the classification of shellfish and requires the assessment of potential pollution sources upstream of shellfish harvesting. The actual classification is based on the fecal indicator bacteria *Escherichia coli* and enterococci which are not species-specific.

In order to distinguish human and animal sources of fecal contamination within environment, microbial source tracking methods have been developed during the last decade. Based on specific microbial or chemical markers from human or animals, they have been successfully applied to field studies to identify the sources of fecal contamination in water,

soil and sediment [7-16]. Among chemical markers, fecal stanols have proven their usefulness as direct fecal markers [17]. Indeed, the distribution of fecal stanols in animal faeces relies on three main factors: i) the animal's diet, ii) the ability of animals to biosynthesize endogenous sterols and iii) the composition of the intestinal flora responsible for sterol biohydrogenation into stanols. Consequently, the fecal stanol fingerprint allows to distinguish between different fecal sources in environmental matrix by the use of stanol ratios [18-20] or multivariate analyses [14, 15, 21, 22].

In Brittany, the main sources of fecal contamination in water are human wastewater treatment effluent, porcine and bovine manure or slurry [12]. In this region, the transfer time of water in coastal watersheds from streams to sea can last for one day [23] and it has been shown that a stanol fingerprint associated with a specific source of contamination can last for six days in fresh and seawaters microcosms [24, 25]. Therefore, the specificity of a stanol fingerprint can be transferred from inland waters to receiving seawater, which could allow the identification of the sources of fecal contamination in water in such areas [14, 20]. In shellfish harvesting areas, shellfish can bio-accumulate microbial pathogens by filtration of contaminated surrounding waters, which enables the identification of contamination sources using microbial markers [7]. However, it is still unknown whether chemical markers such as fecal stanols and corresponding stanol fingerprints allow the identification of fecal contamination sources in oysters.

Indeed, studies dealing with the occurrence of fecal stanols in shellfish mainly have focused on coprostanol as a marker of human fecal contamination in fresh or seawater mussels [26-31]. However, the identification of fecal sources with stanol fingerprints requires the accurate analysis of several compounds [14, 17, 22]. Therefore, it is necessary to develop an analytical pathway that allows the quantification of different fecal stanols in shellfish matrix, which constituted the first goal of the present study.

Among the main studies focusing on coprostanol quantification in mussels, only Cathum and Sabik [27] have tested the extraction efficiency of their method and found recoveries of about 48 % for wet mussel samples. As a consequence, the efficiencies of such methods remain largely unknown. In this present study, the efficiencies and repeatabilities of three analytical pathways have been compared for the stanol extraction of the oyster *Crassostrea gigas* using two recovery standards:

-The first method consisted of three steps: i) extraction of lipids from oyster matrix, ii) purification of lipids on silica gel and iii) analysis of stanol fraction by gas chromatography-mass spectrometry (GC-MS).

-The second method comprised of a washing step of oyster tissue with water prior to lipid extraction. Indeed, shellfish such as oysters can be constituted of more than 90 % of non-lipid compounds such as glycogen and proteins that can potentially interact with lipids and decrease their recoveries [32, 33]. Therefore, a washing step of matrix with water prior to the lipid extraction step allows the removal of the non-lipid compounds and could improve extraction efficiencies of stanols [34, 35].

-The third method comprised of a second purification step of lipids on aminopropyl-bonded silica gel after that on silica gel. The separation of lipid classes from the total extract is mandatory in complex environmental matrix to improve the analysis accuracy of target compounds. The lipid fraction of oysters is a complex mixture containing several lipid classes such as phospholipids, triacylglycerols, free fatty acids, sterols and stanols [36]. Therefore, the addition of a purification step could be particularly interesting in order to remove as much of lipids of non-interest as possible. Aminopropyl-bonded silica gel (NH_2) was chosen as the second sorbent because of its affinity and subsequent selective retention of acidic phospholipids [37].

To the best of our knowledge, no method has been developed for the analysis of several fecal stanols in shellfish. Among the three methods tested here, the one leading to the highest and similar recoveries and highest repeatabilities for both recovery standards was chosen to analyse the concentration of several fecal stanols in oysters from northern Brittany, France. The stanol fingerprint of these oysters were analyzed by principal component analysis (PCA) in order to identify the fecal contamination sources in this shellfish harvesting area.

2. Experimental

2.1. Reagent and chemicals

Organic solvents were of high performance liquid chromatography grade. Dichloromethane (DCM) was purchased from Carlo-Erba SDS (Val de Reuil, France), methanol (MeOH) and cyclohexane were purchased from VWR (West Chester, PA). N,O-bis-(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (99:1, by vol) (BSTFA + TMCS) and SPE disks (Supelco ENVI-18DSK, 47mm diameter) were purchased from Supelco (St. Quentin Fallavier, France). Coprostanol (5β-cholestan-3β-ol), cholestanol (5α-cholestan-3β-ol), 5α-cholestane and aminopropyl-bonded silica gel were purchased from Sigma (St. Quentin Fallavier, France). Sitostanol (24-ethyl-5α-cholestan-3β-ol) was purchased from Steraloids (Newport, RI). 24-Ethylcoprostanol (24-ethyl-5β-cholestan-3β-ol) and 24-ethylepicoprostanol (24-ethyl-5β-cholestan-3α-ol) were purchased from BCP Instruments (Irigny, France). Silica gel (40-63 µm) was purchased from Merck (Darmstadt, Germany). 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).

2.2. Sample preparation

147 To compare the three analytical pathways, 90 oysters (*Crassostrea gigas*) were purchased
148 at Cancale (Brittany, France) in November 2012. After purchasing, oysters were opened, the
149 intervalvular liquid discarded, the flesh of 10 individuals were pooled as one sample (ca. 4 g
150 of dry weight flesh), frozen, freeze-dried and finally ground with an agate mortar for
151 homogenization.

152 As the main goal of this study is to analyse several fecal stanols in shellfish tissues, two
153 recovery standards were used to determine the reliability of the tested methods. The common
154 human marker coprostanol was the first one and sitostanol was chosen as the second recovery
155 standard because it is a fecal stanol rather representative of a bovine contamination [17].
156 Coprostanol and sitostanol were spiked on the freeze-dried flesh pool just before organic
157 extraction (methods 1 and 3, see below) or aqueous extraction (method 2, see below) at a
158 concentration of 10 µg g^{-1} dry weight (DW). This concentration is in the range of coprostanol
159 concentration recorded in bivalves after human fecal contamination [26-31]. For both blanks
160 and spiked samples, each extraction method was performed in triplicates.

161

162 *2.3. Application to a study case: the Fresnaye bay*

163 Among the three methods tested, the one leading to the better recoveries of spiked
164 coprostanol and sitostanol and to better repeatabilities was used to determine the
165 concentration and the distribution of fecal stanols of oysters from the Fresnaye bay (Brittany,
166 France, Figure 1). This bay is an intensive shellfish harvesting area with an annual production
167 of ca. 550 tons of *Crassostrea gigas* intended for human consumption. The Fresnaye
168 watershed covers 121 km² and its number of human inhabitants is estimated at 14 000. The
169 potential sources of fecal contaminations originate from the seven wastewater treatment plants
170 of the watershed and multiple sources of untreated wastewater, and its agricultural area covers
171 ca. 70 % of watershed area with intensive livestock farming of pigs (ca. 235.000 head in

2010), and cows (ca. 5300 head in 2010) [38]. In the last decade, this shellfish harvesting area is subjected to increasing fecal contaminations by these different sources leading to the degradation of the quality of oysters. In February, March and August 2013, oysters were sampled at two locations on the bay and analysed for each sampling date.

2.4. Analytical pathways for stanol analysis

Figure 3 summarizes the different steps involved in the four analytical pathways investigated for the analysis of fecal stanols in oyster tissues. Each step is described in detail below. Briefly, the method 1 consisted of an extraction of lipids with DCM followed by a purification step on silica gel and analysis of fecal stanols by GC-MS. The method 2 consisted of a first purification step of samples with water prior to lipid extraction followed by a purification on silica gel and GC-MS analysis. The method 3 involved a second purification step on aminopropyl-bonded silica gel prior to GC-MS analysis.

2.4.1. Lipid extraction

For the three analytical pathways, lipids were extracted using an Accelerated Solvent Extractor (ASE 200, Dionex, Courtaboeuf, France) with DCM. For each sample, about 4 g of freeze-dried tissue were extracted 3 times in pre-washed (with DCM) 33 mL extraction cells. Each extraction consisted in 2 cycles of 5 min at 100°C and 100 bar followed by a 40 second flush step and a 30 second purge step. Each extract was then concentrated under reduced pressure and the 3 extracts were pooled. Then, total lipid extracts were dried, weighed and dissolved in 20 mL of DCM to obtain a concentration of ca. 20 mg mL^{-1} of lipids compounds and stored at – 20°C until fractionation.

2.4.2. Silica gel purification

About 10 g of silica gel was preconditioned with ca. 50 mL of a mixture of cyclohexane/DCM (2:1, by vol) and loaded into a 35 mL chromatography column equipped with a glass frit and a pre-washed (with DCM) cotton wool at the bottom. Aliquots of 5 mL of total lipid extract, corresponding to ca. 100 mg, were made up to a final volume of 15 mL in cyclohexane to obtain of final ratio of cyclohexane/DCM of 2:1 by volume before loading on the silica column. Nonpolar compounds were eluted with 30 mL of a mixture of cyclohexane/DCM (2:1, by vol) and the stanol-containing polar fraction was eluted with 40 mL of a mixture of DCM/Methanol (MeOH, 1:1, by vol). On average, this fraction accounted for 70 % of total lipids. For all samples, the elution was completed with pressurized air. For methods 1 and 3, the polar fraction of interest containing stanols was then dried under reduced pressure and weighed for quantification.

2.4.3. Aqueous extraction

For the method 2, freeze-dried tissues were extracted with 50 mL of ultra-pure water to obtain a ratio of 10 mL per gram of sample, and extractions were performed with stirrers at ambient temperature overnight [39]. Then the separation of the solid residue from the aqueous extract was performed by centrifugation (2 x 15 min at 3500 rpm and 10°C, Rotenta 460 R centrifuge, Hettich, Tuttlingen, Germany). The solid residue was freeze-dried prior to lipid extraction and the aqueous extract filtered through a 0.7µm glass-fiber filter. In order to analyse the amount of fecal stanols removed from the sample by the water purification step, solid phase extractions were performed on aqueous extracts as described by Jeanneau et al. [39].

2.4.4. Aminopropyl-bonded silica gel purification

For method 3, the polar fraction eluted on the silica gel column was loaded on a chromatography column containing about 10 g of aminopropyl-bonded silica gel (NH_2) preconditioned with ca. 50 mL of a mixture of DCM/MeOH (1:1, by vol). The fraction containing stanols was eluted with 30 mL of a mixture of DCM/MeOH (1:1, by vol), dried under reduced pressure and weighted for quantification.

2.2.5. Stanols analysis by gas chromatography-mass spectrometry (GC-MS)

Stanols were derivatizated using a mixture of BSTFA + TMCS (99:1, by vol) at 60°C during 20 minutes to convert hydroxyl groups into trimethylsilyl ether groups.

Stanols as trimethylsilyl ethers were analysed by GC–MS with a Shimadzu QP2010 + MS gas chromatograph/mass spectrometer (Shimadzu, Tokyo, Japan). 1 µL of samples was injected in splitless mode at 310°C. The temperature of the ionization source was set at 200°C. The temperature of the transfer line was set at 250°C, and molecules were ionized by electron impact using an energy of 70 eV. Separation was achieved using a fused silica column coated with SLB-5 MS (Supelco, 60 m, i.d. 0.25 mm, film thickness 0.25 µm) with helium as carrier gas at a flow of 1 mL min^{-1}. The GC oven temperature was maintained at 70°C for 1 min, then increased to 130°C at 15 °C min^{-1}, then to 300°C at 3°C min^{-1} and held at this temperature for 15 min.

Identification of stanols was based on the comparison with mass spectra and retention times of standards. Analyses were performed in selective ion monitoring mode, the identified and quantified stanols were coprostanol, cholestanol, campestanol, stigmastanol, 5β-stigmastanol, sitostanol, 24-ethylcoprostanol and 24-ethylepicoprostanol and (Table 1 and 2). Figure 2 presents the structures of coprostanol, sitostanol and other stanols involved in this study. As 24-ethylcoprostanol eluted with other compounds, the mass fragmentogram of this

signal (main fragments m/z: 253, 296, 343, 386, 470) was a combination of the mass fragmentogram of those coeluted compounds. Therefore, the 215 fragment used to quantify stanols could originate from another compound and was not used here. As the 398 fragment is used as an identification fragment for 24-ethylcoprostanol and as its intensity was similar to that of the 215 fragment for all calibration solutions, it has been used here as the quantification fragment.

Quantification was based on the internal standard 5α-cholestane, which was added to samples after extraction and fractionation steps and prior to derivatization [12, 14, 15, 22, 39] In opposition to recovery standards spiked in oysters (coprostanol and sitostanol) that were used to quantify the efficiency of extraction procedures and to evaluate matrix effects, the internal standard was used to evaluate losses of sensivity of the detection with GC-MS. The quantification method used a five-point calibration curve (standards: coprostanol, cholestanol and sitostanol) at concentrations of 1, 3, 5, 8, and 10 μg mL^{-1} with a constant internal standard concentration of 5 μg mL^{-1}. Considering the mass of samples and dilutions performed during the analytical procedures, the limits of quantification for stanols analysed in oysters ranged from 5 to 50 μg g^{-1} DW. Linearity of calibration curves, detection limits of GC-MS and fragment used for the quantification of stanols are described in the Table 1.

The recoveries of spiked coprostanol and sitostanol (recovery standards) were calculated as follows:

2.5. Escherichia coli analysis

In order to investigate the level of fecal contamination of oysters from the Fresnaye bay, the concentration of the fecal indicator bacteria *Escherichia coli* was determined by IFREMER (Laboratoire National de Référence , Nantes, France) using the impedance method [41].

2.6. Statistical analyses

The analyses were conducted on three replicates for each sample for the comparison of the efficiencies of the three analytical pathways on the recoveries of both recovery standards and on two replicates for oysters from the Fresnaye bay. As non-parametric tests can lead to the conclusion that observed differences are not significant whereas qualitative differences are evident for low replication, comparison of stanol concentrations between samples were only qualitative.

Stanol fingerprints of oysters from the Fresnaye bay were investigated using the principal component analysis (PCA) model set up by Derrien et al. [22] with XLSTAT 2013 (Addinsoft, Paris, France). Briefly, this model is based on the distribution of six main fecal stanols (i.e., coprostanol, epicoprostanol, 24-ethylcoprostanol, 24 ethylepicoprostanol, campestanol, and sitostanol) of 88 various samples from bovine, porcine (faeces, manures, slurries…) and human origin (raw and diluted waste water treatment plant effluent, sewage sludges). The PCA plot is a two-dimensional graphic representation of the correlations between the 6 stanols (variables). This plan is built on two axis (principal components) F1 and F2, which explain 78.3 % of the total variance of the model. Each of the 6 stanol distribution contributes to F1 and F2 axis. This model distinguish the stanol fingerprints from the three previous origins into three distinctive clusters. Based, on their abundance of the 6 previous stanols, the coordinates of samples on the PCA plots are calculated as follows:

- F1 coordonate = 0.497(%coprostanol) – 0.347(%epicoprostanol) + 0.295(%ethylepicoprostanol) – 0.460(%ethylepicoprostanol) – 0.422(%sitostanol) – 0.395(%campestanol)
- F2 coordonate = – 0.074(%coprostanol) + 0.565(%epicoprostanol) + 0.531(%ethylcoprostanol) – 0.303(%ethylepicoprostanol) – 0.288(%sitostanol) + 0.468(%campestanol).

These equations allow the identification of the origin of fecal contamination in environmental matrix between, bovine, porcine and human contaminations using this PCA model.

3. Results and Discussion

3.1. Method comparison

3.1.1. Water extraction

Non-lipid compounds can interact with lipids and decrease the efficiency of their extraction from the sample matrix or their separation during solid-phase chromatography. Thus, the addition of an extraction step with water is expected to increase the recovery of target lipids. To investigate the effects of water extractions on the recovery of coprostanol and sitostanol in oysters, we compared the method 1 (organic extraction and silica gel purification) to the method 2 (water extraction, organic extraction and silica gel purification). Figure 4 presents recoveries of both coprostanol and sitostanol for the two methods tested.

The recovery of coprostanol extracted with the method 2 (59 ± 10 %, mean ± standard deviation, SD) was lower than that extracted with the method 1 (79 ± 8 %) and the two methods led to similar repeatabilities. Similarly, the recovery of sitostanol extracted with the

314 method 2 (47 ± 13 %) was lower than that extracted with the method 1 (84 ± 8 %) and the
315 repeatability of the method 2 was lower than that of the method 1. Contrary to our hypothesis,
316 the recoveries of both coprostanol and sitostanol extracted with the method 2 tended to be
317 lower than those of the method 1. This result suggests that the extraction step with water
318 removed more coprostanol and sitostanol than it decreased the potential interactions of these
319 two molecules with non-lipid compounds. Interestingly, the addition of coprostanol and
320 sitostanol quantities analysed in aqueous extracts by solid phase extraction to the quantities of
321 coprostanol and sitostanol in oyster tissues extracted with the method 2 led to quite similar
322 recoveries (61 *versus* 59 % for coprostanol and 54 *versus* 47 % for sitostanol) and remained
323 lower than those without the water extraction (method 1). This imbalance could be attributed
324 to the low efficiency of solid phase extraction on aqueous extracts that are very rich in
325 hydrophilic organic compounds, which greatly decrease the efficiency of this method [40].
326 Unfortunately, the efficiency of solid phase extractions could not be checked because of the
327 coelution of the recovery standard cholesterol d_6 with cholesterol.
328
329 *3.1.2. Aminopropyl-bonded silica gel (NH_2) purification*
330 Oysters contain high amounts of lipids from different classes that can potentially interact
331 each other's and decrease the efficiency of their analysis [32, 33, 37]. The effects of a
332 purification step with NH_2 was tested in order to remove as much as compounds of non-
333 interest as possible. The comparison of the efficiency of the methods 1 (organic extraction and
334 silica gel purification) and 3 (organic extraction, silica gel and NH_2 purifications) allows the
335 investigation of the impact of this second purification step.
336 The recoveries of coprostanol were 79 ± 8 % and 89 ± 15 % for the methods 1 and 3,
337 respectively and the recoveries of sitostanol were 84 ± 8 %, 103 ± 70 % for the methods 1 and
338 3, respectively (Figure 4). The addition of this second chromatographic step involving

aminopropyl-bonded silica seems to induce an increase of the recoveries of both coprostanol and sitostanol. However, the standard deviation between the triplicates highlights that the values of the recoveries belong to the same group and that the methods appear to be not different. The repeatability of the methods 1 and 3 can be inferred from the value of the relative standard deviation. For the method 1, the relative standard deviation represented 10 and 9% of the mean value for coprostanol and sitostanol, respectively, while for method 3 it represented 17 and 68% of the mean value for coprostanol and sitostanol, respectively.

3.1.3. Comparison of the three methods

The first goal of the present study was to determine an efficient method for the analysis of fecal stanols in the oyster *Crassostrea gigas*.

Water extraction led to opposite trends on coprostanol and sitostanol recoveries and increased their respective standard deviation. Thus, the addition of this step on the extraction pathway i) did not increase the recovery of both recovery standards and ii) decreased their repeatabilities. As a consequence, water extraction prior to organic extraction is not reliable for the analysis of fecal stanols in oysters.

The addition of a purification step on NH_2 increased the recoveries of coprostanol and sitostanol. Nevertheless, this step strongly decreased the repeatabilities of the methods tested, especially for sitostanol. Therefore, this step does not appear to be reliable for the analysis of fecal stanols in oysters.

Finally, among the three methods tested, , the method 1, which included a lipid extraction step with organic solvent, a purification step on silica gel and analysis by GC-MS, led to i) statistically similar recoveries than the others two methods, ii) the higher repeatability and iii) similar recoveries for both recovery standards. Moreover, the recovery of coprostanol with the

363 method 1 (79%) is higher than that found by Cathum and Sabik [27] (48%) probably because
364 these authors analysed coprostanol by GC-MS as underivatized compound.

365 In order to further improvement of this method and solvent and sorbent savings, stanol
366 extraction efficiency could be investigate using pre-packed silica cartridges available for
367 solid-phase extraction.

368 Finally, the method 1 has been chosen to analyse the concentration of fecal stanols in
369 natural oysters sampled at the Fresnaye bay.

370

371 *3.2. Stanol occurrence and concentrations in natural oysters from the Fresnaye bay*

372 Table 2 presents the concentrations of the stanols quantified in oysters sampled in
373 February, March and August 2013.

374 Eight stanols were detected and quantified in the samples analysed. Cholestanol was the
375 major compound and ranged from 58.4 µg g^{-1} DW (August) to 221.8 µg g^{-1} DW (February).
376 As these concentrations were above the upper limit of quantification of our method (i.e. 50 µg
377 g^{-1} DW), they were just qualitatively discussed in comparison to the concentrations of the
378 other stanol found in oysters. The other stanols detected and quantified were coprostanol, 5β-
379 stigmastanol, 24-ethylcoprostanol, 24-ethylepicoprostanol, campestanol, stigmastanol and
380 sitostanol and their concentrations ranged from 7.5 to 21.7 µg g^{-1} DW. The predominance in
381 *Crassostrea gigas* of cholestanol compared to other stanols is not surprising since its
382 precursor, cholesterol, is the main sterol in oysters [42-44]. Dunstan et al. [43] found
383 concentration of cholestanol of ca. 112 µg g^{-1} DW for *Crassostrea gigas* that is our range of
384 concentrations for cholestanol. Since cholestanol have been rarely found or in very low
385 concentrations in the diet of bivalves largely dominated by phytoplankton [45, 46], its high
386 relative abundance in bivalves might be due to the bioconversion of cholesterol during the
387 digestive process by the presence of gut bacteria [47]. However, cholesterol might not be the

388 only dietary sterol supplied by food leading to the formation of cholestanol within bivalves.
389 Indeed, it has been shown that marine bivalves are able to bioconvert several dietary sterols
390 into cholesterol for physiological needs [43, 48]. Furthermore, the high variability of
391 cholestanol concentrations between oyster samples from date to date are accompanied by high
392 variations of total stanol concentrations, which ranged from 145.0 µg g^{-1} DW (August) to
393 297.6 µg g^{-1} DW (February). These variations might be due to the differences in physico-
394 chemical conditions of surrounding seawater between sampling dates that could have led to
395 different metabolic responses of oysters resulting in different stanol concentrations.
396
397 *3.3. Fecal contamination and stanol fingerprint of oysters*
398 The concentration of *Escherichia coli* in oysters sampled in February and March was
399 respectively 67 and 220 Most Probable Number 100 g^{-1} (Table 2). According to the European
400 Shellfish Directive on shellfish harvesting (854/2004/EC), these amounts of *Escherichia coli*
401 classified the two previous samples in the A class and oysters collected in February and
402 March were considered as non-fecally contaminated. With a concentration of *Escherichia coli*
403 of ca. 9150 Most Probable Number 100 g^{-1}, oysters sampled in August were classified in the
404 B class and considered here as fecally contaminated.
405 In order to investigate the ability of oysters to record a species-specific fecal
406 contamination by bioaccumulation using their stanol fingerprint, the relative proportions of
407 fecal stanols of the three samples were injected in the PCA developed by Derrien et al. [14].
408 Stanol fingerprints of oysters sampled in February (F1 and F2) and March (M1 and M2) were
409 located between the bovine and the human clusters (Figure 5). This absence of a specific
410 fingerprint is consistent with the absence of a fecal contamination of these samples measured
411 with *Escherichia coli*. By contrast, the fecally contaminated oysters sampled in August (A1
412 and A2) showed specific stanol fingerprints located in the bovine cluster (Figure 5). The

absence of a specific stanol fingerprint of oysters when they are not fecally contaminated in addition to the specific stanol fingerprint of oysters fecally contaminated suggests that these organisms could be able to record a species-specific stanol fingerprint when they are exposed to a fecal contamination high enough. The bovine fingerprint of oysters sampled in August suggests that the fecal contaminations transferred from the watershed to seawater and bioaccumulated by oysters during this period would mainly originate from bovine sources. This hypothesis is consistent with the agricultural activity of the watershed where livestock farming of cows is not negligible with ca. 5300 heads of livestock in 2010 [38]. The contamination of oysters by bovine sources suggested by their specific stanol fingerprint in August could be explained by agricultural practices and manure spreading calendar. Indeed, during summer, cows are grazing on grassland and thought lixiviation and erosion of soils during raining events, even low, their faeces can be directly transported to streams that flow into the bay. In August, pig slurry spreading is forbidden, so the large quantities of pig slurry produced by pig farming (ca. 235.000 pigs in 2010 in the watershed) remain stored, limiting the fecal contamination of soils, streams and finally shellfish by this source.

In conclusion, the method developed here enables to analyse the concentration of eight fecal stanols in oysters and to record and identify the main source of fecal contamination of oysters using their stanol fingerprint with PCA. Stanol fingerprint could then be used as a microbial source tracking tool in oysters to track the origin of the fecal contamination in shellfish in order to enhance watershed management and reduce health risks linked to the consumption of contaminated shellfish.

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442

References

1. Fong T-T, Lipp EK (2005) Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. Microbiol Mol Biol Rev 69:357–71. doi: 10.1128/MMBR.69.2.357-371.2005
2. Hundesa A, Maluquer de Motes C, Bofill-Mas S, et al. (2006) Identification of human and animal adenoviruses and polyomaviruses for determination of sources of fecal contamination in the environment. Appl Environ Microbiol 72:7886–93. doi: 10.1128/AEM.01090-06
3. Soller J, Embrey M, Tuhela L, et al. (2010) Risk-based evaluation of Escherichia coli monitoring data from undisinfected drinking water. J Environ Manage 91:2329–35. doi: 10.1016/j.jenvman.2010.06.017
4. Zatta P, Gobbo S, Rocco P, et al. (1992) Evaluation of heavy metal pollution in the Venetian lagoon by using Mytilus galloprovincialis as biological indicator. Sci Total Environ 119:29–41. doi: 10.1016/0048-9697(92)90253-O
5. Baudrimont M, Schäfer J, Marie V, et al. (2005) Geochemical survey and metal bioaccumulation of three bivalve species (Crassostrea gigas, Cerastoderma edule and Ruditapes philippinarum) in the Nord Médoc salt marshes (Gironde estuary, France). Sci Total Environ 337:265–280.
6. Goulletquer P, Le Moine O (2002) Shellfish farming and coastal zone management (CZM) development in the Marennes-Oleron Bay and Charentais Sounds (Charente Maritime, France): A review of recent developments. Aquac Int 10:507–525. doi: 10.1023/A:1023975418669

7. Mieszkin S, Caprais MP, Le Mennec C, et al. (2013) Identification of the origin of fecal contamination in estuarine oysters using Bacteroidales and F-specific RNA bacteriophage markers. J Appl Microbiol 115:897–907. doi: 10.1111/jam.12260
8. Simpson JM, Santo Domingo JW, Reasoner DJ (2002) Microbial Source Tracking: State of the Science. Environ Sci Technol 36:5279–5288. doi: 10.1021/es026000b
9. Glassmeyer ST, Furlong ET, Kolpin DW, et al. (2005) Transport of Chemical and Microbial Compounds from Known Wastewater Discharges: Potential for Use as Indicators of Human Fecal Contamination. Environ Sci Technol 39:5157–5169. doi: 10.1021/es048120k
10. Cimenti M, Hubberstey A, Bewtra J, Biswas N (2007) Alternative methods in tracking sources of microbial contamination in waters. Water SA 33:183–194.
11. Field KG, Samadpour M (2007) Fecal source tracking, the indicator paradigm, and managing water quality. Water Res 41:3517–38. doi: 10.1016/j.watres.2007.06.056
12. Jardé E, Gruau G, Mansuy-Huault L, et al. (2006) Using Sterols to Detect Pig Slurry Contribution to Soil Organic Matter. Water Air Soil Pollut 178:169–178. doi: 10.1007/s11270-006-9188-9
13. Ahmad F, Tourlousse DM, Stedtfeld RD, et al. (2009) Detection and Occurence of Indicator Organisms and Pathogens. Water Environ Res 81:959–980. doi: 10.2175/106143009X12445568399299
14. Derrien M, Jardé E, Gruau G, et al. (2012) Origin of fecal contamination in waters from contrasted areas: stanols as Microbial Source Tracking markers. Water Res 46:4009–16. doi: 10.1016/j.watres.2012.05.003
15. Biache C, Philp RP (2013) The use of sterol distributions combined with compound specific isotope analyses as a tool to identify the origin of fecal contamination in rivers. Water Res 47:1201–8. doi: 10.1016/j.watres.2012.11.037

16. Chan K-H, Lam MHW, Poon K-F, et al. (1998) Application of sedimentary fecal stanols and sterols in tracing sewage pollution in coastal waters. Water Res 32:225–235. doi: 10.1016/S0043-1354(97)00175-9
17. Leeming R, Ball A, Ashbolt N, Nichols P (1996) Using fecal sterols from humans and animals to distinguish fecal pollution in receiving waters. Water Res 30:2893–2900. doi: 10.1016/S0043-1354(96)00011-5
18. Bull ID, Lockheart MJ, Elhmmali MM, et al. (2002) The origin of faeces by means of biomarker detection. Environ Int 27:647–654. doi: 10.1016/S0160-4120(01)00124-6
19. Tyagi P, Edwards DR, Coyne MS (2009) Distinguishing between human and animal sources of fecal pollution in waters: a review. Int J Water 5:15. doi: 10.1504/IJW.2009.023080
20. Gourmelon M, Caprais MP, Le Mennec C, et al. (2010) Application of library-independent microbial source tracking methods for identifying the sources of fecal contamination in coastal areas. Water Sci Technol 61:1401. doi: 10.2166/wst.2010.033
21. Shah VG, Dunstan RH, Geary PM, et al. (2007) Evaluating potential applications of fecal sterols in distinguishing sources of fecal contamination from mixed fecal samples. Water Res 41:3691–700. doi: 10.1016/j.watres.2007.04.006
22. Derrien M, Jarde E, Gruau G, Pierson-Wickmann A-C (2011) Extreme variability of steroid profiles in cow feces and pig slurries at the regional scale: implications for the use of steroids to specify fecal pollution sources in waters. J Agric Food Chem 59:7294–302. doi: 10.1021/jf201040v
23. Water National Agency of Loire-Brittany. (accessed Jan. 2014). http://www.eau-loire-bretagne.fr/informations_et_donnees/reseaux_de_mesure

516 24. Solecki O, Jeanneau L, Jardé E, et al. (2011) Persistence of microbial and chemical
517 pig manure markers as compared to fecal indicator bacteria survival in freshwater and
518 seawater microcosms. Water Res 45:4623–33. doi: 10.1016/j.watres.2011.06.012
519 25. Jeanneau L, Solecki O, Wéry N, et al. (2012) Relative decay of fecal indicator bacteria
520 and human-associated markers: a microcosm study simulating wastewater input into
521 seawater and freshwater. Environ Sci Technol 46:2375–82. doi: 10.1021/es203019y
522 26. Sherwin MR, Van Vleet ES, Fossato VU, Dolci F (1993) Coprostanol (5β-cholestan-
523 3β-ol) in lagoonal sediments and mussels of Venice, Italy. Mar Pollut Bull 26:501–
524 507. doi: 10.1016/0025-326X(93)90467-X
525 27. Cathum S, Sabik H (2001) Determination of steroids and coprostanol in surface water,
526 effluent and mussel using gas chromatography-mass spectrometry. Chromatographia
527 53:S394–S399. doi: 10.1007/BF02490364
528 28. Gagné F, Blaise C, Aoyama I, et al. (2002) Biomarker study of a municipal effluent
529 dispersion plume in two species of freshwater mussels. Environ Toxicol 17:149–59.
530 doi: 10.1002/tox.10046
531 29. Gagné F, Blaise C, Lachance B, et al. (2001) Evidence of coprostanol estrogenicity to
532 the freshwater mussel Elliptio complanata. Environ Pollut 115:97–106. doi:
533 10.1016/S0269-7491(01)00089-6
534 30. Hellou J, Yeats P, Steller S, Gagné F (2003) Chemical contaminants and biological
535 indicators of mussel health during gametogenesis. Environ Toxicol Chem 22:2080.
536 doi: 10.1897/02-396
537 31. Yeats P, Gagné F, Hellou J (2008) Body burden of contaminants and biological effects
538 in mussels: an integrated approach. Environ Int 34:254–64. doi:
539 10.1016/j.envint.2007.08.009

32. Linehan L., O'Connor T., Burnell G (1999) Seasonal variation in the chemical composition and fatty acid profile of Pacific oysters (Crassostrea gigas). Food Chem 64:211–214. doi: 10.1016/S0308-8146(98)00144-7
33. Costil K, Royer J, Ropert M, et al. (2005) Spatio-temporal variations in biological performances and summer mortality of the Pacific oyster Crassostrea gigas in Normandy (France). Helgol Mar Res 59:286–300. doi: 10.1007/s10152-005-0004-5
34. Danger M, Allard B, Arnous M, et al. (2012) Effects of food-web structure on the quantity and the elemental quality of sedimenting material in shallow lakes. Hydrobiologia 679:251–266. doi: 10.1007/s10750-011-0890-2
35. Guenet B, Danger M, Harrault L, et al. (2014) Fast mineralization of land-born C in inland waters: first experimental evidences of aquatic priming effect. Hydrobiologia 721:35–44. doi: 10.1007/s10750-013-1635-1
36. Lacaze J-PCL, Stobo LA, Turrell EA, Quilliam MA (2007) Solid-phase extraction and liquid chromatography--mass spectrometry for the determination of free fatty acids in shellfish. J Chromatogr A 1145:51–7. doi: 10.1016/j.chroma.2007.01.053
37. Pernet F, Pelletier CJ, Milley J (2006) Comparison of three solid-phase extraction methods for fatty acid analysis of lipid fractions in tissues of marine bivalves. J Chromatogr A 1137:127–37. doi: 10.1016/j.chroma.2006.10.059
38. French Ministry of Agriculture, Agri-food and Forests (accessed Jan. 2014). http://www.acces.agriculture.gouv.fr/cartostat/#v=map2;i=stru1.saumoy10;l=fr
39. Jeanneau L, Jardé E, Gruau G (2011) 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. J Chromatogr A 1218:2513–20. doi: 10.1016/j.chroma.2011.02.066

564 40. Landgraf D, Leinweber P, Makeschin F (2006) Cold and hot water-extractable organic
565 matter as indicators of litter decomposition in forest soils. J plant Nutr ans soil Sci
566 169:76–82. doi: 10.1002/jpin.200521711
567 41. Dupont J, Dumont F, Menanteau C, Pommepuy M (2004) Calibration of the
568 impedance method for rapid quantitative estimation of Escherichia coli in live marine
569 bivalve molluscs. J Appl Microbiol 96:894–902. doi: 10.1111/j.1365-
570 2672.2004.02218.x
571 42. Gordon DT, Collins N (1982) Anatomical distribution of sterols in oysters
572 (Crassostrea gigas). Lipids 17:811–817. doi: 10.1007/BF02535358
573 43. Dunstan GA, Volkman JK, Barrett SM (1993) The effect of lyophilization on the
574 solvent extraction of lipid classes, fatty acids and sterols from the oysterCrassostrea
575 gigas. Lipids 28:937–944. doi: 10.1007/BF02537504
576 44. Knauer J, Kerr RG, Lindley D, Southgate PC (1998) Sterol Metabolism of Pacific
577 Oyster (Crassostrea gigas) Spat. Comp Biochem Physiol Part B Biochem Mol Biol
578 119:81–84. doi: 10.1016/S0305-0491(97)00269-1
579 45. Nishimura M, Koyama T (1977) The occurrence of stanols in various living organisms
580 and the behavior of sterols in contemporary sediments. Geochim Cosmochim Acta
581 41:379–385. doi: 10.1016/0016-7037(77)90265-4
582 46. Volkman JK (2003) Sterols in microorganisms. Appl Microbiol Biotechnol 60:495–
583 506. doi: 10.1007/s00253-002-1172-8
584 47. Harris JM (1993) The presence, nature, and role of gut microflora in aquatic
585 invertebrates - a synthesis. Microb Ecol 25:195–231.
586 48. Berenberg CJ, Patterson GW (1981) The relationship between dietary phytosterols and
587 the sterols of wild and cultivated oysters. Lipids 16:276–278. doi:
588 10.1007/BF02535028

589 Figure legends:

590

591 Figure 1: Location of oyster sampling sites, streams, towns and waste water treatment plants
592 (WWTP) in the Fresnaye watershed, Brittany, France.

593

594 Figure 2: Generalized structure of a stanol. The distinction between main fecal stanols
595 involves four points: i) the orientation (α or β) of the hydrogen atom at the position C-5
596 (mediated by the anaerobic reduction of the double bond located at the same position in the
597 corresponding unsaturated sterol precursor), ii) the orientation (α or β) of the hydroxyl group
598 at the position C-3, iii) the occurrence of methyl or ethyl groups at position C-24 (denoted by
599 R), and iv) the occurrence of a double bond at position C-22.

600

601 Figure 3: Schematic representation of the three analytical pathways used for the extraction
602 and the purification of fecal stanols from oyster samples (blanks and spiked ones) and their
603 subsequent analysis by GC–MS.

604

605 Figure 4: Comparison of coprostanol and sitostanol recoveries in oysters for the three
606 extraction methods. Error bars are standard deviations (n = 3). Values under the error bars are
607 relative standard deviations. The dotted line represents the 100% recovery threshold.

608

609 Figure 5: Plot of the principal component analysis comparing the 88 source samples and the 6
610 oyster samples using the 6 most discriminant stanol compounds proposed by Derrien et al.

611 (2012). Each source samples was used as individual and oyster samples were used as

612 supplementary individuals. F1 axis: principal component 1; F2 axis: principal component 2.

Figure 1 :

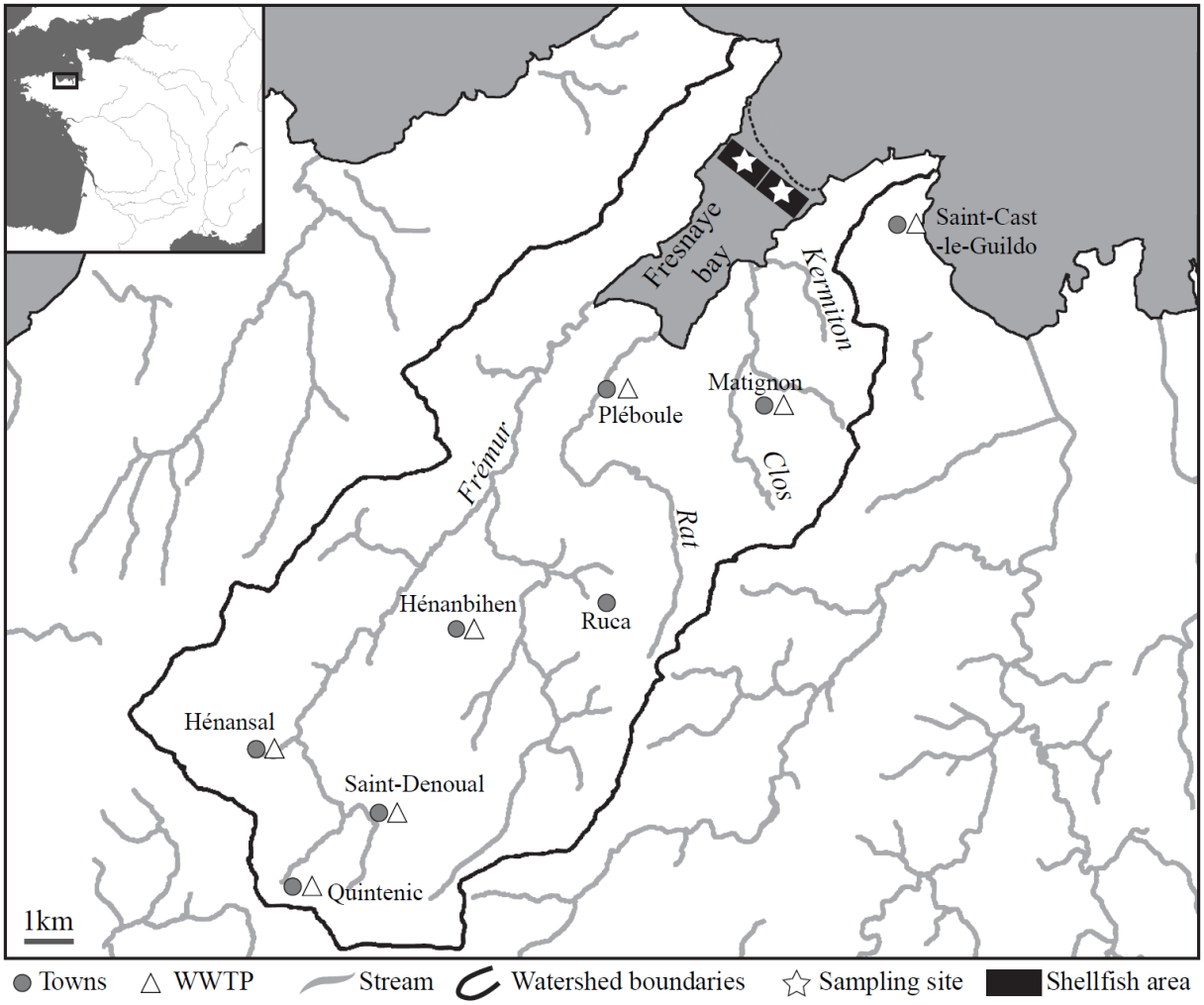
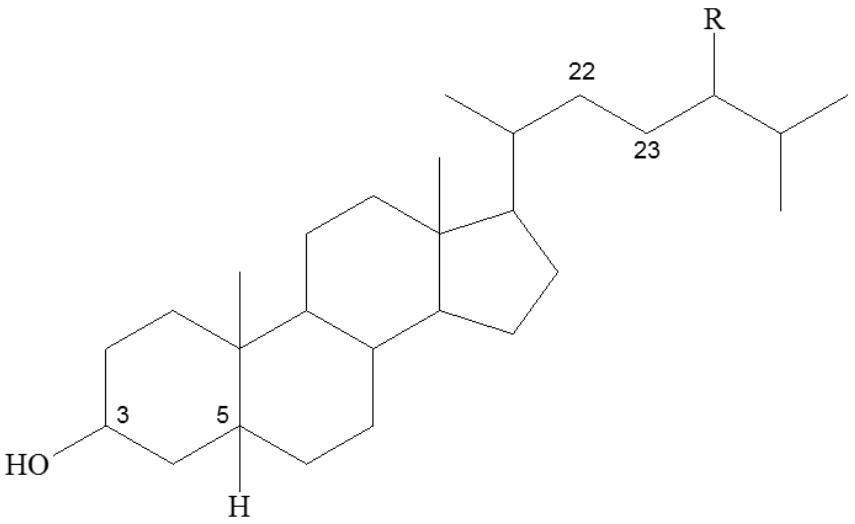

Saint-Cast
-le-Guildo
Fresnaye
bay
Kerniton
Matignon
Pléboule
Frémur
Clos
Rat
Hénanbihen
Ruca
Hénansal
Saint-Denoual
Quintenic
1km
Towns
WWTP
Stream
Watershed boundaries
Sampling site
Shellfish area

Figure 2 :



R = H
3C-OH = β
5C-H = β
Coprostanol

R = C_2H_5
3C-OH = β
5C-H = β
24-Ethylcoprostanol

R = H
3C-OH = β
5C-H = α
Cholestanol

R = C_2H_5
3C-OH = α
5C-H = β
24-Ethylepicoprostanol

R = CH_3
3C-OH = β
5C-H = α
Campestanol

R = C_2H_5
3C-OH = β
5C-H = α
Sitostanol

R = C_2H_5
3C-OH = β
5C-H = α
22C-23C = double bond
Stigmastanol

R = C_2H_5
3C-OH = β
5C-H = β
22C-23C = double bond
5β-Stigmastanol

Figure 3 :

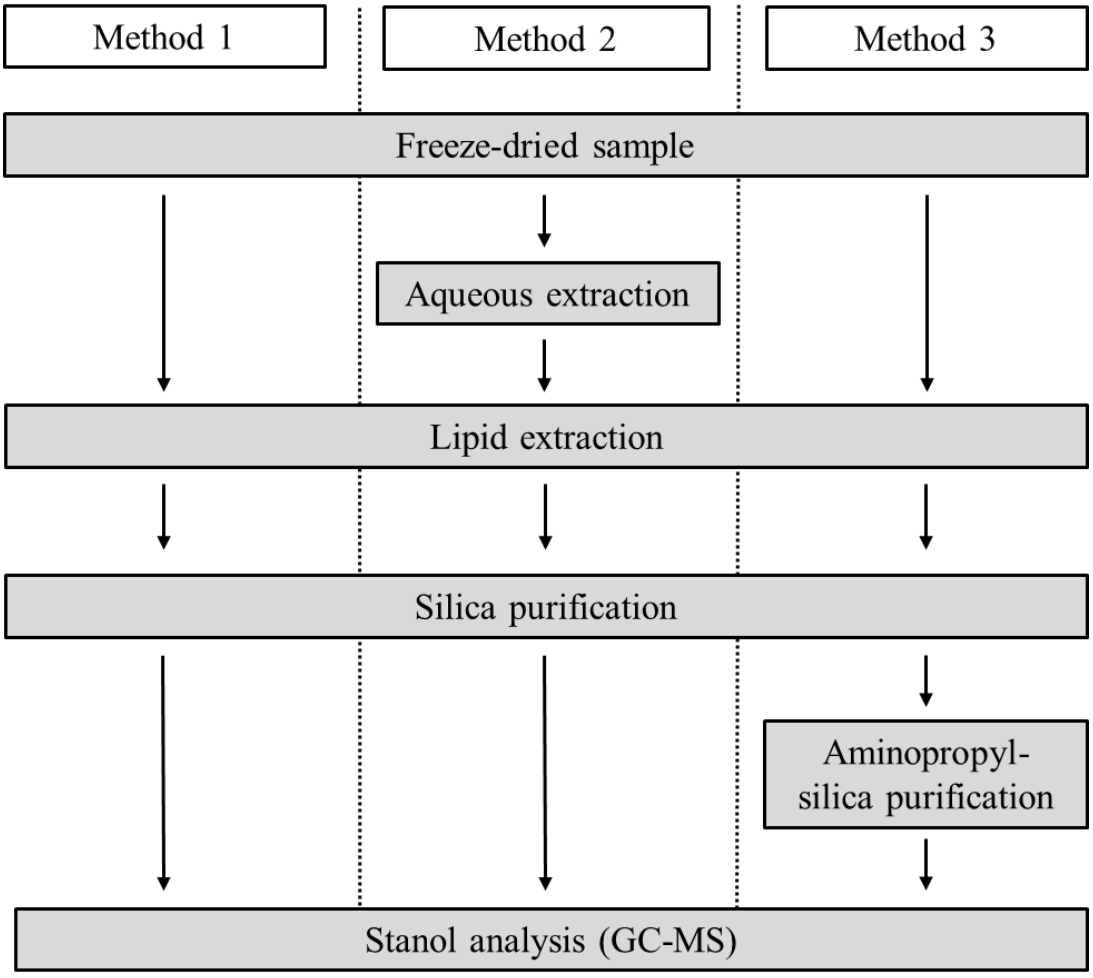

Method 1
Method 2
Method 3
Freeze-dried sample
Aqueous extraction
Lipid extraction
Silica purification
Aminopropyl-
silica purification
Stanol analysis (GC-MS)

Figure 4 :

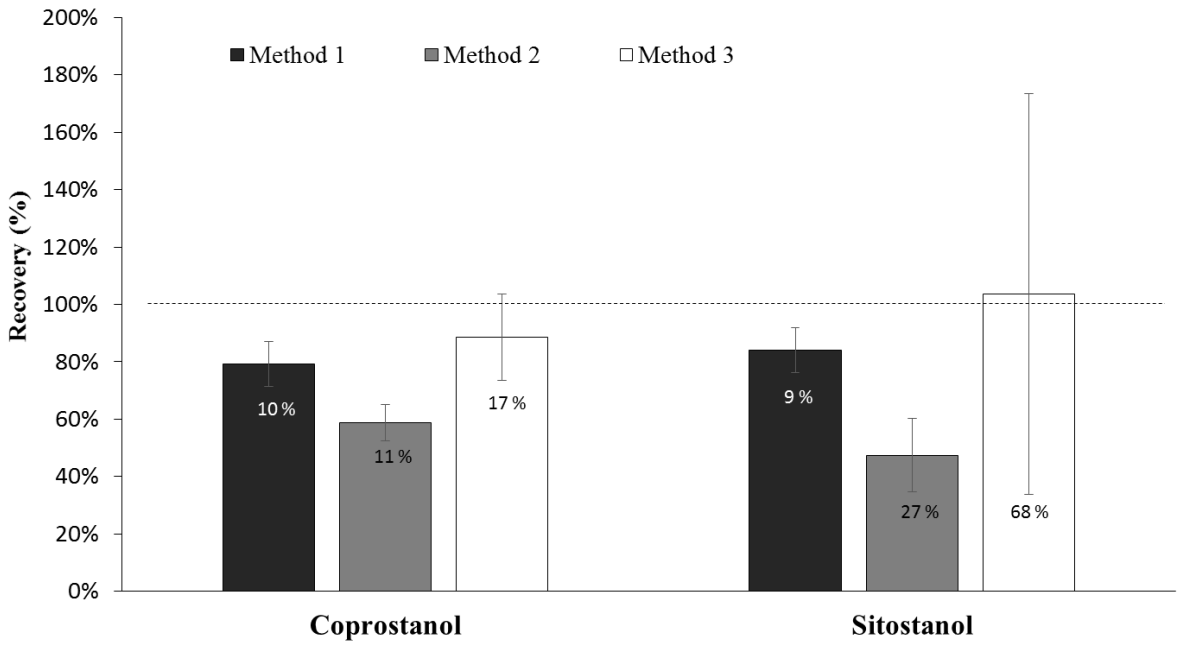

200%
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
Recovery (%)
Method 1
Method 2
Method 3
10 %
11 %
17 %
9 %
27 %
68 %
Coprostanol
Sitostanol

Figure 5 :

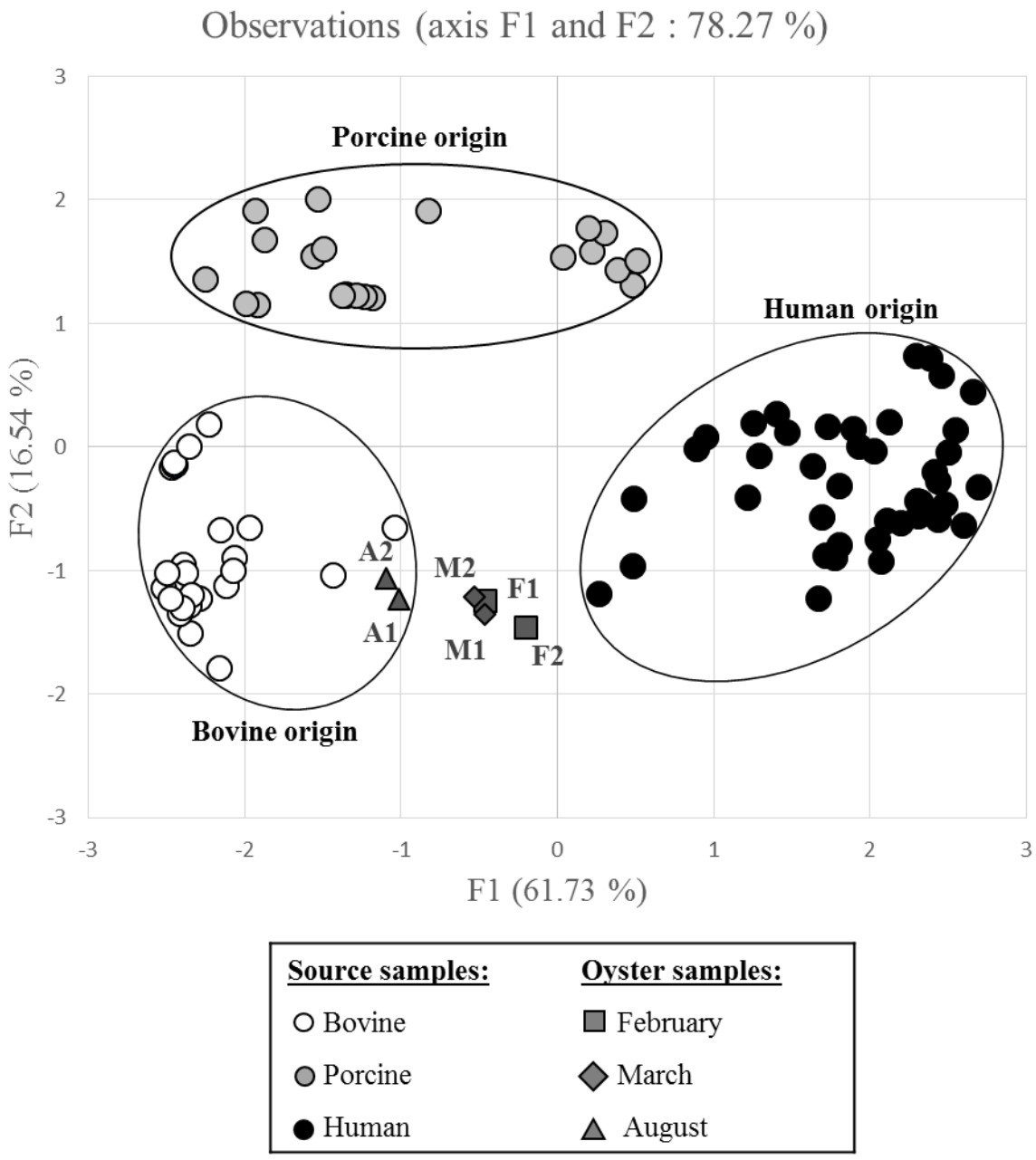

Observations (axis F1 and F2 : 78.27 %)
Porcine origin
Human origin
Bovine origin
A2
A1
M2
M1
F1
F2
F2 (16.54 %)
F1 (61.73 %)
3
2
1
0
-1
-2
-3
-3
-2
-1
0
1
2
3
Source samples:
Bovine
Porcine
Human
Oyster samples:
February
March
August

Table 1: Trivial and IUPAC names, retention times relative to cholestanol (RRT), m/z values used for the identification and quantification of stanols, and information on quantification compounds (standard used, linearity of the corresponding calibration curve, and limit of detection).

Trivial name	IUPAC name	RRT	Fragment m/z		Quantification details		
			Identification	Quantification	Standard	Linearity	LD[a] (ppb)
Coprostanol	5β-Cholestan-3β-ol	0.956	257, 355, 370	215	Coprostanol	0.998	5
Cholestanol	5α-Cholestan-3β-ol	1.000	257, 355, 384	215	Cholestanol	0.997	10
Campestanol	24-Methyl-5α-cholestan-3β-ol	1.050	369, 398, 484	215	Coprostanol	0.998	5
Stigmastanol	24-Ethyl-5α-cholesta-22-en-3β-ol	1.063	215, 383	215	Coprostanol	0.998	5
5β-Stigmastanol	24-Ethyl-5β-cholesta-22-en-3β-ol	1.019	257, 353, 486	215	Coprostanol	0.998	5
Sitostanol	24-Ethyl-5α-cholestan-3β-ol	1.097	383, 398, 473	215	Sitostanol	0.995	10
24-Ethylcoprostanol	24-Ethyl-5β-cholestan-3β-ol	1.041	257, 383, 398	398	Coprostanol	0.994	10
24-Ethylepicoprostanol	24-Ethyl-5β-cholestan-3α-ol	1.047	257, 283, 398	215	Coprostanol	0.998	5

[a] Limit of Detection

Table 2 : Stanol (µg g^{-1} DW) and *Escherichia coli* (Most Probable Number 100 g^{-1} of meat and shell liquor) concentrations of oysters from the Fresnaye bay. Errors are standard deviations (n = 2).

Concentration	Sampling date		
	February	March	August
Coprostanol	7.9 ± 1.4	7.4 ± 0.1	10.4 ± 1.2
Cholestanol	221.8 ± 83.0	110.3 ± 16.3	58.4 ± 12.0
Campestanol	16.1 ± 0.7	10.8 ± 0.2	12.2 ± 0.3
Stigmastanol	8.0 ± 2.0	8.9 ± 1.0	10.7 ± 0.8
5β-Stigmastanol	9.6 ± 0.8	7.8 ± 0.1	10.6 ± 1.0
Sitostanol	13.1 ± 1.8	13.9 ± 1.2	21.7 ± 0.3
24-Ethylcoprostanol	7.6 ± 1.7	7.5 ± 0.5	9.6 ± 1.3
24-Ethylepicoprostanol	13.4 ± 0.2	9.4 ± 0.5	11.4 ± 0.6
Total	297.6 ± 91.7	175.9 ± 19.7	145.0 ± 17.5
Escherichia coli	67 ± 0	220 ± 0	9150 ± 9687