

1 Lipids:

2

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

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17 **Abstract**

18 The objective of this work was to study the effects of washing and purification steps
19 on qualitative and quantitative analysis of fecal stanols in the oyster *Crassostrea gigas* using
20 either single or combination of lipid purification steps on silica gel or aminopropyl bonded
21 silica gel (NH₂) or a washing step. Among the three analytical pathways compared, the two
22 including water extraction or NH₂ purification did not lead to higher recoveries and decreased
23 repeatabilities of extractions compared to the single purification on silica gel. This latter led to
24 similar recoveries (ca. 80%) and repeatabilities (ca. 10%) for both spiked standards
25 (coprostanol and sitostanol). This analytical pathway has been applied to oysters collected in a
26 harvesting area in Brittany (France) where fecal contaminations are important and allowed to
27 quantify eight stanols in oysters. The relative proportions of fecal stanols of these oysters
28 were combined with principal component analysis in order to investigate the usefulness of
29 their stanol fingerprints to record a fecal contamination and to distinguish its source between
30 human, porcine and bovine contaminations. Oysters non-fecally contaminated by *Escherichia*
31 *coli* did not present specific stanol fingerprints while oysters fecally contaminated had a
32 bovine fingerprint, suggesting a contamination of these samples by bovine sources. As a
33 consequence, the method developed here allows the use of stanol fingerprints of oysters as a
34 microbial source tracking tool that can be applied to shellfish harvesting areas subjected to
35 fecal contaminations in order to identify the different sources of contamination and improve
36 watershed management.

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38

39	Abbreviations	
40	BSTFA	N,O-bis- (trimethylsilyl)trifluoroacetamide
41	DCM	Dichloromethane
42	DW	Dry weight
43	GC-MS	Gas chromatography- mass spectrometry
44	MeOH	Methanol
45	NH ₂	Aminopropyl-bonded silica gel
46	PCA	Principal component analysis
47	TMCS	Trimethylchlorosilane
48		

50 **1. Introduction**

51

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

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

65 For this purpose, the actual European Shellfish Directive on shellfish harvesting
66 (854/2004/EC) imposes the classification of shellfish and requires the assessment of potential
67 pollution sources upstream of shellfish harvesting. The actual classification is based on the
68 fecal indicator bacteria *Escherichia coli* and enterococci which are not species-specific.

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

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

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

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

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

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

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

113 -The third method comprised of a second purification step of lipids on aminopropyl-
114 bonded silica gel after that on silica gel. The separation of lipid classes from the total
115 extract is mandatory in complex environmental matrix to improve the analysis accuracy of
116 target compounds. The lipid fraction of oysters is a complex mixture containing several
117 lipid classes such as phospholipids, triacylglycerols, free fatty acids, sterols and stanols
118 [36]. Therefore, the addition of a purification step could be particularly interesting in order
119 to remove as much of lipids of non-interest as possible. Aminopropyl-bonded silica gel
120 (NH₂) was chosen as the second sorbent because of its affinity and subsequent selective
121 retention of acidic phospholipids [37].

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

128

129 **2. Experimental**

130

131 *2.1. Reagent and chemicals*

132 Organic solvents were of high performance liquid chromatography grade.
133 Dichloromethane (DCM) was purchased from Carlo-Erba SDS (Val de Reuil, France),
134 methanol (MeOH) and cyclohexane were purchased from VWR (West Chester, PA). N,O-bis-
135 (trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (99:1, by vol) (BSTFA + TMCS)
136 and SPE disks (Supelco ENVI-18DSK, 47mm diameter) were purchased from Supelco (St.
137 Quentin Fallavier, France). Coprostanol (5 β -cholestan-3 β -ol), cholestanol (5 α -cholestan-3 β -
138 ol), 5 α -cholestane and aminopropyl-bonded silica gel were purchased from Sigma (St.
139 Quentin Fallavier, France). Sitostanol (24-ethyl-5 α -cholestan-3 β -ol) was purchased from
140 Steraloids (Newport, RI). 24-Ethylcoprostanol (24-ethyl-5 β -cholestan-3 β -ol) and 24-
141 ethylepicoprostanol (24-ethyl-5 β -cholestan-3 α -ol) were purchased from BCP Instruments
142 (Irigny, France). Silica gel (40-63 μ m) was purchased from Merck (Darmstadt, Germany).
143 Cholesterol d₆ ([2,2,3,4,4,6-²H₆]-cholest-5-en-3 β -ol) was purchased from CDN Isotopes
144 (Pointe-Claire, Quebec, Canada).

145

146 *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 $10 \mu\text{g g}^{-1}$ 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

172 2010), and cows (ca. 5300 head in 2010) [38]. In the last decade, this shellfish harvesting area
173 is subjected to increasing fecal contaminations by these different sources leading to the
174 degradation of the quality of oysters. In February, March and August 2013, oysters were
175 sampled at two locations on the bay and analysed for each sampling date.

176

177 *2.4. Analytical pathways for stanol analysis*

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

185

186 *2.4.1. Lipid extraction*

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

195

196 *2.4.2. Silica gel purification*

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

208

209 *2.4.3. Aqueous extraction*

210 For the method 2, freeze-dried tissues were extracted with 50 mL of ultra-pure water to
211 obtain a ratio of 10 mL per gram of sample, and extractions were performed with stirrers at
212 ambient temperature overnight [39]. Then the separation of the solid residue from the aqueous
213 extract was performed by centrifugation (2 x 15 min at 3500 rpm and 10°C, Rotenta 460 R
214 centrifuge, Hettich, Tuttlingen, Germany). The solid residue was freeze-dried prior to lipid
215 extraction and the aqueous extract filtered through a 0.7µm glass-fiber filter. In order to
216 analyse the amount of fecal stanols removed from the sample by the water purification step,
217 solid phase extractions were performed on aqueous extracts as described by Jeanneau et al.
218 [39].

219

220 *2.4.4. Aminopropyl-bonded silica gel purification*

221 For method 3, the polar fraction eluted on the silica gel column was loaded on a
222 chromatography column containing about 10 g of aminopropyl-bonded silica gel (NH₂)
223 preconditioned with ca. 50 mL of a mixture of DCM/MeOH (1:1, by vol). The fraction
224 containing stanols was eluted with 30 mL of a mixture of DCM/MeOH (1:1, by vol), dried
225 under reduced pressure and weighted for quantification.

226

227 *2.2.5. Stanols analysis by gas chromatography-mass spectrometry (GC-MS)*

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

230 Stanols as trimethylsilyl ethers were analysed by GC-MS with a Shimadzu QP2010 +
231 MS gas chromatograph/mass spectrometer (Shimadzu, Tokyo, Japan). 1 µL of samples was
232 injected in splitless mode at 310°C. The temperature of the ionization source was set at
233 200°C. The temperature of the transfer line was set at 250°C, and molecules were ionized by
234 electron impact using an energy of 70 eV. Separation was achieved using a fused silica
235 column coated with SLB-5 MS (Supelco, 60 m, i.d. 0.25 mm, film thickness 0.25 µm) with
236 helium as carrier gas at a flow of 1 mL min⁻¹. The GC oven temperature was maintained at
237 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
238 at this temperature for 15 min.

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

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

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

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

264

265 2.5. *Escherichia coli* analysis

266 In order to investigate the level of fecal contamination of oysters from the Fresnaye bay,
267 the concentration of the fecal indicator bacteria *Escherichia coli* was determined by
268 IFREMER (Laboratoire National de Référence , Nantes, France) using the impedance method
269 [41].

270

271 *2.6. Statistical analyses*

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

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

290 • F1 coordonate = 0.497(%coprostanol) - 0.347(%epicoprostanol) +
291 0.295(%ethylepicoprostanol) - 0.460(%ethylepicoprostanol) - 0.422(%sitostanol) -
292 0.395(%campestanol)

293 • F2 coordonate = - 0.074(%coprostanol) + 0.565(%epicoprostanol) +
294 0.531(%ethylcoprostanol) - 0.303(%ethylepicoprostanol) - 0.288(%sitostanol) +
295 0.468(%campestanol).

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

299

300 **3. Results and Discussion**

301 *3.1. Method comparison*

302

303 *3.1.1. Water extraction*

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

311 The recovery of coprostanol extracted with the method 2 (59 ± 10 %, mean \pm standard
312 deviation, SD) was lower than that extracted with the method 1 (79 ± 8 %) and the two
313 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 (NH₂) 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 NH₂ 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 NH₂ 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

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

346

347 *3.1.3. Comparison of the three methods*

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

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

355 The addition of a purification step on NH₂ increased the recoveries of coprostanol and
356 sitostanol. Nevertheless, this step strongly decreased the repeatabilities of the methods tested,
357 especially for sitostanol. Therefore, this step does not appear to be reliable for the analysis of
358 fecal stanols in oysters.

359 Finally, among the three methods tested, , the method 1, which included a lipid extraction
360 step with organic solvent, a purification step on silica gel and analysis by GC-MS, led to i)
361 statistically similar recoveries than the others two methods, ii) the higher repeatability and iii)
362 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. Cholesterol was the
375 major compound and ranged from 58.4 $\mu\text{g g}^{-1}$ DW (August) to 221.8 $\mu\text{g g}^{-1}$ 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, 5 β -
379 stigmastanol, 24-ethylcoprostanol, 24-ethylepicoprostanol, campestanol, stigmastanol and
380 sitostanol and their concentrations ranged from 7.5 to 21.7 $\mu\text{g g}^{-1}$ DW. The predominance in
381 *Crassostrea gigas* of cholesterol 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 cholesterol of ca. 112 $\mu\text{g g}^{-1}$ DW for *Crassostrea gigas* that is our range of
384 concentrations for cholesterol. Since cholesterol 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 $\mu\text{g g}^{-1}$ DW (August) to
393 297.6 $\mu\text{g g}^{-1}$ 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

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

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

434

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442

443

444 **References**

445

- 446 1. Fong T-T, Lipp EK (2005) Enteric viruses of humans and animals in aquatic
447 environments: health risks, detection, and potential water quality assessment tools.
448 *Microbiol Mol Biol Rev* 69:357–71. doi: 10.1128/MMBR.69.2.357-371.2005
- 449 2. Hundesa A, Maluquer de Motes C, Bofill-Mas S, et al. (2006) Identification of human
450 and animal adenoviruses and polyomaviruses for determination of sources of fecal
451 contamination in the environment. *Appl Environ Microbiol* 72:7886–93. doi:
452 10.1128/AEM.01090-06
- 453 3. Soller J, Embrey M, Tuhela L, et al. (2010) Risk-based evaluation of *Escherichia coli*
454 monitoring data from undisinfected drinking water. *J Environ Manage* 91:2329–35.
455 doi: 10.1016/j.jenvman.2010.06.017
- 456 4. Zatta P, Gobbo S, Rocco P, et al. (1992) Evaluation of heavy metal pollution in the
457 Venetian lagoon by using *Mytilus galloprovincialis* as biological indicator. *Sci Total*
458 *Environ* 119:29–41. doi: 10.1016/0048-9697(92)90253-O
- 459 5. Baudrimont M, Schäfer J, Marie V, et al. (2005) Geochemical survey and metal
460 bioaccumulation of three bivalve species (*Crassostrea gigas*, *Cerastoderma edule* and
461 *Ruditapes philippinarum*) in the Nord Médoc salt marshes (Gironde estuary, France).
462 *Sci Total Environ* 337:265–280.
- 463 6. Gouletquer P, Le Moine O (2002) Shellfish farming and coastal zone management
464 (CZM) development in the Marennes-Oleron Bay and Charentais Sounds (Charente
465 Maritime, France): A review of recent developments. *Aquac Int* 10:507–525. doi:
466 10.1023/A:1023975418669

- 467 7. Mieszkin S, Caprais MP, Le Mennec C, et al. (2013) Identification of the origin of
468 fecal contamination in estuarine oysters using Bacteroidales and F-specific RNA
469 bacteriophage markers. *J Appl Microbiol* 115:897–907. doi: 10.1111/jam.12260
- 470 8. Simpson JM, Santo Domingo JW, Reasoner DJ (2002) Microbial Source Tracking:
471 State of the Science. *Environ Sci Technol* 36:5279–5288. doi: 10.1021/es026000b
- 472 9. Glassmeyer ST, Furlong ET, Kolpin DW, et al. (2005) Transport of Chemical and
473 Microbial Compounds from Known Wastewater Discharges: Potential for Use as
474 Indicators of Human Fecal Contamination. *Environ Sci Technol* 39:5157–5169. doi:
475 10.1021/es048120k
- 476 10. Cimenti M, Hubberstey A, Bewtra J, Biswas N (2007) Alternative methods in tracking
477 sources of microbial contamination in waters. *Water SA* 33:183–194.
- 478 11. Field KG, Samadpour M (2007) Fecal source tracking, the indicator paradigm, and
479 managing water quality. *Water Res* 41:3517–38. doi: 10.1016/j.watres.2007.06.056
- 480 12. Jardé E, Gruau G, Mansuy-Huault L, et al. (2006) Using Sterols to Detect Pig Slurry
481 Contribution to Soil Organic Matter. *Water Air Soil Pollut* 178:169–178. doi:
482 10.1007/s11270-006-9188-9
- 483 13. Ahmad F, Tourlousse DM, Stedtfeld RD, et al. (2009) Detection and Occurrence of
484 Indicator Organisms and Pathogens. *Water Environ Res* 81:959–980. doi:
485 10.2175/106143009X12445568399299
- 486 14. Derrien M, Jardé E, Gruau G, et al. (2012) Origin of fecal contamination in waters
487 from contrasted areas: stanols as Microbial Source Tracking markers. *Water Res*
488 46:4009–16. doi: 10.1016/j.watres.2012.05.003
- 489 15. Biache C, Philp RP (2013) The use of sterol distributions combined with compound
490 specific isotope analyses as a tool to identify the origin of fecal contamination in
491 rivers. *Water Res* 47:1201–8. doi: 10.1016/j.watres.2012.11.037

- 492 16. Chan K-H, Lam MHW, Poon K-F, et al. (1998) Application of sedimentary fecal
493 stanols and sterols in tracing sewage pollution in coastal waters. *Water Res* 32:225–
494 235. doi: 10.1016/S0043-1354(97)00175-9
- 495 17. Leeming R, Ball A, Ashbolt N, Nichols P (1996) Using fecal sterols from humans and
496 animals to distinguish fecal pollution in receiving waters. *Water Res* 30:2893–2900.
497 doi: 10.1016/S0043-1354(96)00011-5
- 498 18. Bull ID, Lockheart MJ, Elhmmali MM, et al. (2002) The origin of faeces by means of
499 biomarker detection. *Environ Int* 27:647–654. doi: 10.1016/S0160-4120(01)00124-6
- 500 19. Tyagi P, Edwards DR, Coyne MS (2009) Distinguishing between human and animal
501 sources of fecal pollution in waters: a review. *Int J Water* 5:15. doi:
502 10.1504/IJW.2009.023080
- 503 20. Gourmelon M, Caprais MP, Le Mennec C, et al. (2010) Application of library-
504 independent microbial source tracking methods for identifying the sources of fecal
505 contamination in coastal areas. *Water Sci Technol* 61:1401. doi:
506 10.2166/wst.2010.033
- 507 21. Shah VG, Dunstan RH, Geary PM, et al. (2007) Evaluating potential applications of
508 fecal sterols in distinguishing sources of fecal contamination from mixed fecal
509 samples. *Water Res* 41:3691–700. doi: 10.1016/j.watres.2007.04.006
- 510 22. Derrien M, Jarde E, Gruau G, Pierson-Wickmann A-C (2011) Extreme variability of
511 steroid profiles in cow feces and pig slurries at the regional scale: implications for the
512 use of steroids to specify fecal pollution sources in waters. *J Agric Food Chem*
513 59:7294–302. doi: 10.1021/jf201040v
- 514 23. Water National Agency of Loire-Brittany. (accessed Jan. 2014). [http://www.eau-loire-
bretagne.fr/informations_et_donnees/reseaux_de_mesure](http://www.eau-loire-
515 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 (5 β -cholestan-
523 3 β -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

- 540 32. Linehan L., O'Connor T., Burnell G (1999) Seasonal variation in the chemical
541 composition and fatty acid profile of Pacific oysters (*Crassostrea gigas*). *Food Chem*
542 64:211–214. doi: 10.1016/S0308-8146(98)00144-7
- 543 33. Costil K, Royer J, Ropert M, et al. (2005) Spatio-temporal variations in biological
544 performances and summer mortality of the Pacific oyster *Crassostrea gigas* in
545 Normandy (France). *Helgol Mar Res* 59:286–300. doi: 10.1007/s10152-005-0004-5
- 546 34. Danger M, Allard B, Arnous M, et al. (2012) Effects of food-web structure on the
547 quantity and the elemental quality of sedimenting material in shallow lakes.
548 *Hydrobiologia* 679:251–266. doi: 10.1007/s10750-011-0890-2
- 549 35. Guenet B, Danger M, Harrault L, et al. (2014) Fast mineralization of land-born C in
550 inland waters: first experimental evidences of aquatic priming effect. *Hydrobiologia*
551 721:35–44. doi: 10.1007/s10750-013-1635-1
- 552 36. Lacaze J-PCL, Stobo LA, Turrell EA, Quilliam MA (2007) Solid-phase extraction and
553 liquid chromatography--mass spectrometry for the determination of free fatty acids in
554 shellfish. *J Chromatogr A* 1145:51–7. doi: 10.1016/j.chroma.2007.01.053
- 555 37. Pernet F, Pelletier CJ, Milley J (2006) Comparison of three solid-phase extraction
556 methods for fatty acid analysis of lipid fractions in tissues of marine bivalves. *J*
557 *Chromatogr A* 1137:127–37. doi: 10.1016/j.chroma.2006.10.059
- 558 38. French Ministry of Agriculture, Agri-food and Forests (accessed Jan. 2014).
559 <http://www.acces.agriculture.gouv.fr/cartostat/#v=map2;i=stru1.saumoy10;l=fr>
- 560 39. Jeanneau L, Jardé E, Gruau G (2011) Influence of salinity and natural organic matter
561 on the solid phase extraction of sterols and stanols: application to the determination of
562 the human sterol fingerprint in aqueous matrices. *J Chromatogr A* 1218:2513–20. doi:
563 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 and soil Sci*
566 169:76–82. doi: 10.1002/jpin.200521711
- 567 41. Dupont J, Dumont F, Menanteau C, Pommepeuy 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 oyster *Crassostrea*
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 :

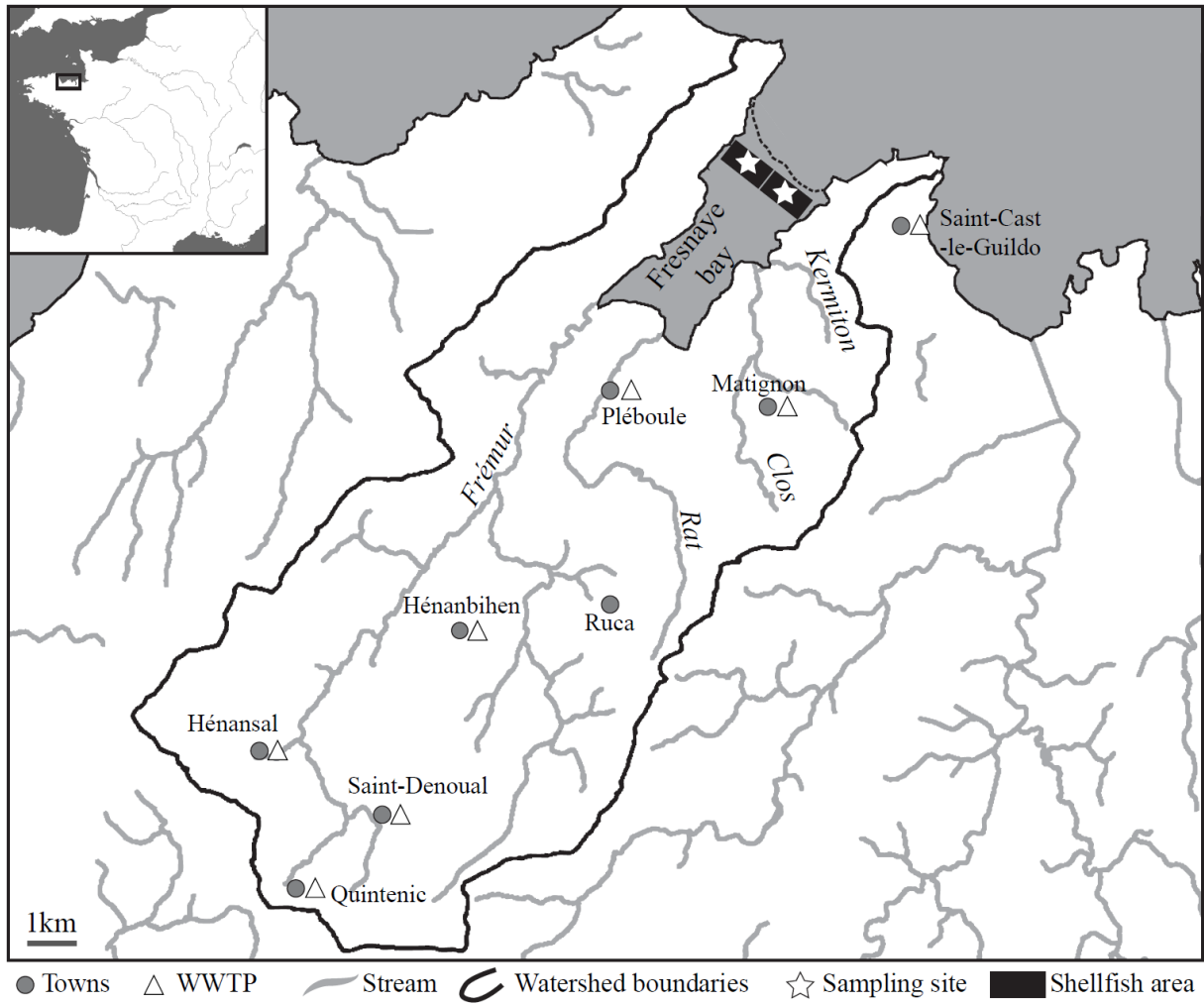
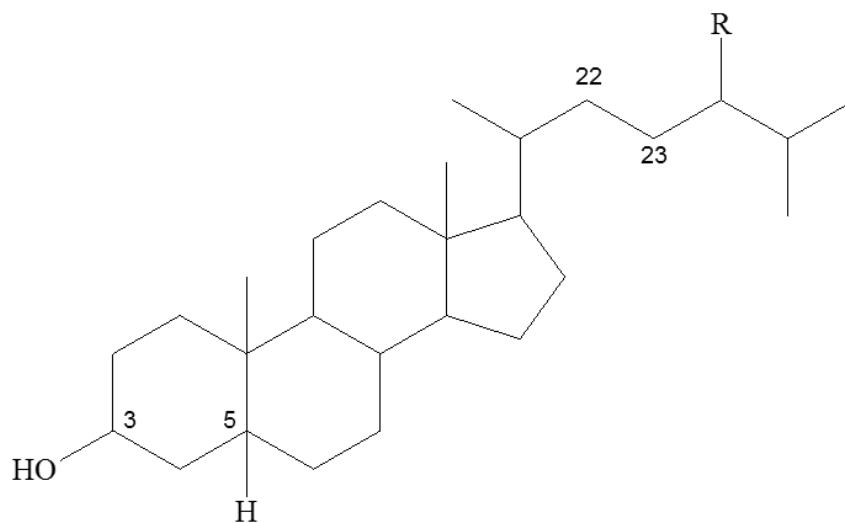


Figure 2 :



R = H 3C-OH = β 5C-H = β	<u>Coprostanol</u>	R = C ₂ H ₅ 3C-OH = β 5C-H = β	<u>24-Ethylcoprostanol</u>
R = H 3C-OH = β 5C-H = α	<u>Cholestanol</u>	R = C ₂ H ₅ 3C-OH = α 5C-H = β	<u>24-Ethylepicoprostanol</u>
R = CH ₃ 3C-OH = β 5C-H = α	<u>Campestanol</u>	R = C ₂ H ₅ 3C-OH = β 5C-H = α	<u>Sitostanol</u>
R = C ₂ H ₅ 3C-OH = β 5C-H = α 22C-23C = double bond	<u>Stigmastanol</u>	R = C ₂ H ₅ 3C-OH = β 5C-H = β 22C-23C = double bond	<u>5β-Stigmastanol</u>

Figure 3 :

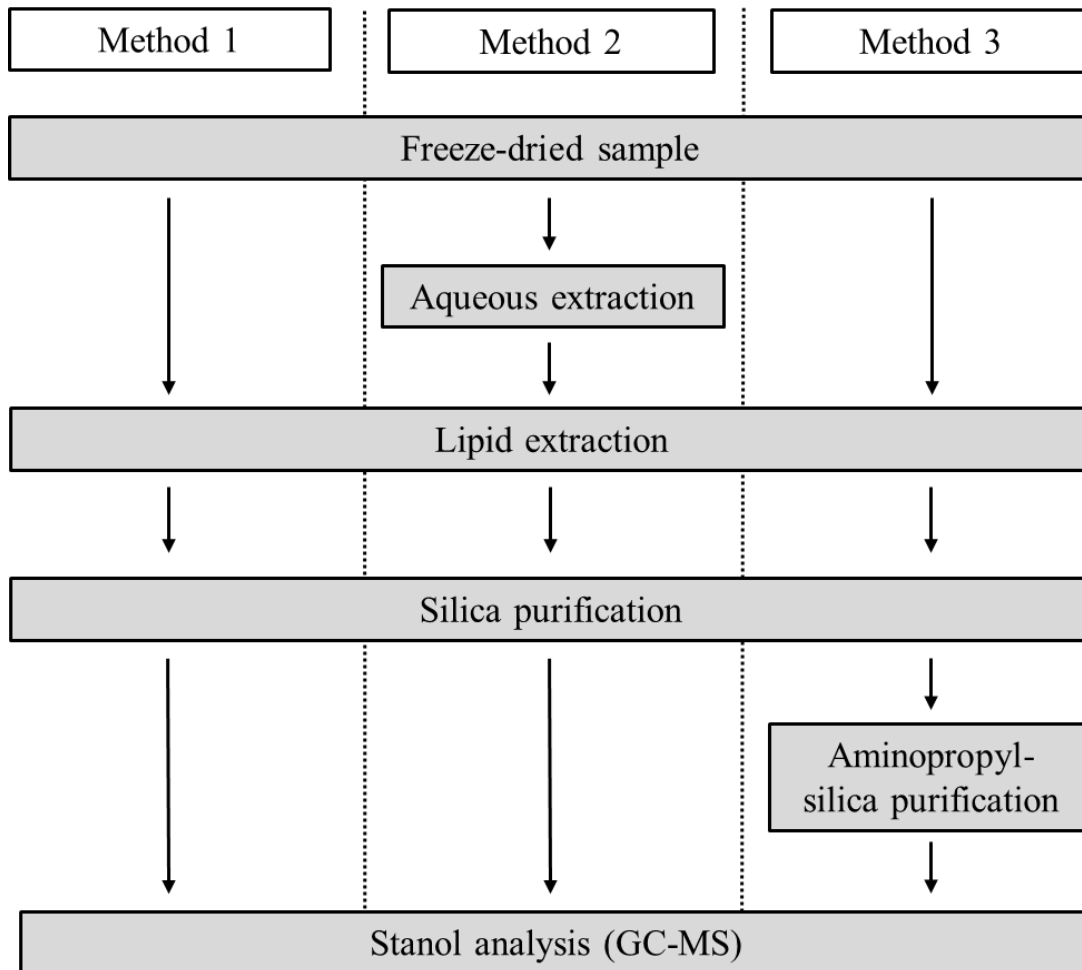


Figure 4 :

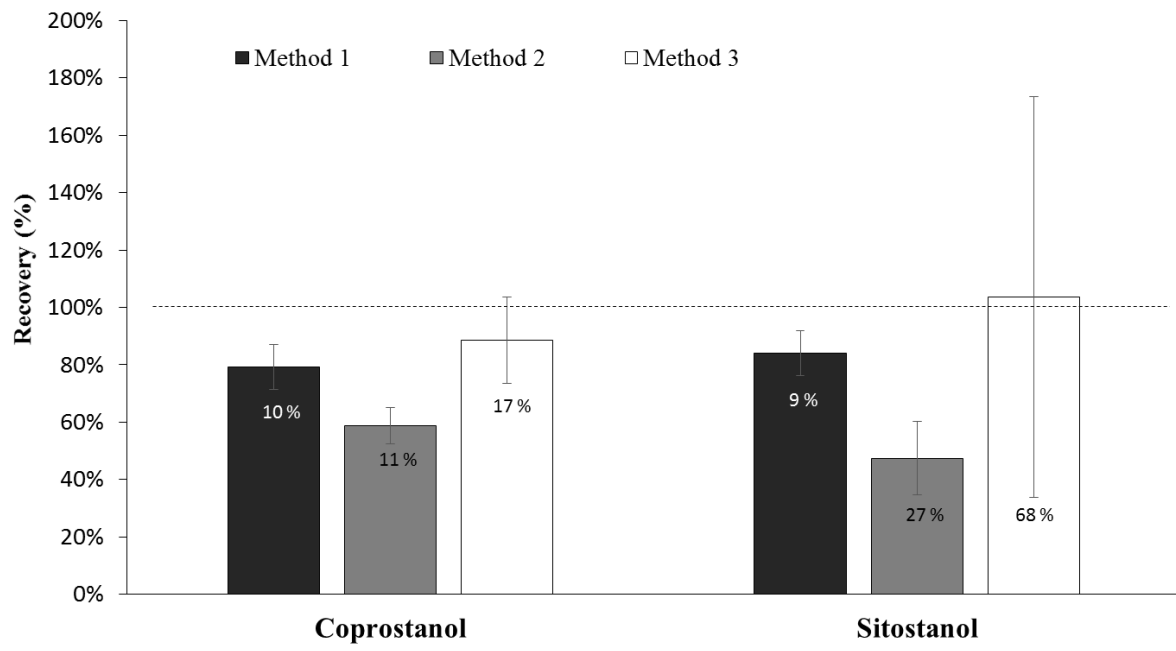
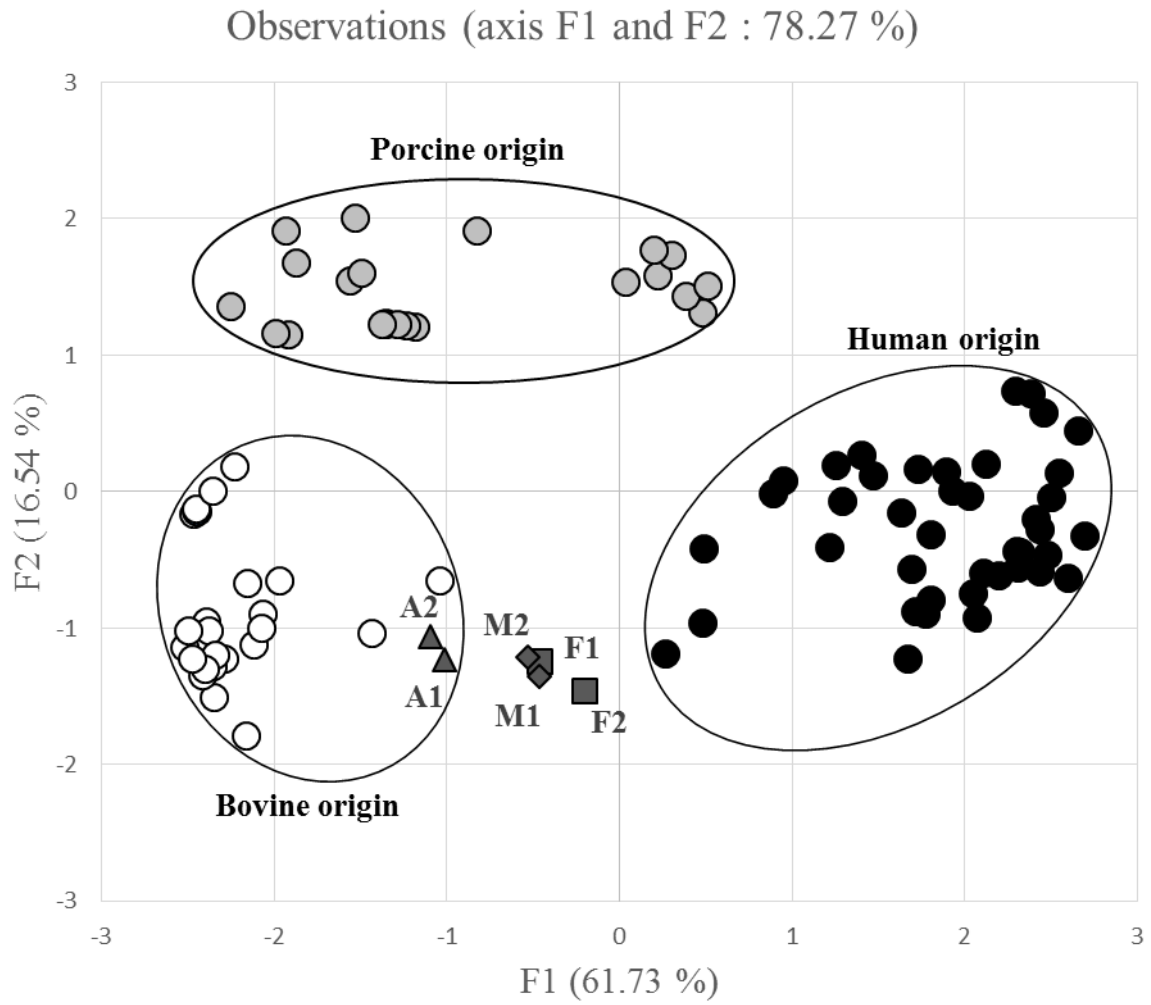


Figure 5 :



<u>Source samples:</u>	<u>Oyster samples:</u>
○ Bovine	■ February
● Porcine	◆ March
● Human	▲ 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	5 β -Cholestan-3 β -ol	0.956	257, 355, 370	215	Coprostanol	0.998	5
Cholestanol	5 α -Cholestan-3 β -ol	1.000	257, 355, 384	215	Cholestanol	0.997	10
Campestanol	24-Methyl-5 α -cholestan-3 β -ol	1.050	369, 398, 484	215	Coprostanol	0.998	5
Stigmastanol	24-Ethyl-5 α -cholesta-22-en-3 β -ol	1.063	215, 383	215	Coprostanol	0.998	5
5 β -Stigmastanol	24-Ethyl-5 β -cholesta-22-en-3 β -ol	1.019	257, 353, 486	215	Coprostanol	0.998	5
Sitostanol	24-Ethyl-5 α -cholestan-3 β -ol	1.097	383, 398, 473	215	Sitostanol	0.995	10
24-Ethylcoprostanol	24-Ethyl-5 β -cholestan-3 β -ol	1.041	257, 383, 398	398	Coprostanol	0.994	10
24-Ethylepicoprostanol	24-Ethyl-5 β -cholestan-3 α -ol	1.047	257, 283, 398	215	Coprostanol	0.998	5

^a Limit of Detection

Table 2 : Stanol ($\mu\text{g g}^{-1}$ 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
5 β -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
<i>Escherichia coli</i>	67 ± 0	220 ± 0	9150 ± 9687