Tapping the archives: The sterol composition of marine sponge species, as determined non-invasively from museum preserved specimens, reveals biogeographical features

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Abstract

Over 8,600 species are currently recorded in the phylum Porifera (sponges). They produce a large diversity of biochemical compounds including sterols, with more than 250 different sterols identified. Some of these sterols are of great interest, due to their use for fingerprinting in ecological and biomarker (molecular fossil) studies. As a large number of identified extant species from biodiversity surveys are housed in museum collections, preserved in ethanol, these present a potentially rich source of identified specimens for comparative lipid analyses. Here, we show that, in at least one species, sterol distributions obtained from the ethanol used to preserve specimens of sponges were representative, and comparable to the sterol distribution obtained from wet frozen, and from freeze dried tissue from the same species. We employed both GC-MS as well as two-dimensional gas chromatography – time of flight mass spectrometry (GC×GC-TOFMS), with an improved signal-to-noise ratio for even minor constituents. Analysis of two additional specimens of the same species, but of different provenance, resulted in detection of marked differences in sterol composition which could be attributed to variations in geography, environmental conditions, microbial communities, diet or cryptic speciation. The possibility of using ethanol from identified, preserved museum sponges could drastically increase the number of available samples. This could enable the study of their sterol complements, and the detailed investigation of differences due to geographical and oceanographic, phylogenetic and other factors in unprecedented detail.

Introduction

The number of species in the phylum Porifera (sponges) is rapidly rising with over 8,600 currently recognized species, and suggestions that there could be more than twice as many species globally (Van Soest *et al.*, 2012). Sponges are widespread in many shallow and deep water reef systems, and, as filter feeders, they occupy a key role in the carbon cycle of marine ecosystems (Van Soest *et al.*, 2012). The phylum is deeply branching in the Metazoa and their phylogeny is of great interest to evolutionary biologists (Wörheide *et al.*, 2012). Sponges are known to produce a vast number of highly diverse natural products (Genta-Jouve & Thomas, 2012), including over 200, often unusual triterpenoids and steroids (Bergmann, 1949; D'Auria *et al.*, 1993).

Djerassi and Silva (1991) concluded that the composition of most sponges consists of fairly common sterols, while some contain unusual sterols. This was a result of their analyses of sponge sterols (the most common types of steroids, with a hydroxyl group on C-3, Fig. 1) by mass spectrometry and nuclear magnetic resonance (NMR) in different specimens (De Rosa *et al.*, 1973; Bergquist *et al.*, 1980; Kerr & Baker, 1991). Unusual sterols include the cyclopropyl-side chain containing sterols found in sponges of the order Haplosclerida (Proudfoot & Djerassi, 1987; Gauvin *et al.*, 1998; Giner *et al.*, 1999), the unusual 19-*nor*sterols present in some members of the genus *Axinella* (Minale & Sodano, 1974; Crist & Djerassi, 1983), or the multiply alkylated side chains produced by members of the order Halichondrida (Stoilov *et al.*, 1986a, 1986b). Of particular interest to geobiologists is 24-*iso*propylcholesterol, which was isolated first from *Pseudaxinyssa* sp. (Hofheinz & Oesterhelt, 1979), now accepted as *Axinyssa* sp., family Halichondriidae, order Suberitida. Sponges are the only known extant organisms where this compound is present in large amounts (McCaffrey *et al.*, 1994; Love & Summons, 2015). This finding resulted in the interpretation of high abundances of its geologically stable derivative, 24-*iso*propylcholestane

compared to 24-*n*-propylcholestane in the rock record as a proxy for the abundance of Demosponges (Love *et al.*, 2009; Kelly *et al.*, 2011). However, the validity of this biomarker is debated (Antcliffe, 2013; Love & Summons, 2015) and should be applied with caution as small amounts of it are also produced by marine algae. Molecular clock studies of the biosynthetic genes though have recently shown that pelagophyte algae evolved the gene for the synthesis of this particular sterol later than the Cryogenian, when the first massive occurrence of this molecular fossil is observed (Gold *et al.*, 2016). It provides a tantalizing possibility for determining the rise of animal life.

As only a select number of sponge species and specimens has been analysed so far, the relationship of sterol composition with phylogeny is not entirely clear: Bergquist *et al.* (1991) reported a correlation, but others such as Fromont *et al.* (1994), concluded that sterol composition was not necessarily related to phylogeny. Future opportunities lie in combining DNA based phylogeny and elucidation of biosynthetic pathways, but in order to provide comprehensive results, a representative number of species and specimens needs to be analysed (Erpenbeck & van Soest, 2007). This is particularly important when considering that sponges do not only employ *de novo* biosynthesis of sterols, but are also capable of modifying dietary sterols (Bergquist, 1978; Silva *et al.*, 1991; Silva & Djerassi, 1992). Consequently, the determining factors on the sterol composition of sponges are of high interest to geochemists and geobiologists.

Analysis of sterols usually entails the extraction of collected or cultivated sponge tissue, followed by purification through a gravity column or high performance liquid column chromatography (HPLC) procedures (Popov *et al.*, 1976). Analysis by gas chromatography-mass spectrometry (GC-MS) is then usually carried out on the derivatized sterols, carrying either a trimethylsilyl group or an acetyl group (Goad & Akihisa, 1997). These derivatizations have been reported to affect the distribution of measured sterols (Mitrevski *et*

87 *al.*, 2008). However, sponge tissue can be difficult to obtain due to the necessity of sampling
88 permits, as many locations are marine protected areas, and due to logistical reasons for
89 sampling in deep waters (trawls, remote operated vehicles have to be employed).
90 Identification of these sponge samples requires a taxonomist, is very time consuming and
91 presents one of the main bottlenecks in sponge research. Therefore, analyzing large numbers
92 of identified sponge samples for sterols would be useful for investigating and comparing
93 sterol distributions with respect to phylogenetic relationships, identifying unusual sterols of
94 potential biomedical interest, and of biomarker potential as a chemotaxonomy tool e.g. in the
95 field of paleontology (Erpenbeck & van Soest, 2007).

96 Hence, here we investigate the potential of using ethanol that has been used to preserve
97 sponge specimens in museum collections (a standard procedure), for sterol analyses. Sponge
98 tissue was stored in ethanol in glass jars for several years, causing polar extractable organic
99 compounds to be leached into the solution. As the samples are usually stored in the dark and
100 at a controlled temperature, chemical alteration is reduced to a minimum. Therefore, these
101 collections present a valuable resource for the analysis of natural products, allowing non-
102 invasive sampling of identified specimens. We employed conventional GC-MS and two
103 dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-
104 TOFMS; Liu and Phillips (1991)), the latter in order to circumvent interferences due to co-
105 elutions of other polar compounds in the first dimension. Previous application of GC×GC
106 coupled to flame ionization detection allowed unprecedented resolving power for sterols in
107 environmental samples (Truong *et al.*, 2003), and quantification of steroids in a urine sample
108 (Mitrevski *et al.*, 2008).

109 In order to determine the suitability of ethanol, used to preserve sponge specimens, for the
110 analysis of sterols, we analyzed ethanol from a preserved specimen of *Agelas* sp. collected off
111 the Western Australian coast and subsequently stored in the dark and at 18 °C and compared

it to an extract of samples of the same specimen, one of which was freeze dried and stored at 18 °C and one frozen at –20 °C. We also analyzed two other species preserved by the above three methods, however, the specimens had been collected from various locations at different times. Here, we resolve these sterols by GC×GC-TOFMS; demonstrate that the ethanol collections of museum specimens can be a valuable resource for lipid and potentially other natural products research, or for large scale studies in marine chemical ecology, and discuss the differences between sponges of the same species but collected at different locations.

Methodology

Sampling

A specimen of the sponge *Agelas* sp. MF1 (family Agelasidae, order Agelasida) was collected off the south-western Australian coastline during cruises and surveys as specified (Table 1, Fig. S1). A part of the sponge was wet frozen at - 20°C, one part was preserved in 75% ethanol on board, and one part was wet frozen on board and lyophilized at the Western Australian Museum. One specimen of *Petrosia* sp. 1 (family Petrosiidae, order Haplosclerida) and one specimen of *Ecionemia* sp. SS1 (family Ancorinidae, order Tetractinellida) were collected at Ningaloo (Table 1, Fig. S1) and stored in ethanol, and two specimens of each were collected at Kalbarri (Table 1, Fig. S1) and stored wet frozen at -20°C and freeze dried, respectively.

The ethanol preserved (6 to 9 years, analysis in 2014, see date of collection in Table 1) and lyophilized tissue was stored in the dark at 18°C, while the frozen tissue was stored in the dark and at -20°C. The frozen, freeze dried and ethanol preserved tissue was extracted as outlined in section 2.2. 10 – 20 mL of the ethanol was sampled, dried under a stream of N_2, dissolved in dichloromethane (DCM) / methanol (MeOH) 1:1 (*v/v)* and dried over $MgSO_4$, dissolved to a concentration of 10 mg/mL and purified as detailed below.

136 *Tissue extraction*

137 Analysis steps are summarized in Fig. 2. Ethanol preserved tissue was dried under
138 atmospheric pressure at 22°C, wet frozen tissue was lyophilized, and lyophilized tissue
139 obtained from the museum was used without modification. The extraction protocol for the
140 aliquots of the *Agelas* MF1 specimen are also represented in Fig. 1. Dry tissue (0.5 – 1 g) was
141 ground with a pestle and mortar and sonicated in 10 mL DCM/MeOH 1:1 (v/v) (10 min).
142 After centrifugation at 3,000 rpm (5 min), the supernatant was collected. This procedure was
143 repeated twice; the combined supernatant was dried under N_2 and over anhydrous $MgSO_4$,
144 and constituted the total lipid extract (TLE).

145 For analysis of free sterols, 2.5 mg of TLE was subjected to gravity column chromatography,
146 and the polar fraction was eluted from 0.8 g activated 60 mesh SiO_2 with 4 mL DCM/MeOH
147 1:1 after the apolar and aromatic compounds had been eluted with 4 mL hexane and 4 mL
148 Hex/DCM 3:7 (v/v). The polar fraction was dried under a stream of N_2 and dissolved in *n*-
149 hexane prior to analysis by GC-MS and GC×GC-TOFMS. For some of the extracts, free
150 hydroxyl groups were converted to trimethylsilyl (TMS)-ethers by reaction with 50 µL
151 pyridine and 50 µL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 70°C for one hour,
152 and evaporated to dryness under a stream of N_2 before dissolving in hexane.

153 In addition, for the *Agelas* samples, the total extracted, i.e. free and bound, sterols were
154 determined. For this, 2.5 mg of the TLE was saponified: it was dissolved in 2 mL 1N KOH in
155 MeOH and refluxed for 2 h at 80°C. 2 mL of water and 2 mL of cyclohexane were added,
156 shaken, and the cyclohexane containing the sterols was collected. This was repeated twice
157 and the combined cyclohexane fractions were dried, dissolved in diethylether/ethylacetate 1:1
158 (v/v), eluted over SiO_2 and dissolved in hexane for analysis.

In order to determine the sterols bound in the biomass residue of the ethanol preserved *Agelas*, the residue of the extracted biomass was dried and refluxed in 25 mL 1N KOH in MeOH (1 h). The pH was adjusted to 6 with 2N HCl in MeOH, water was added in equal amounts to the MeOH, and the aqueous phase was extracted three times with 10 mL DCM. The combined DCM phases were dried under N_2 and over $MgSO_4$, dissolved in diethylether/ethylacetate 1:1 (v/v) and purified by elution from a silica (SiO_2) column.

GC-MS and GC×GC-TOFMS analyses of sterols

For GC-MS, an Agilent 5973 mass-selective detector coupled to a 6890 gas chromatograph was employed, using a 30 m x 0.25 mm ID x 0.25 um film capillary column of type DB5-MS, with a temperature programmed from 40 to 325°C at 10°C . min^{-1} and held at the final temperature for 20 min. Samples were injected in ethylacetate on a split/splitless injector in pulsed splitless mode at 320°C. The carrier gas was He at a constant flow of 1.1 mL/min. Ionization was carried out at 70 eV, with an electron multiplier voltage of 1800 V and the source kept at 230°C. Masses scanned ranged from 50 to 750 Da. Data analysis of GC-MS data was carried out using Wsearch32 (www.wsearch.com.au).

For GC×GC-TOFMS, splitless injection at 310°C inlet temperature was employed, on an 7890 Agilent GC modified for GC×GC, coupled to a Pegasus 4D TOF-MS with linear modulation (LECO Corporation, St. Joseph, MI) employing electron ionization (EI). Primary column was a 30 m Restek CP5-Sil of 0.25 mm inner diameter and 0.25 μm film thickness and secondary column a 1.5 m 17Sil-MS (equivalent to 50 % phenyl) of dimensions 0.18 mm / 0.18 μm with helium as a carrier gas at a flow rate of 1.05 mL. min^{-1}. Modulation was carried out directly on the secondary column and modulation time was 5s (0.8 s hot jet, 1.70 s cold jet). The temperature was ramped from 40°C to 300°C at a rate of 3°C . min^{-1}, with the modulator at a 15°C and the secondary column at a 40°C offset. The Pegasus 4D was

operated at 100 Hz and at a mass range of 50-650 Daltons, with the transfer line at 290°C and the ion source at 230°C. This configuration and program was optimized on the secondary column separation, as using the small differences in polarity of the sterols analyzed allowed for separation of several co-eluting compounds under these conditions. Data analysis was conducted using ChromaTOF automatic peak detection with a signal to noise ratio and peak width of 20 and 0.1 s, respectively, for small peaks and 300/0.4 s for larger peaks, areas of TIC were used in order to calculate the area percentages of individual sterols of the total sterol area.

Results and Discussion

Sterol abundances

The structures of the sterols detected are shown in Fig. 1 and their identification is described in the supplementary material.

The ethanol from *Agelas* sp. MF1 contained sterols with 27, 28 and 29 carbon atoms (Fig. 3, Fig. 4, Table 2). The sterols from the cholestane series consisted of **2**, **3**, **4** and **7**, the sterols from the ergostane series of **6**, **10**, **13**, **14** and **17**, and the ones from the stigmasterane series **9, 15**, **16**, **17**, **23** and **26**. The two sterols present in largest abundance were **4** and **20**, other major sterols included **2**, **6**, **10**, **13**, **23** and **26**.

The ethanol from *Ecionemia* sp. SS1 contained a large variety of sterols, including **1 - 8**, **11**, **12**, **15**, **18**, **19**, and **22-25** (Fig. 3, Fig. 5, Table 3). However, the sterols of both the frozen and the freeze dried samples consisted mainly of **22**, with some other minor constituents (Fig. 5). The sterols obtained from the ethanol of the *Petrosia* specimen consisted of **2**, **4**, **6**, **11**, **15**, **19**, **21** and **22**, in a distribution largely similar to the preserved specimen. The wet frozen and lyophilized *Petrosia* sp. 1 contained a slightly larger variety of sterols, with also **1**, **3**, **5** and **8**

present in considerable proportions (Table 3). It is possible that some of these were not detected in the ethanol due to a very large peak of **4** and **17**. The ethanol of *Petrosia* sp. 1 also contained a number of 3-oxosterols, which were not observed in the wet frozen and lyophilized specimens. It is possible that these were degradation products, however, they were not observed in any of the other ethanol preserved samples and it is thus more likely that they were present in the sponge. No sterols of less than 27 and more than 29 carbon atoms were detected, but it is possible that these were present in minor amounts.

Comparison of extraction methods using one specimen of Agelas preserved in three different ways

The *Agelas* MF1 specimen was split into aliquots when collected in 2007, and analysis of the wet frozen and the lyophilized samples thus allowed for a direct comparison of the sterol composition to the ethanol preserved aliquot. The same sterols were detected, with the exception of **3**, which was only present in ethanol (Fig. 3 A). The distribution was slightly different, with **20** being present in larger proportions in the wet frozen, and even larger proportions in the lyophilized sample. Other differences in proportional amounts were minor. This confirms that a representative amount of sterols is leached into the preservation fluid; and that alteration during storage is minimal.

In order to analyze the completeness of extraction achieved by storage in ethanol, we also extracted some of the sponge tissue that had been preserved in ethanol, and had leached the sterols. This resulted in similar sterol compositions to those observed in the ethanol (Fig. 4B). However, we obtained a slightly larger amount of sterol **20** (22.1 / 25.7 %), which, in conjunction with the larger amounts present in the wet frozen and lyophilized samples, suggests that ethanol might not completely extract **20**. No $\Delta^{5,7}$ sterols were detected in any of the samples. Whilst these sterols are known to be chemically rather labile, they were not

230 detected in the frozen and the lyophilized samples either, therefore this is probably not an
231 artefact of the preservation method.

232 When the residue of the ethanol preserved sponge after tissue extraction was subjected to
233 saponification in order to release the more strongly bound sterols, a similar distribution to the
234 ethanol extracted sterols was observed (Fig. 4B). However, interestingly, it was also observed
235 that a large number of (unidentified) triterpenoids were released, as exemplified by the
236 extracted ion current (EIC) for *m/z* 191, a common ion observed in many triterpenoids (Fig.
237 S4). These compounds were thus present as more strongly bound, non-extractable lipids, or
238 potentially were associated with symbionts. Many sponges are able to source carbon and
239 energy from a number of symbionts they harbor within their tissue (Webster & Blackall,
240 2009; Thacker & Freeman, 2012), many of which are known to produce bacteriohopanoids
241 (Ourisson & Albrecht, 1992).

242 In addition to the analysis of the free sterols, we also determined whether significant amounts
243 of sterol esters had been extracted by ethanol leaching or DCM/MeOH extraction, and
244 saponified the extracts of *Agelas* sp. MF1 in order to obtain the sum of free and bound sterols
245 (= total). Negligible changes in their distributions were observed (Table 2, Fig. 4 B),
246 suggesting that (i) the sterol esters are present in similar proportions to the free sterols, that
247 (ii) there are no sterol esters, or that (iii) sterol esters are not leached into the ethanol during
248 preservation. Distributions of extracts gained by wet or lyophilized tissue extraction in
249 DCM/MeOH similarly showed only negligible changes in distribution upon saponification
250 (Table 2), thus suggesting reason (i) or (ii) was the cause.

251 Our results show that the ethanol taken from preserved museum specimens contains sterols
252 that can be representative in type and distribution for an individual sponge. This technique
253 could be more widely applicable and make a pool of samples accessible for larger screening

studies for identification of new compounds for biomedical research, for geochemical research relying on biomarkers ('unique' compounds), or for ecological and phylogenetic studies investigating sterol distributions and their determining factors.

Enhancement of sterol analysis by GC×GC

GC×GC was first used by, and consists of the employment of two capillary columns of orthogonal selectivity, e.g. an apolar column effecting separation by volatility, followed by a polar column where retention increases with increasing polarity. The eluting compounds from the primary column are frozen for a certain period of time (the "modulation period"), usually from 2-10 seconds, and then released onto the secondary column which is shorter in length by a steep increase in temperature. This technique has, especially in the past decade, been extensively developed and applied to many fields as reviewed by e.g. Adachour *et al.* (2008). The advantages include an improved signal-to-noise ratio, increased separation efficiency and structured chromatograms, in which structurally similar compounds elute in roof-tile like sections, which can substantially improve compound identification, without the need for separation procedures (Eiserbeck *et al.*, 2012; Naeher *et al.*, 2016), and separation of structural and stereoisomers (Eiserbeck *et al.*, 2011). Sterols are amenable to GC, show specific and varied polarities, and many potential isomers occur, which can be difficult to fully separate by one-dimensional GC without extensive pre-fractionation steps. This makes them very suitable for GC×GC-TOFMS, which allows separation not only by boiling point, but also by polarity, and hence results in a structured, two-dimensional chromatogram with grouped compound classes. Handling of the samples for identification and voucher sample preservation in ethanol instead of for lipid analysis could introduce a number of contaminants, which can unnecessarily complicate GC-chromatograms, but can easily be separated by GC×GC. It also allows for simple separation of the 3-oxo compounds from the 3-hydroxy compounds (Fig. 3 B, C), which is not possible employing one-dimensional

analysis (Fig. S2, S3) as the former exhibit a higher retention time in the second dimension (polar column; Rt_2). This results in additional confidence in structural identifications. Moreover, a number of different isomers were detected, such as compounds **6** / **7**, which were co-eluting in one dimensional analysis (Fig. S2, S3). If some of these compounds are present in trace amounts, the signals could be difficult to deconvolute. GC×GC chromatograms also allow for sophisticated untargeted comparison of samples, thus potentially allowing untargeted cross sample comparison (Reichenbach *et al.*, 2011; Marney *et al.*, 2013). Further, whilst here, analysis was conducted following simple gravity column chromatography separation, GC×GC also allows the analysis of an untreated extract, thereby removing any possibilities of bias and loss of compounds present in low concentrations during the workup. With appropriate derivatization, it could also be possible to determine a range of other compounds of interest in these extracts, and of potential interest, such as alkaloids or terpenoids (cf. Erpenbeck and van Soest, 2007; Genta-Jouve and Thomas, 2012).

Differences in specimens from different locations

While *Agelas* sp. showed distributions which were unaffected by the preservation method, the sterol compositions obtained from *Ecionemia* sp. and *Petrosia* sp. specimens largely differed between the ethanol and the lyophilized and preserved specimens. In *Ecionemia* sp., the diversity of sterols was higher in the ethanol preserved sponge, while in *Petrosia* sp. the diversity was higher in the wet frozen and lyophilized sponges. This is in contrast to the results obtained from the *Agelas* sp. specimen. It is thus less likely that preservation methods were causing these differences, however it is possible that differences in the sponges such as proportions of spicules, and thus silica, in *Ecionemia* and *Petrosia* sp. compared to *Agelas* sp., (with comparatively fewer siliceous spicules) could have resulted in more pronounced changes in sterol composition in the two former species. In addition, sponges of the genus *Petrosia* are known to form reactive polyacetylenes (Cimino *et al.*, 1989) which could be

responsible for the conversion of the sterols to ketones, but are unlikely to have caused all of these differences.

A more likely reason for these differences is that the results are not directly comparable as they were not derived from the same specimen, but rather from three different specimens (Table 1), of which the one preserved in ethanol was obtained from a completely different location (Fig. S1). This is in contrast to previous studies, where sterol composition was found to be species specific and independent of location (Bergquist *et al.*, 1980; Fromont *et al.*, 1994). As sponges employ both *de novo* biosynthesis along with uptake and modification of dietary and symbiont produced sterols (Bergquist, 1978) these sterol differences between specimens of one species are not surprising. Habitat, depth, or times of collection are unlikely to have caused these differences: for example, all specimens of *Ecionemia* sp. were collected around 100 m depth in the same year. In the case of *Petrosia*, both the freeze dried and the ethanol preserved specimens were collected at a similar depth (around 100 m depth), while the wet frozen specimen was from 253 m depth, yet it was the ethanol preserved specimen that contained different sterols from the other two. The collection time was austral summer for all specimens (Table 1). Subtle differences were seen in the sponge color and spicule dimensions of the ethanol preserved specimen of *Petrosia*, which was darker brown and had thinner spicules that the wet frozen and freeze dried samples (260 x 12 μm compared to 270 x 20 μm for the largest size category of oxeas). It is possible that *Petrosia* sp. 1 is a species complex (i.e. a group of two or more closely related cryptic species), but this could only be determined with more detailed morphological analyses and molecular data.

However, in both *Petrosia* sp. and *Ecionemia* sp., the wet frozen and lyophilized samples, which had differing sterol complements, had been collected at the same location, while the ethanol preserved sponge had been collected in a different area (Fig. S1). It is thus most likely that the sterol distributions are related to geographical or ecosystem differences, and

329 that the sterol composition varies moderately between species across their biogeographic
330 distributions. Ethanol preserved specimens of *Petrosia* and *Ecionemia* were collected in the
331 tropics at Ningaloo Reef (Carnarvon Shelf, NW Australia, 22°S) and the wet frozen and
332 lyophilized specimens of these species were collected from Kalbarri/Zuytdorp (Dirk Hartog
333 Shelf, Central Western Australia). The latter region is subtropical (27°S) and to ≤ 250 m
334 depth exposed to the Leeuwin current, potentially a rich source of particulates for filter
335 feeders such as sponges (Fromont *et al.*, 2012), that could influence the dietary sterol uptake
336 *via* organic matter supply (Silva *et al.*, 1991; Silva & Djerassi, 1992). It is thus possible that
337 sterol and sterane biomarker distributions derived from sponges can be indicative of
338 environmental factors such as their diet. Also other factors varying between localities and
339 individuals (nutrient regimes, a difference in symbionts, or microbial defense) could play a
340 major role in activating *de novo* biosynthesis or modification after uptake. This could explain
341 observations made by Kerr *et al.* (1991), who saw a strong variation in the sterol composition
342 of *Xestospongia muta* specimens collected in close proximity, although this could also reflect
343 cryptic speciation.

344 Our results suggest that inferences about *de novo* sterol biosynthesis from the sterol
345 composition of a sponge sample can be difficult. Moreover, there are strong indications that
346 the sterol composition of specimens of the same species of sponges could relate to their
347 biogeographical and oceanographic environment. Regardless of whether *de novo* synthesis or
348 dietary modification lead to the presence of a certain sterol in a sponge specimen, it appears
349 that their sterol composition is shaped by additional factors which might also need to be taken
350 into account when interpreting the sterane biomarker record, and could provide more
351 information about depositional environments.

352 *Comparison of sterol compositions with the literature*

353 Sponges of the genus *Agelas* have been investigated for sterol composition on many
354 occasions: Santalova *et al.* (2004) analyzed *A. mauritiana*, and reported 20 sterols, including
355 **1** (trace amounts = tr), **2** (4.48 %), **3** (2.51 %), **4** (28.72 %), **5** (tr), **6** (tr), **7** (9.45 %), **8** (tr), **11**
356 (tr), **13** (9.03 %), **15** (tr), **16**(1.46 %), **17** (6.01 %), **19** (2.23 %), **20** (2.23 %), **23** (tr) and **26**
357 (20.57 %), in addition to a number of other sterols, including 5α-25-desmethyl-ergost-22-en-
358 3β-ol (tr), 5α-cholesta-7,22-dien-3β-ol (tr), 5α-ergosta-7,22-dien-3β-ol (5.22 %), 5α-23-
359 methyl-ergost-22-en-3β-ol (2.04 %). This profile resembles the one for the *Agelas* species
360 analyzed here, but differed slightly in relative amounts (Fig. 4). Also, sponges of the order
361 Petrosiidae have been extensively investigated for sterol composition, and were found to
362 contain a number of unusual, often cyclopropyl-containing sterols (Wahid Khalil *et al.*, 1980;
363 Gauvin *et al.*, 1998; Giner *et al.*, 1999; Reddy *et al.*, 1999), which were not detected in this
364 study. This was in agreement with Fromont *et al.* (1994) and Bergquist *et al.* (1980), who
365 examined various species of the genus *Petrosia*, but could not detect any of these unusual
366 sterols. Instead, *P. pigmentosa* and *P. australis* contained **1** (0.7 / 2.4 %), **3** (4.2 / 10 %), **4** (12
367 / 0.4 %), **5** (7.9 / 8.1 %), **6** (0.5 / 0 %), **7** (4.5 / 0 %), **8** (0 / 47 %), **11** (2.2 / 0.7 %), **12** (0 / 1.8
368 %), **13** (0.4 / 0 %), **15** (1.3 / 2.6 %), **19** (31 / 2.3 %), **22** (0.3 / 13 %), **23** (1.6 / 0 %) and **26** (13
369 / 0 %). Other sterols detected in these specimens were (*E*)-stigmasta-5,24(24^1)-dien-3β-ol
370 (0.1 / 7.6 %), 26-desmethyl-cholesta-5,22-dien-3β-ol (0.7 / 0.5 %), 26-desmethyl-cholest-22-
371 en-3β-ol (1.4 / 0 %), and a number of $\Delta^{5,7}$ sterols, which were not detected in our study.
372 These are known to be particularly labile and it is thus possible that they had been present in
373 the live sponge, but could not be detected in our samples. Similarity between the sterol
374 compositions of the two *Petrosia* species reported by Fromont *et al.* (1994) was not high, and
375 the samples investigated here also show little similarity to these species (Fig. 4). No sterol
376 composition for the genus *Ecionemia* has been reported.

A literature comparison of the sterols from the same genera as the species analyzed here demonstrates the similarity of *Agelas* sp. MF1 to *A. mauritiana,* and confirms the comparability of our method with results gained by more traditional methods. It is possible that sponges of the genus *Agelas* are so similar to each other because they rely more strongly on *de novo* biosynthesis, while sponges of the genus *Petrosia* (and *Ecionemia*) rely on modified dietary sterols, which causes greater variation in sterols at the genus and to a lesser extent, species level. This is in agreement with Silva *et al.* (1992), who attributed the unusual sterols of *P. ficiformis*, which were not detected in the specimens investigated here, to dietary modification in line with biosynthetic observations. However, contrastingly, Gold *et al.* (2016) suggested that this species does possess all the genes necessary for their production.

The lack of unequivocal resolution of the phylogeny of the Haplosclerida further complicates comparison of *Petrosia* sp. 1 sterols with other species. Recent advances in sponge phylogeny have suggested that *Petrosia* is indeed a paraphyletic group (Redmond *et al.*, 2011), which might also cause the strong differences in sterol composition when comparing our results to the literature, and explain the absence of the unusual sterols of *P. ficiformis* in *Petrosia* sp. 1 and other *Petrosia* species (Fromont *et al.*, 1994). In accordance with ongoing advances in sponge phylogeny, more detailed analysis of sterol complements, combined with molecular analysis, with replicates of the same species from the same and different locations or oceanographic and ecological conditions could provide valuable information for the interpretation of sterol distributions, the sterane geological record and the evolution of Porifera and the Metazoa.

Conclusions

The sterol composition obtained from the ethanol of museum voucher specimens presents a new method for non-invasive sampling of archived, identified sponge specimens. This can

facilitate comparative studies in geochemistry, phylogeny, marine biogeography, and geobiology. While we cannot completely exclude the possibility that preservation method impacts sterol recovery, comparative analysis of different specimens of the same species of *Petrosia* sp. 1 and *Ecionemia* sp. SS1 most likely showed strong intraspecies variability, potentially due to differences in geographical location, nutrient regimes, microbial communities, the acquisition of sterols *via* their diet, or cryptic speciation. The relationship of biogeographical and oceanographic environment with sterol composition warrants further investigation in terms of the transfer of these features to the geological record. The sampling methodology presented here opens up the potential for non-destructive, non-invasive sampling of preserved museum specimens for analysis of sterols and potentially other compounds - currently an underutilized but vast resource for large scale biochemical studies.

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565

566 Figure captions

567 Figure 1. Sterol structures. A - Identified sterols in the three species analyzed. Sterols were
568 named according to IUPAC nomenclature and listed in Table 2. B – Numbering of the sterol
569 skeleton.

570 Figure 2. Flow chart of the extractions of the different *Agelas* sp. MF1 aliquots. Steps for
571 analysis of free sterols are shown in white boxes, and for analysis of free and bound or bound
572 sterols (including saponification), in grey boxes.

573 Figure 3. GC×GC chromatograms of the ethanol extracts of the three sponges. A – *Agelas* sp.
574 MF1, B – *Ecionemia* sp. SS1, C – *Petrosia* sp. 1; dotted lines indicate 3-oxosterols. Inserts
575 show the 1D-GC-MS chromatograms.

576 Figure 4. Comparison of sterol distribution in extracts obtained from *Agelas* sp. MF1. Free
577 sterols refers to sterols obtained from ethanol of the preserved specimen, from the wet frozen
578 and the lyophilized sample. Total sterols includes sterols detected in the extracts after
579 saponification, and bound sterols include those obtained from the preserved tissue after
580 saponification of the extracted residue.

581 Figure 5. Sterol composition of the three sponges analyzed, in comparison to published
582 species from the same genera. *Agelas* sp. MF1 is compared to *A. mauritiana*, for *Petrosia* sp.
583 1, the composition determined from the three differently preserved specimens is shown, and
584 compared to *P. australis* and *P. pigmentosa*. For *Ecionemia* sp. SS1, sterol composition is as
585 determined from the three differently preserved specimens. [1] Composition as determined by
586 Santalova *et al.* (2004), [2] composition as determined by Fromont *et al.* (1994).

Tables

Table 1. Sample details. All sponges were collected during the following cruises and surveys: WA Marine Futures Biodiversity Project Survey Oct 2007 (WA-MFBPS), AIMS-WAM RV "Solander" Ningaloo Survey III Jan/Feb 2008 (AIMS-WAM III) and CSIRO RV "Southern Surveyor" Cruise SS1005 Nov/Dec 2005 (CSIRO SS1005).

Species	Museum registr. numbers	Preservation method	Location			Station	Depth [m]	Date collected	Cruise/Survey
				Start of trawl	End of trawl				
Agelas sp. MF1 Agelasidae, Agelasida, Demospongiae	Z49312	Ethanol	Broke Inlet	35°08'23"S 116°16'10"E	35°08'03"S 116°16'14"E	Trawl 1	65	2007/10/15	WA-MFBPS
	Z49312	Wet frozen	Broke Inlet	35°08'23"S 116°16'10"E	35°08'03"S 116°16'14"E	Trawl 1	65	2007/10/15	WA-MFBPS
	Z49312	Freeze dried	Broke Inlet	35°08'23"S 116°16'10"E	35°08'03"S 116°16'14"E	Trawl 1	65	2007/10/15	WA-MFBPS
Petrosia sp. 1 Petrosiidae Haplosclerida, Demospongiae	Z45259	Ethanol	Ningaloo Reef	22°36'53"S 113°34'55"E	22°36'52"S 113°34'55"E	RVS4545/2008	100	2008/02/05	AIMS-WAM III
	Z35817	Wet frozen	Kalbarri	27°55'42"S 113°08'16"E	27°56'01"S 113°08'38"E	SS1005/099	253.5	2005/12/04	CSIRO SS1005
	Z35811	Freeze dried	Kalbarri	27°48'48"S 113°18'39"E	27°49'05"S 113°18'39"E	SS1005/102	97	2005/12/05	CSIRO SS1005
Ecionemia sp. SS1 Ancorinidae, Tetractinellida Demospongiae	Z35069	Ethanol	Ningaloo South	22°04'00"S 113°48'40"E	22°04’15"S 113°48'54"E	SS1005/144	103.5	2005/12/10	CSIRO SS1005
	Z35949	Wet frozen	Zuytdorp	27°03'07"S 113°04'51"E	27°02'52"S 113°04'37"E	SS1005/110	106	2005/12/06	CSIRO SS1005
	Z35808	Freeze dried	Zuytdorp	27°03'06"S 113°06'03"E	27°02'56"S 113°05'59"E	SS1005/104	97	2005/12/05	CSIRO SS1005

Table 2. Percentages of identified sterols in Agelas samples. Pres. BM –sponge biomass preserved in ethanol. n.d. = not detected.

Nr.	Sterols	Free sterols					Total sterols			
		Ethanol	Frozen	Lyophil.	Pres. BM		Ethanol	Frozen	Lyophil.	Pres. BM*
1	Cholesta-5,22-dien-3β-ol									
2	5α-Cholest-22-en-3β-ol	7.3	5.8	4.2	3.6	1.6	12.5	7.3	5.8	4.2
3	Cholest-5-en-3β-ol	3.7	2.5	n.d.	n.d.	n.d.	2.7	3.7	2.5	n.d.
4	5α-Cholestan-3β-ol	18.7	16.9	10.7	10.4	7.0	22.1	18.7	16.9	10.7
5	Ergosta-5,22-dien-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	5α-Ergost-22-en-3β-ol	8.4	7.8	6.1	5.9	4.5	7.3	8.4	7.8	6.1
7	5α-Cholest-7-en-3β-ol	2.0	2.1	2.0	1.5	1.9	2.4	2.0	2.1	2.0
8	5α-Ergosta-5,24(24^1)-dien-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9	C_{29}Δ-Sterol	3.6	4.8	7.1	5.7	7.4	5.9	3.6	4.8	7.1
10	23,24^1-Cycloergost-5-en-3β-ol	4.8	6.0	n.d.	n.d.	n.d.	n.d.	4.8	6.0	n.d.
11	Ergost-5-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	5α-Ergost-24(24^1)-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
13	5α-Ergostan-3β-ol	6.5	8.0	2.9	2.1	1.5	3.9	6.5	8.0	2.9
14	Ergostatrien-3β-ol	0.3	0.6	3.0	1.3	0.1		0.3	0.6	3.0
15	Stigmasta-5,22-dien-3β-ol	4.0	3.6	7.5	2.2	1.7	4.2	4.0	3.6	7.5
16	5α-Stigmast-22-en-3β-ol	0.7	0.8	0.5	0.6	0.7	0.9	0.7	0.8	0.5
17	5α-Ergost-7-en-3β-ol	3.1	3.5	4.2	3.1	4.0	4.7	3.1	3.5	4.2
18	23,24^1-Cyclostigmast-5-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
19	Stigmast-5-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20	5α-Stigmasta-7,22-dien-3β-ol	21.9	22.0	34.5	51.0	54.0	24.8	21.9	22.0	34.5
21	(E)-Stigmast-24(24^1)-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22	Stigmasta-5,24(24$^{1)}$-dien-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
23	5α-Stigmastan-3β-ol	6.1	6.7	7.1	3.3	5.8	4.2	6.1	6.7	7.1
24	(Z)-Stigmast-24(24^1)-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
25	5α-Stigmast-8-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
26	5α-Stigmast-7-en-3ß-ol	9.0	9.0	10.1	9.5	9.8	4.4	9.0	9.0	10.1

*Abundances represent the bound sterols only, which were obtained by saponification of the residue from extraction.

Table 3. Percentages of identified sterols in Ecionemia and Petrosia specimens. Pres. BM –sponge biomass preserved in ethanol.

		***Ecionemia* sp. SS1**			***Petrosia* sp. 1**		
Nr.	**Sterol name**	**Ethanol**	**Frozen**	**Lyophil.**	**Ethanol**	**Frozen**	**Lyophil.**
1	Cholesta-5,22-dien-3β-ol	2.0	1.3	2.0	n.d.	1.1	2.3
2	5α-Cholest-22-en-3β-ol	2.7	n.d.	0.3	1.2	n.d.	n.d.
3	Cholest-5-en-3β-ol	34.1	4.1	3.5	n.d.	3.2	3.7
4	5α-Cholestan-3β-ol	10.1	1.0	0.9	10.1	3.5	8.7
5	Ergosta-5,22-dien-3β-ol	3.2	6.4	7.5	n.d.	3.4	8.2
6	5α-Ergost-22-en-3β-ol	1.3	n.d.	0.5	0.9	n.d.	0.6
7	5α-Cholest-7-en-3β-ol	1.1	n.d.	n.d.	n.d.	n.d.	n.d.
8	5α-Ergosta-5,24(24^1)-dien-3β-ol	1.9	n.d.	0.3	n.d.	21.9	18.7
9	C_{29}Δ-Sterol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	23,24^1-Cycloergost-5-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
11	Ergost-5-en-3β-ol	1.4	n.d.	0.4	68.7	37.3	50.3
12	5α-Ergost-24(24^1)-en-3β-ol	3.4	n.d.	n.d.	n.d.	n.d.	n.d.
13	5α-Ergostan-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
14	Ergostatrien-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15	Stigmasta-5,22-dien-3β-ol	1.7	1.5	1.2	1.8	2	2.8
16	5α-Stigmast-22-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
17	5α-Ergost-7-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18	23,24^1-Cyclostigmast-5-en-3β-ol	1.6	n.d.	0.4	n.d.	n.d.	tr
19	Stigmast-5-en-3β-ol	13.4	3.4	1.8	11.3	24.6	n.d.
20	5α-Stigmasta-7,22-dien-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	4.2
21	(E)-Stigmast-24(24^1)-en-3β-ol	n.d.	n.d.	n.d.	2.1	n.d.	n.d.
22	Stigmasta-5,24($24^{1)}$-dien-3β-ol	19.8	79.0	81.3	4.0	3	0.0
23	5α-Stigmastan-3β-ol	0.2	n.d.	n.d.	n.d.	n.d.	n.d.
24	(Z)-Stigmast-24(24^1)-en-3β-ol	1.8	n.d.	n.d.	n.d.	n.d.	n.d.
25	5α-Stigmast-8-en-3β-ol	0.3	3.3	n.d.	n.d.	n.d.	n.d.
26	5α-Stigmast-7-en-3ß-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Figure 1.

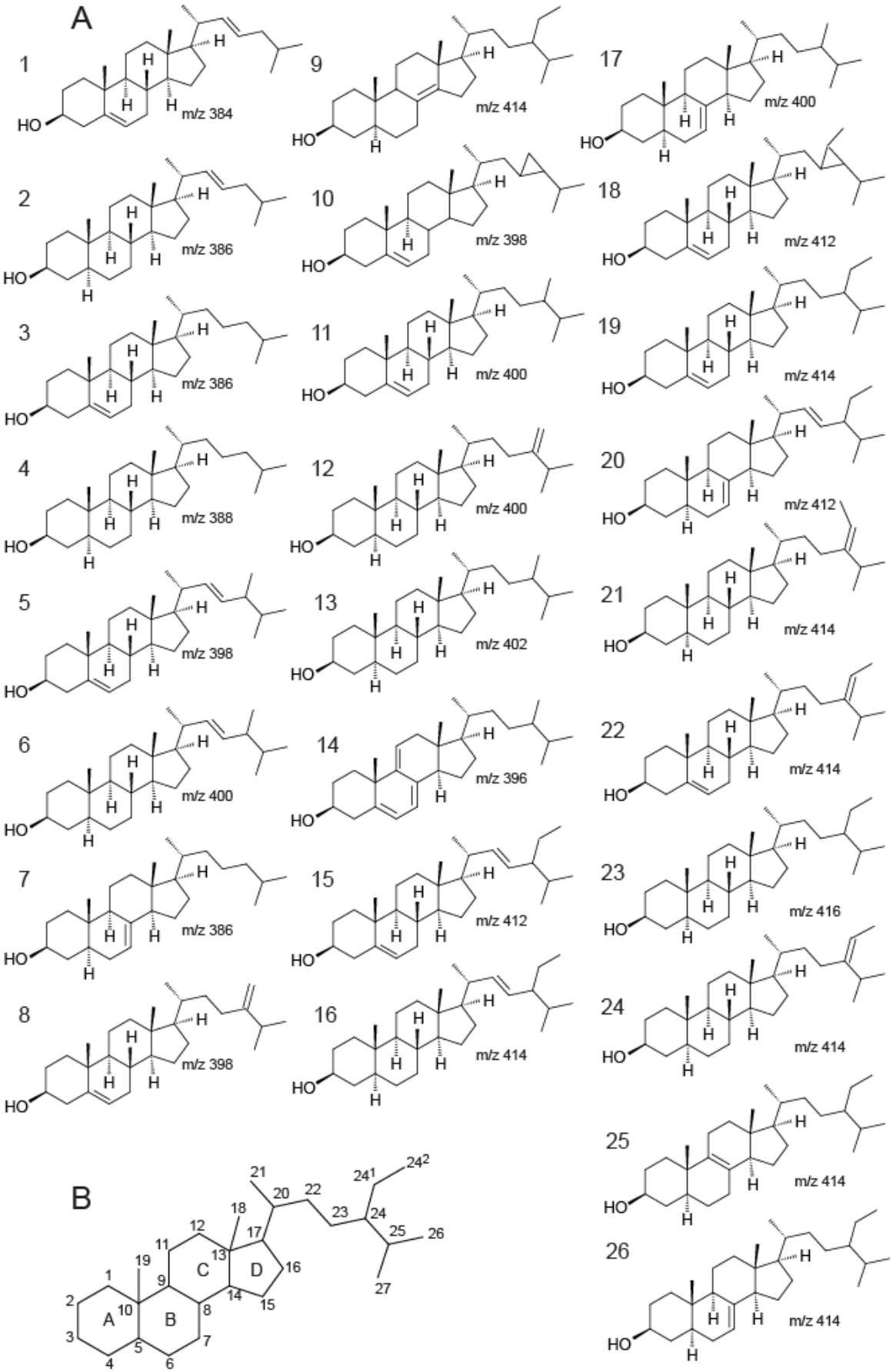

A
1
m/z 384
2
m/z 386
3
m/z 386
4
m/z 388
5
m/z 398
6
m/z 400
7
m/z 386
8
m/z 398
9
m/z 414
10
m/z 398
11
m/z 400
12
m/z 400
13
m/z 402
14
m/z 396
15
m/z 412
16
m/z 414
17
m/z 400
18
m/z 412
19
m/z 414
20
m/z 412
21
m/z 414
22
m/z 414
23
m/z 416
24
m/z 414
25
m/z 414
26
m/z 414
HO
B
A
B
C
D

Figure 2.

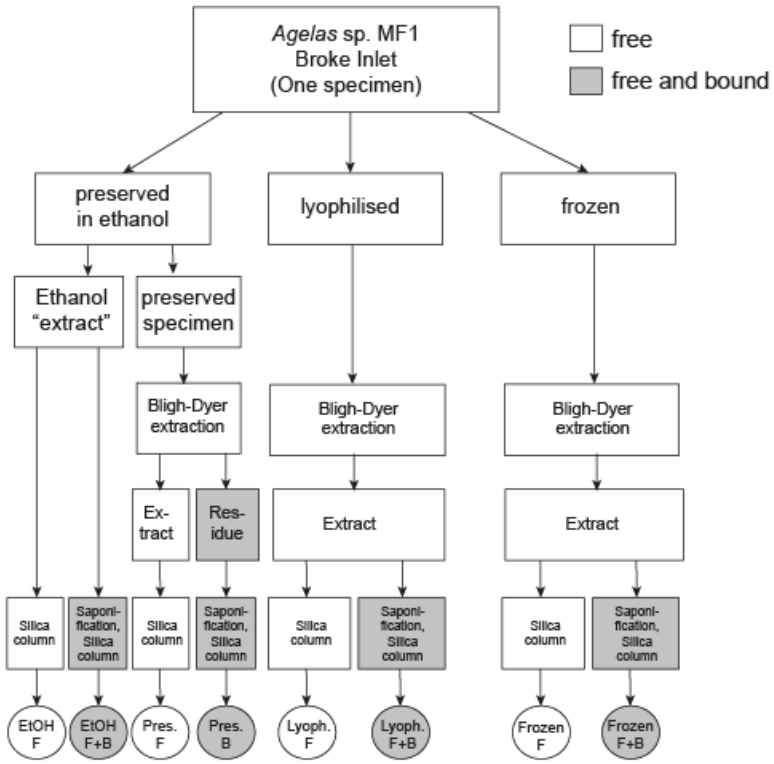

Agelas sp. MF1
Broke Inlet
(One specimen)
free
free and bound
preserved in ethanol
lyophilised
frozen
Ethanol "extract"
preserved specimen
Bligh-Dyer extraction
Bligh-Dyer extraction
Bligh-Dyer extraction
Ex-tract
Res-idue
Extract
Extract
Silica column
Saponi-fication, Silica column
Silica column
Saponi-fication, Silica column
Silica column
Saponi-fication, Silica column
Silica column
Saponi-fication, Silica column
EtOH F
EtOH F+B
Pres. F
Pres. B
Lyoph. F
Lyoph. F+B
Frozen F
Frozen F+B

Figure 3.

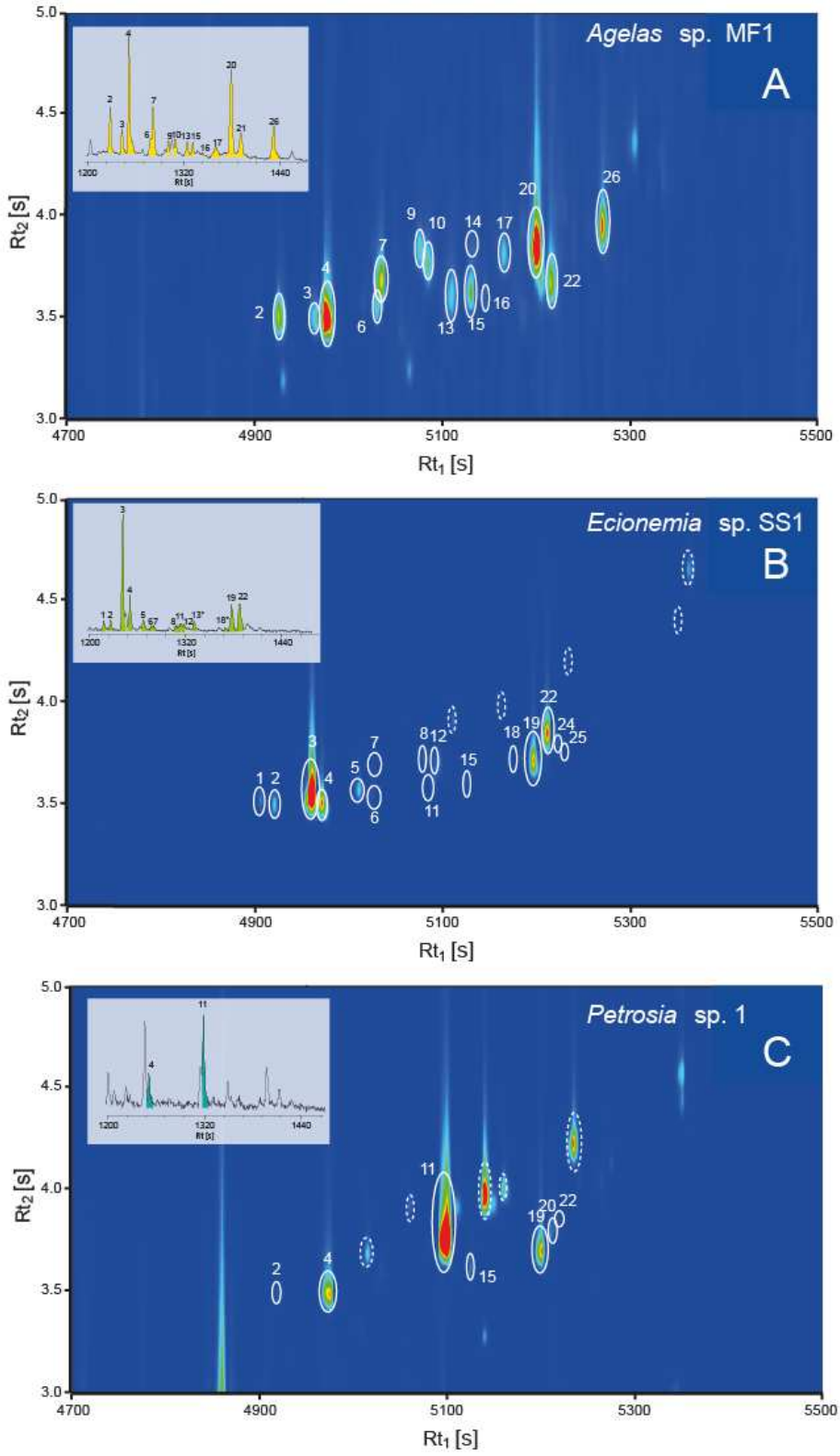

Agelas sp. MF1
A
Ecionemia sp. SS1
B
Petrosia sp. 1
C
Rt2 [s]
Rt1 [s]
5.0
4.5
4.0
3.5
3.0
4700
4900
5100
5300
5500
1200
1320
1440
Rt [s]

Figure 4.

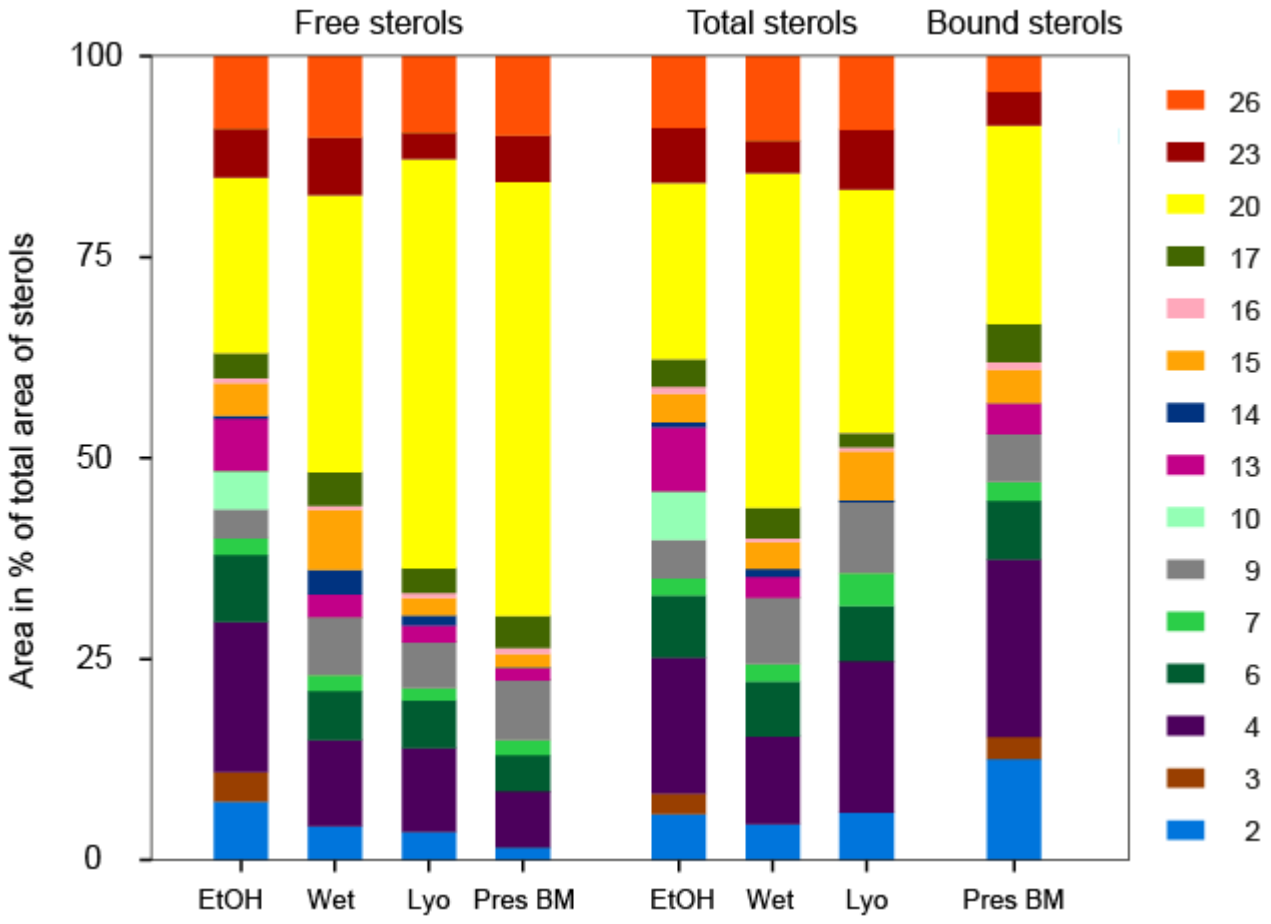
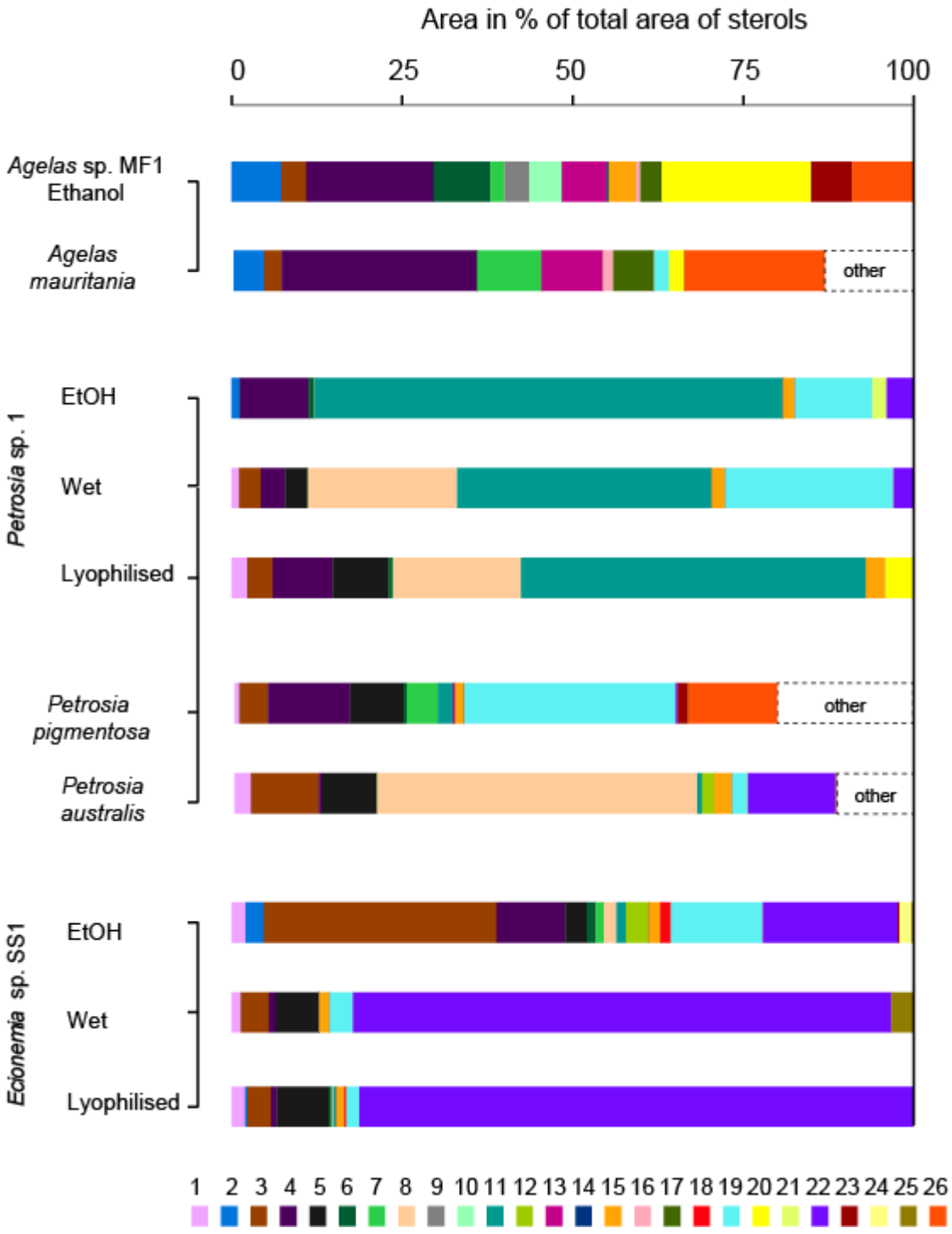

Free sterols
Total sterols
Bound sterols
Area in % of total area of sterols
100
75
50
25
0
EtOH
Wet
Lyo
Pres BM
EtOH
Wet
Lyo
Pres BM
26
23
20
17
16
15
14
13
10
9
7
6
4
3
2

Figure 5.


Area in % of total area of sterols
0
25
50
75
100
Agelas sp. MF1
Ethanol
Agelas
mauritania
other
Petrosia sp. 1
EtOH
Wet
Lyophilised
Petrosia
pigmentosa
other
Petrosia
australis
other
Ecionemia sp. SS1
EtOH
Wet
Lyophilised
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

Supplementary 1. *Sterol identification, mass spectra and retention times*

Analysis by GC-MS (underivatized and as TMS ethers) allowed the identification of the main sterol constituents (Fig. S2, S3). However, analysis by GCxGC-TOFMS showed an increased signal-to-noise ratio particularly for the ethanol samples, where some contaminants or oxosterols had previously concealed the presence of smaller, in the first dimension partially co-eluting compounds in the extracts. It was thus further used for identification and semi-quantification. In order to increase the separation efficiency on the polar, secondary column, underivatized sterol fractions rather than TMS ethers were used.

Sterols in the sponge extracts were identified based on GC retention times (Primary, or 1st column: Rt_1; Secondary, or 2nd column: Rt_2) and mass spectra, *via* comparison to library spectra or previously published spectra (as referenced below). 26 different sterols were detected in the three sponges (Table S1). Most sterols could be identified based on their mass spectra, such as the sterols from the cholestane series with 27 carbon atoms (**1-4**, Fig. 1, Table S1), of which cholesta-5,22-dien-3β-ol (**1**), 5α-cholest-22-en-3β-ol (**2**), cholest-5-en-3β-ol (**3**) and 5α-cholestan-3β-ol (**4**). 5α-cholest-7-en-3β-ol (**7**) showed a very similar mass spectrum to (**3**), but a higher Rt_1, which indicates that the double bond was present at the 7-position (Gerst et al., 1997). The Rt_2 of sterols were around 3.5 to 3.8 seconds, and generally around 0.5 seconds lower than the corresponding 3-oxosterols, with a ketone group instead of the hydroxyl group at position 3 (Fig. 2), a valuable feature in distinguishing 3-oxo and 3-hydroxy compounds. A number of sterols from the ergostane series (28 carbon atoms) could be identified, with 5α-ergostan-3β-ol (**13**) as the fully saturated representative, and with a saturated side chain but unsaturation in the ring system (**11**, **17**). The latter showed a molecular ion ($M^{+\cdot}$) at *m/z* 400, indicating one unsaturation, and a fragment at *m/z* 213 produced after loss of the D-ring, the side chain and H_2O, which indicates an unsaturated ring system. As with the cholestane series, the location of unsaturation was determined from the elution order as specified by Gerst et al. (1997): the sterol with the double bond between carbon 8 and 14 [$=\Delta^{8(14)}$] eluted first, followed by Δ^5, $\Delta^{8(9)}$ and then Δ^7 sterols. Ergostane-type sterols with one double bond on the side chain (SC) (**6**, **12**) showed a molecular ion at *m/z* 400. 5α-ergost-22-en-3β-ol (**6**) additionally showed strong peaks for the fragments *m/z* 273, 257 and 213

(M^{+}·-SC-42-H_2O), while the base peak of 5α-ergost-24(24^1)-en-3β-ol was observed at 316, a result of a McLafferty fragmentation and loss of part of the side chain. Similarly, C_{28} sterols with two unsaturations could be determined: the mass spectrum of ergosta-5,22-dien-3β-ol showed the characteristic fragments 271, 255 and 213 (M-SC-42-H_2O), while the base peak in the mass spectrum of ergosta-5,24(24^1)-dien-3β-ol was at *m/z* 314, diagnostic for Δ^{24} sterols. A triunsaturated sterol could also be resolved by GC×GC, tentatively assigned as ergostatrienol, with all double bonds located in the A, B and C rings in the ring system (numbering see Fig. 1B), as such presumably $\Delta^{5,7,9(11)}$. **10** was only detected in the ethanol of Agelas sp. MF1, and could thus be an artefact or a contaminant. The mass spectrum indicates an unsaturated core, and a cyclic side chain, and is thus possibly 23,24^1-cycloergost-5-en-3β-ol. A large number of different sterols from the stigmastane series were also identified: 5α-stigmastan-3β-ol (**24**) was the fully saturated sterol, while sterols with one unsaturation in the ring system (stigmast-5-en-3β-ol, **19**, 5α-stigmast-8-en-3β-ol, **25**, and 5α-stigmast-7-en-3β-ol, **26**) were also detected. Due to the similarity of their mass spectra, the positions of the double bond were assigned based on retention times, with Rt_1 of $\Delta^{8(14)} < \Delta^5 < \Delta^{8(9)} < \Delta^7$. C_{29} sterols with one unsaturation in the side chain were are also detected and included 5α-stigmast-22-en-3β-ol (**16**), and two isomers of 5α-stigmast-24(24^1)-en-3β-ol (M^{+}· *m/z* 414, base peak *m/z* 316), which were identified as the 24(*E*)- (**21**) and the 24(*Z*)- (**24**) isomers based on retention times, which are greater for the latter. It has to be noted that, in the first dimension, we observed co-elution of the 24(*E*)-isomer with stigmasta-5,24(24^1)-dien-3β-ol (**22** M^{+}· *m/z* 412, base peak *m/z* 314), while the compounds could be resolved in the second dimension. Other isomers with two unsaturations included stigmasta-5,22-dien-3β-ol (**15**), 5α-stigmast-7,22-dien-3β-ol (**20**). An unusual sterol that was tentatively identified in *Ecionemia* sp. SS1 (ethanol and lyophilized) by spectral matching was 23,24^1-cyclostigmasta-5-en-3β-ol (**18**, = dihydrocalysterol, Li et al. 1982). Identification of this sterol was based on its molecular ion of *m/z* 412 of low abundance, the presence of an ion of *m/z* of 314, m/z 271 and *m/z* 213 and comparison to the spectra published by Li et al. (1982). An unusually early eluting C_{29} sterol (**9**) with an M+· of *m/z* 414, and a strong peak at *m/z* 213, were also detected and tentatively assigned as a C_{29} sterol with one unsaturation in the core.

Table S1. Identified sterols in the compounds, retention times and *m/z* used for identification. 'Compound nr.' indicates the number of the compound used in Fig. 1, 'sterol name' the name according to IUPAC nomenclature (IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN), 1989), 'Rt_1' the retention time in the first dimension in seconds, 'Rt_2' the retention time in the second dimension in seconds, with a 5 second modulation period, and MS data the fragments that were observed and used for identification.

Sterol nr. (see Fig. 2)	**Sterol name**	**[$M^{+}\cdot$]**	Rt_1	Rt_2	**MS data (typical fragments)**
1	Cholesta-5,22-dien-3β-ol	384	4905	3.52	384, 366, 351, 300, 285, 271, 255, 229, 213, 199
2	5α-Cholest-22-en-3β-ol	386	4920	3.49	386, 371, 353, 302, 287, 273, 257, 245, 232, 215, 201
3	Cholest-5-en-3β-ol	386	4960	3.56	386, 368, 353, 301, 275, 255, 231, 213, 199
4	5α-Cholestan-3β-ol	388	4970	3.50	388, 373, 355, 262, 233, 215
5	Ergosta-5,22-dien-3β-ol	398	5010	3.57	398, 365, 355, 337, 300, 271, 255, 213, 199
6	5α-Ergost-22-en-3β-ol	400	5025	3.54	400, 339, 316, 302, 287, 273, 257, 242, 215
7	5α-Cholest-7-en-3β-ol	386	5025	3.66	386, 371, 353, 301, 273, 255, 231, 213, 199
8	5α-Ergosta-5,24(24^1)-dien-3β-ol	398	5080	3.71	398, 314, 299, 281,271, 255, 229, 213, 199
9	C_{29}Δ-Sterol	414	5070	3.83	414, 399, 382, 381, 301, 283, 273, 269, 213
10	23,24^1-Cycloergost-5-en-3β-ol	398	5080	3.73	398, 383, 271, 255, 229, 213
11	Ergost-5-en-3β-ol	400	5090	3.62	400, 382, 367, 340, 327, 315, 289, 255, 231, 213, 199
12	5α-Ergost-24(24^1)-en-3β-ol	400	5095	3.70	400, 385, 367, 316, 301, 283, 273, 255, 233, 215, 201
13	5α-Ergostan-3β-ol	402	5110	3.66	402, 387, 369, 327, 299, 276, 233, 215
14	Ergostatrien-3β-ol	396	5130	3.86	408, 390, 375, 277, 267, 251, 235, 225, 209
15	Stigmasta-5,22-dien-3β-ol	412	5125	3.61	412, 397, 379, 300, 271, 255, 229, 213
16	5α-Stigmast-22-en-3β-ol	414	5145	3.59	414, 257, 215
17	5α-Ergost-7-en-3β-ol	400	5165	3.81	400, 357, 327, 273, 255, 213
18	23,24^1-Cyclostigmast-5-en-3β-ol	412	5185	3.72	412, 394, 379, 352, 338, 327, 314, 301, 281, 271, 255, 231, 213, 199
19	Stigmast-5-en-3β-ol	414	5195	3.80	414, 396, 381, 367, 339, 329, 303, 273, 255, 241, 231, 213, 199
20	5α-Stigmasta-7,22-dien-3β-ol	412	5200	3.85	412, 369, 351, 300, 271, 255, 229, 213
21	(E)-Stigmast-24(24^1)-en-3β-ol	414	5210	3.77	414, 399, 381, 316, 301, 283, 273, 233, 215, 203
22	Stigmasta-5,24($24^{1)}$-dien-3β-ol	412	5210	3.85	412, 397, 379, 314, 299, 281, 271, 255, 253, 229, 213, 211, 199
23	5α-Stigmastan-3β-ol	416	5215	3.67	416, 401, 383, 355, 316, 290, 248, 233, 215
24	(Z)-Stigmast-24(24^1)-en-3β-ol	414	5220	3.80	414, 399, 381, 316, 301, 283, 273, 233, 215, 203, 201, 199
25	5α-Stigmast-8-en-3β-ol	414	5225	3.73	414, 396, 381, 355, 329, 315, 303, 267, 255, 231, 213, 199
26	5α-Stigmast-7-en-3ß-ol	414	5270	3.95	414, 399, 381, 273, 255, 231, 213, 201, 199

References

Gerst N, Ruan B, Pang J, Wilson WK, Schroepfer GJ (1997) An updated look at the analysis of unsaturated C_{27} sterols by gas chromatography and mass spectrometry. *Journal of Lipid Research* **38**, 1685–1701.

IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN) (1989) The nomenclature of steroids. *European Journal of Biochemistry* **186**, 429–458.

Li LN, Li HT, Lang RW, Itoh T, Sica D, Djerassi C (1982) Minor and trace sterols in marine invertebrates. 31. Isolation and structure elucidation of 23H-isocalysterol, a naturally occurring cyclopropene. Some comparative observations on the course of hydrogenolytic ring opening of steroidal cyclopropenes and cyclopropanes. *Journal of the American Chemical Society* **104**, 6726–6732.

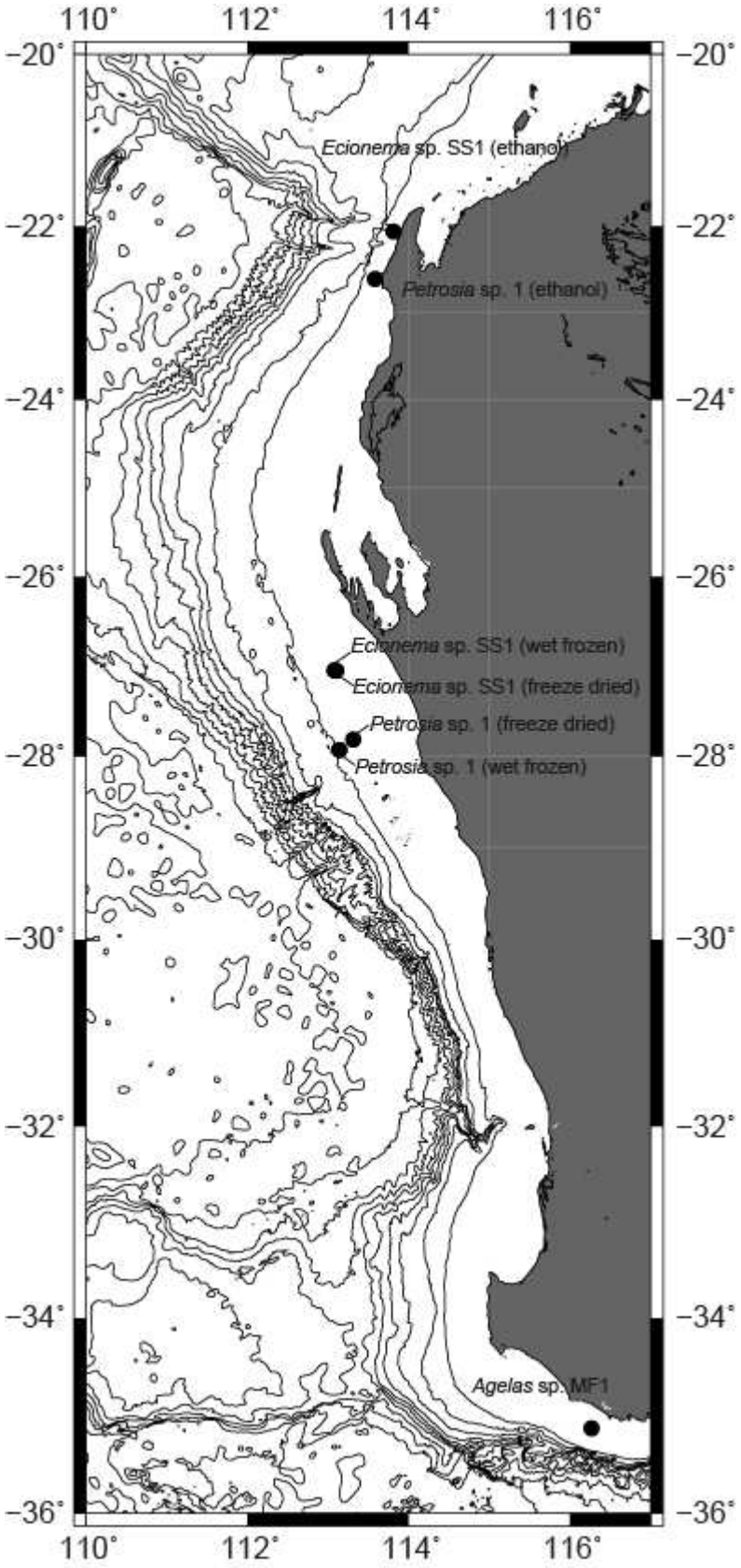


Figure S1. Map showing the sampling locations for all 9 sponge specimens. Bathymetry is shown with 500 m contour lines.

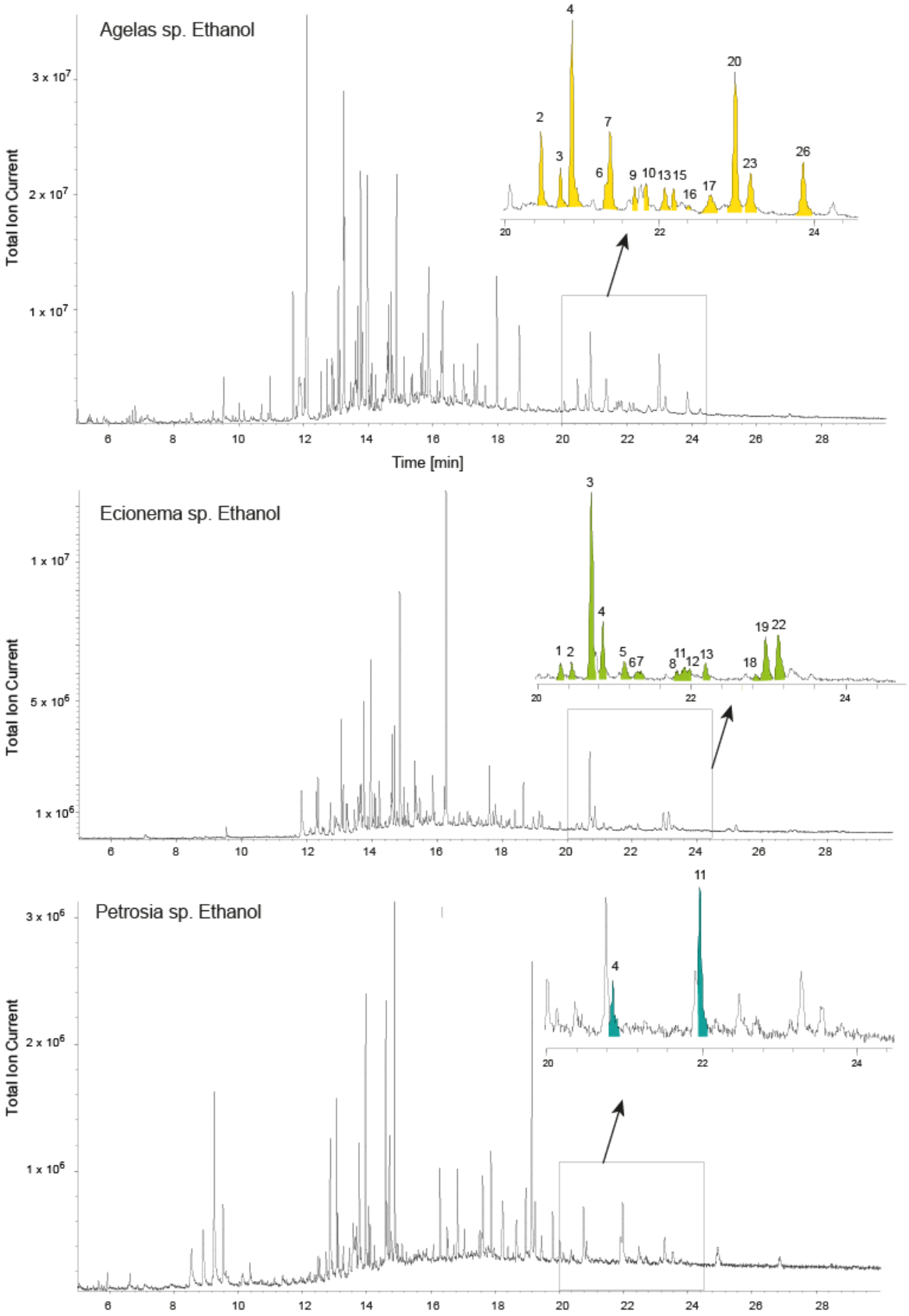


Figure S2. Chromatograms of the underivatized polar fraction obtained by GC-MS. A – *Agelas* sp. MF1, B – *Ecionemia* sp. SS1, C – *Petrosia* sp. 1.

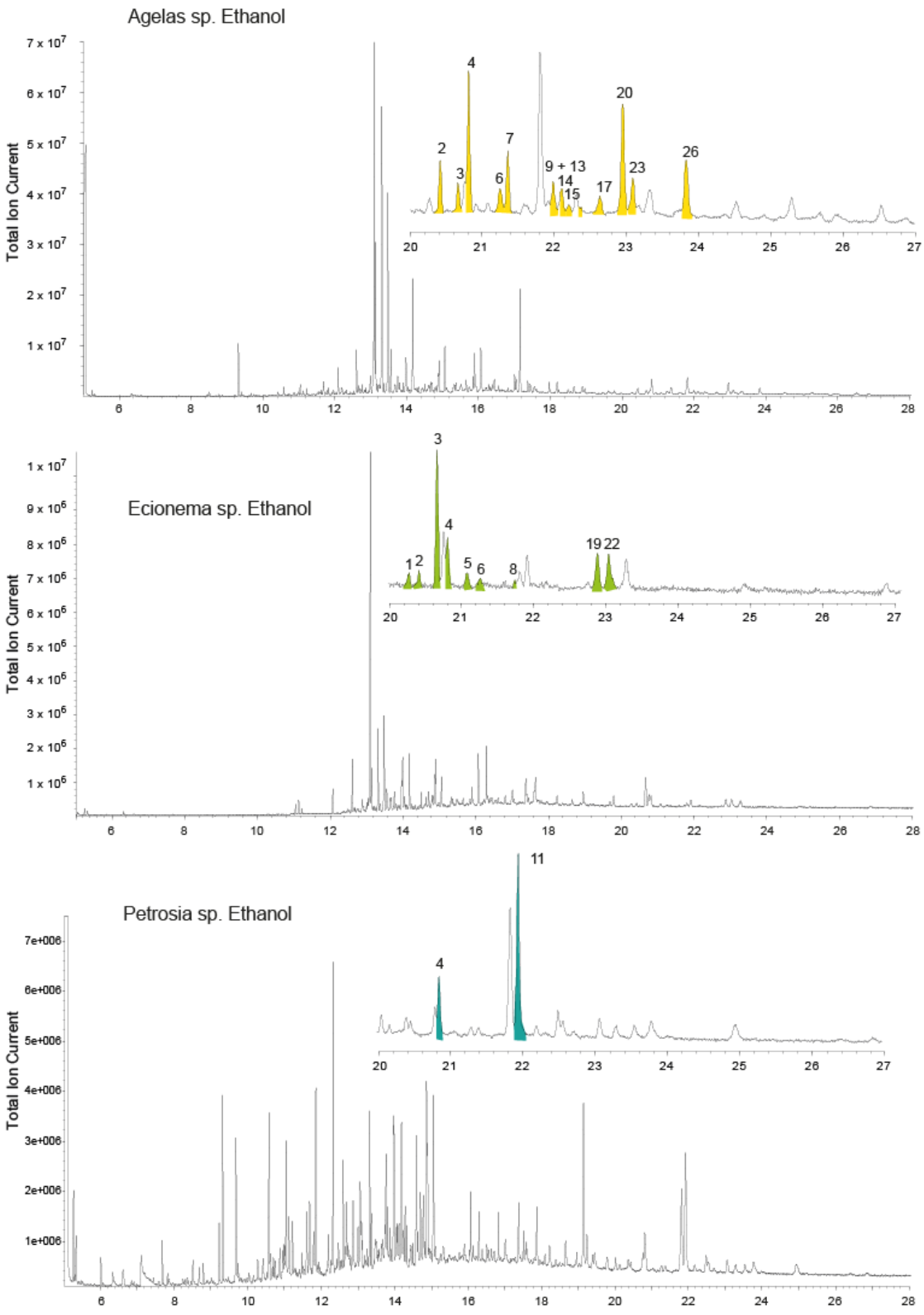


Figure S3. Chromatograms of the BSTFA-derivatized polar fraction (TMS ethers) obtained by GC-MS. A – *Agelas* sp. MF1, B – *Ecionemia* sp. SS1, C – *Petrosia* sp. 1.

Figure S4.

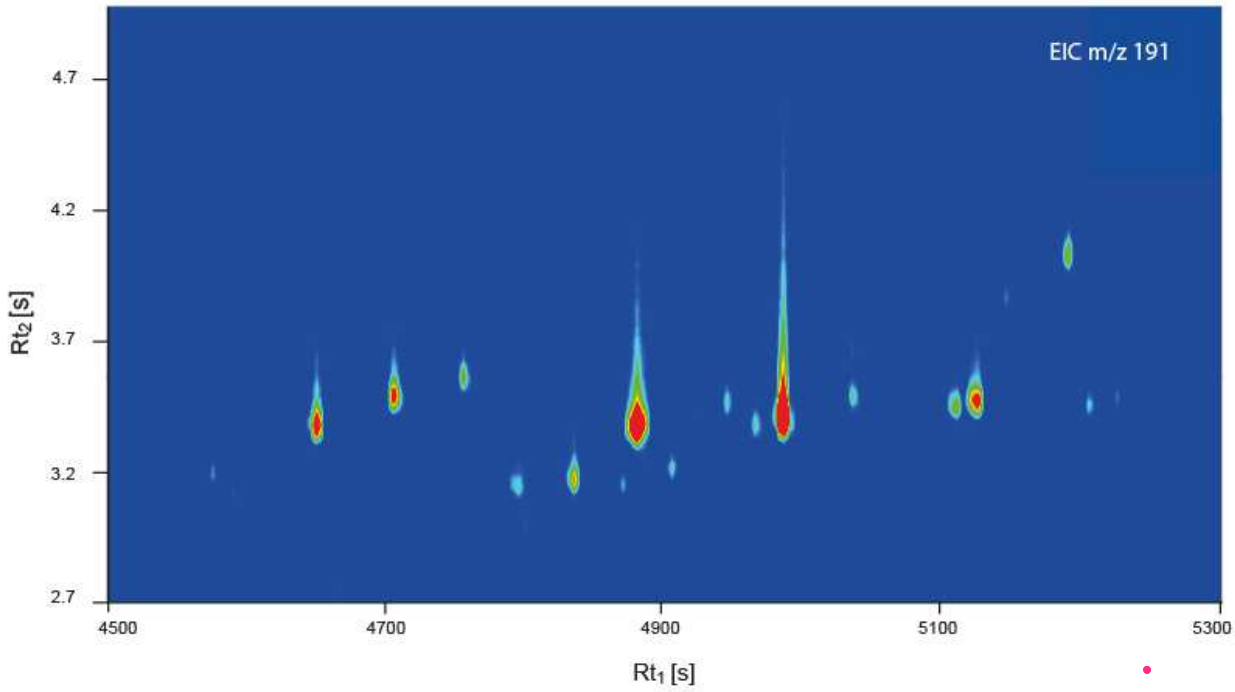


Figure S4. Extracted ion current of *m/z* 191 for the saponified biomass showing possible triterpenoid compounds which were not present in any of the extracts.