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Rapid Profiling of Saponin Extracts from *Stichopus horrens* Sea Cucumbers by Mass Spectrometry

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Sea cucumbers produce bioactive compounds known as saponins. Structurally, these triterpene glycosides contain different sugar moieties that are attached to a lanostane-based aglycone backbone. This study aims to rapidly profile the saponins of the Philippine sea cucumber, *Stichopus* cf. *horrens*, by mass spectrometry (MS). MS and tandem MS (MS/MS) were acquired using a UPLC-QToF (ultra-performance liquid chromatography–quadrupole time-of-flight) mass spectrometer. The aglycone and glycan components of the saponins were determined from the corresponding MS^2 spectra. A total of 22 saponins were detected, nine of which were similar to known sea cucumber saponins. Some of these saponins – such as holothurinoside C, holothurinoside H, impatienoside A, and stichloroside A2/B2 – have been shown to be effective as antitumor, anti-cancer, or antifungal compounds. The other 13 saponins we detected appear to be unique as these have not been previously reported. Glycan and potential aglycone components of the new saponins based on MS/MS are proposed. The methods used in this study may be used to rapidly profile saponin compounds in sea cucumbers and quickly identify bioactive compounds or new saponins that may be of interest.

Keywords: mass spectrometry, sea cucumbers, saponins

INTRODUCTION

Holothuroid sea cucumbers possess saponins that function as part of chemical defense against predators. These triterpene glycosides accumulate in the body walls and Cuverian tubules of sea cucumbers and have been reported to have antimicrobial, antiviral, hemolytic, cytotoxic, and antitumor activities (Bahrami and Franco 2016; Bordbar *et al*. 2011; van Dyck *et al*. 2010; Zhao *et al*. 2018; Olavides *et al.* 2010). These bioactivities are primarily attributed to the inherent structure of saponins that allow them to interact and disrupt cellular membranes (Popov 2002). These structures are diverse and have evolved to have varying aglycone backbone structures and modified glycans (Bondoc *et al*. 2013; Kalinin *et al*. 2005, 2015, 2016). Interestingly, the saponin structures have been shown to vary between families or even genera and have been used extensively as chemotaxonomic markers (Avilov *et al*. 2000, 2003; Bahrami and Franco 2016; Omran *et al*. 2020; Kalinin *et al*. 2005, 2016; Moraes *et al.* 2005).

The diversity of saponins in a species can be rapidly profiled using MS techniques. The coupling of liquid chromatography (LC) with MS enables the study of numerous compounds in a high-throughput manner. Information about the aglycone and glycan components of

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saponins can be obtained through tandem MS. Profiling of sea cucumber saponins studies has increased in recent years and, as a result, has expanded the list of reported saponin structures (Mondol *et a*l. 2017).

There are over 170 species of sea cucumbers in the Philippines, with around 41 species being commercially sold (Choo 2008; Olavides *et al.* 2010). *Stichopus horrens* contributes largely to the Philippine sea cucumber trading and is used as raw material for pharmaceutical products (Akamine 2001; Bordbar *et al.* 2011; Choo 2008; Purcell *et al*. 2014). There are very few studies conducted to analyze the saponins produced by local sea cucumbers. Studies on the toxicity of the crude holothurin content of various sea cucumber species have been conducted before by Pocisidio, but no further identification has been made aside from holothurins A and B from *Holothuria pulla* Selenka (Pocsidio 1987, 1988, 1990). Bondoc *et al.* (2013) studied three species – *Holothuria scabra*, *Holothuria impatiens*, and *Holothuria fuscocinerea* – using MALDI-FTICR (matrix-assisted laser desorption ionization–Fourier transform ion cyclotron resonance) MS to characterize their saponins. Their study revealed that the different species produced different saponin profiles and further suggested that the differences in the saponins may be used as chemotaxonomic markers (Bondoc *et al.* 2013). In this study, we profiled saponins obtained from *S. horrens* crude extracts using MS/MS.

MATERIALS AND METHODS

Animal Handling

Wild live adults were collected from Anda, Pangasinan, Philippines. Animals were kept and acclimatized in culture tanks at the Bolinao Marine Laboratory, Marine Science Institute in Bolinao, Pangasinan for at least 24 h. For further analysis, animals were killed by freezing and were transported in ice and stored at –80 °C.

Saponin Extraction

Solvents used in the study are analytical grade except for methanol, water, and acetonitrile – all of which are LC-MS grade. The dermal mass of 25 animals *sans* muscles and digestive organs was soaked in 70% ethanol (1 g tissue/mL EtOH) for one day. The resulting solution was lyophilized and resuspended in 90% LC-MS grade methanol prior to solvent partitioning (van Dyck *et al*. 2009; Bondoc *et al.* 2013). The resuspended extract was then partitioned against equal volumes of n-hexane. The methanolic portion was collected and partitioned against equal volumes of dichloromethane, followed by chloroform, and then dried under vacuum using a rotary evaporator at 40 °C. The resulting mass was dissolved in water and finally partitioned against isobutanol. The isobutanol fractions contain saponins. The samples were then stored as is at –20 °C until further analysis.

LC-MS/MS Analysis

MS/MS analyses were performed using a Xevo G2-XS ESI-QToF mass spectrometer equipped with a UPLC ACQUITY H-Class system. Saponin extracts were resuspended in methanol at a concentration of 2 mg/mL. One microliter (1 µL) of the sample solution was loaded onto a C18 column (130 Å, 1.7 µm, 2.1 mm x 50 mm) at a flow rate of 300 uL/min, and compounds were eluted into the mass spectrometer during a 3.5-min gradient from 5–60% B (solvent A: H_2O + 0.1% formic acid; solvent B: ACN + 0.1% formic acid) followed by a subsequent increase to 95% B from 3.50–5.00 min. High organic wash was added by increasing % B to 100% from 5.00–5.50 min and holding the composition until 8.00 min. A re-equilibration step was added by decreasing % B to 5% from 8.00–8.10 min and holding this gradient until 10.00 min. The mass spectrometer was set to acquire data in both positive and negative modes within a mass range of 500–2000. MS/MS was performed using fast data-dependent acquisition for ions with intensities greater than 10,000 counts and using a voltage ramp of 30–40V for low masses and 60–80V for high masses. MS/MS spectra were generated by acquiring ions from *m/z* 50–2000. Deconvoluted and centroided MS and MS/MS peak lists were generated using MassLynx V4.1 software and mMass (version 5.5.0). A signal-to-noise ratio of 3.0 or better was used for peak picking. MS/MS spectra were manually interpreted based on known saponin fragmentation schemes (*i.e.* neutral loss of glycosidic moieties).

RESULTS AND DISCUSSION

The saponin profiles of the semi-purified samples were obtained *via* LC-MS/MS. Mass spectra obtained in positive mode showed peaks that were predominantly sodiated adducts of putative saponins $[M + Na]^+$, whereas negative ion mode spectra showed deprotonated saponins $[M – H]^–$ (Bondoc *et al*. 2013). The combined mass spectra obtained from the LC-MS chromatogram of the butanolic fractions are shown in Figure 1. Saponins generally have molecular weights ranging from 1000–1500 (Table 1).

We observed a total of 22 unique masses within the molecular weight (MW) range of putative saponins, 13 of which have not been reported in the literature and with nine masses similar to previously identified Stichopodidae saponins. These were 26-Nor-25-oxo-holotoxin A1 (1394.6680 Da), variegatuside E/F (1412.6732 Da), variegatuside C (1072.5910 Da), stichloroside A/B

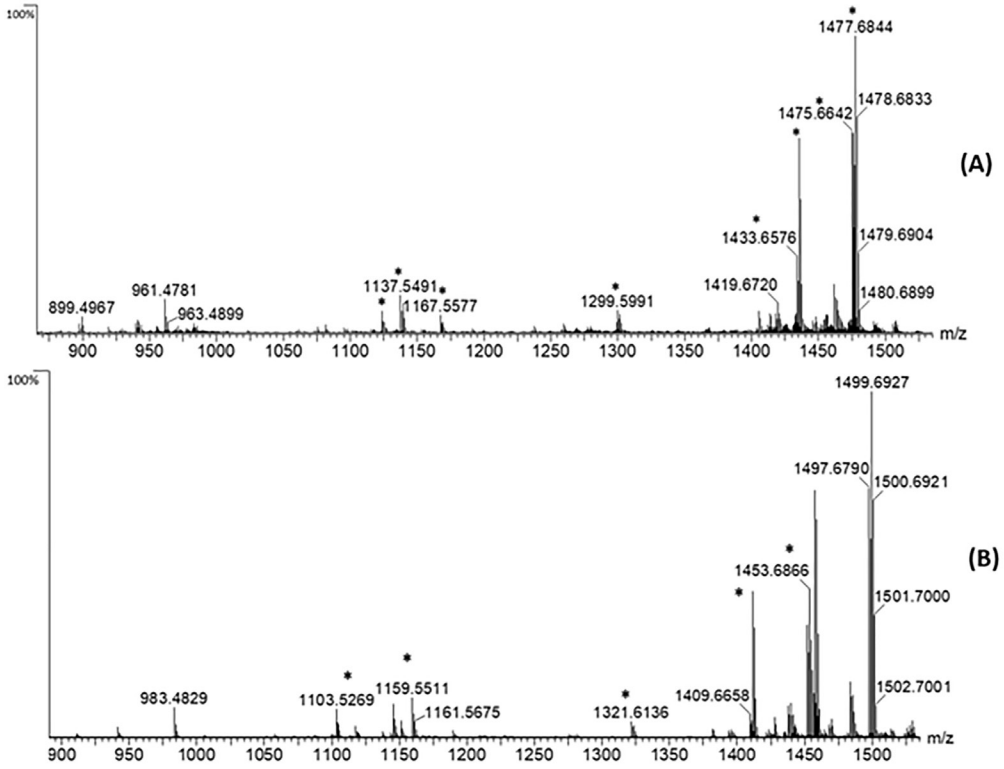

Figure 1. Representative mass spectrum of the semi-purified *S. cf. horrens* saponin extract. Masses acquired were from *m/z* 500 to 2000 in the positive mode (A) and negative mode (B). Putative saponin peaks are marked with an asterisk (*). Some peaks in the positive mode were also detected in the negative mode, such as $[M + Na]^+ = 1475.6642$ showing up as $[M - H]^- = 1451.6729$.

Table 1. List of unique masses identified in *S.* cf. *horrens* saponin extracts. The positive monoisotopic masses ($[M + Na]^+$) were compared with literature values and compounds were assigned within 5.00 ppm tolerance.

MW	$[M + Na]^+$	Compound name	Previously reported (Y/N)	Sea cucumber species
784.5032	807.5022	Unidentified	N	
786.4953	809.4943	Unidentified	N	
1072.5910	*1095.59	Variegatuside C	Y	*Stichopus variegatus* (Wang *et al.* 2014)
1088.5615	1111.5605	Unidentified	N	*Holuthuria scabra* (Bondoc *et al.* 2013)
1089.4990	1112.498	Unidentified	N	
1100.5720	1123.571	Thelenotoside A	Y	*Thelenota ananas* (Stonik *et al.* 1982)
1102.5659	1125.5649	Holothurinoside C	Y	*Holuthuria* sp. (Bondoc *et al.* 2013)
1132.5911	1155.5901	Unidentified	N	
1140.6730	1163.672	Unidentified	N	
1144.5918	1167.5908	Unidentified	N	
1146.6200	1169.619	Unidentified	N	
1391.6981	1414.6971	Unidentified	N	
1394.6680	1417.667	26-Nor-25-oxo-holotoxin A1	Y	*Apostichopus japonicus* (Wang *et al.* 2014)

MW	$[M + Na]^+$	Compound name	Previously reported (Y/N)	Sea cucumber species
1410.6586	1433.6576	Unidentified	N	
1412.6732	1435.6722	Variegatuside E/F	Y	*Stichopus variegatus* (Wang *et al.* 2014)
1424.6739	1447.6729	Impatienside A	N	*Holothuria impatiens* (Sun *et al.* 2007)
1440.7091	1463.7081	Holothurinoside H	N	*Holothuria forskalii* (van Dyck *et al.* 2009)
1443.6839	1466.6829	Unidentified	N	
1452.7558	1475.7548	Stichloroside A2/B2	Y	*Stichopus chloronotus* (Stonik *et al.* 1982)
1454.6854	1477.6844	Stichoposide E	Y	*Stichopus chloronotus* (Mal'tsev *et al.* 1983)
1484.7594	1507.7584	Unidentified	N	
1485.6970	1508.696	Unidentified	N	

Ions that eluted in more than one peak at different r.t. values are marked with an asterisk (*).

(1452.7558 Da), stichoposide E (1454.6854 Da), thelenotoside A (1100.5720 Da), holothurinoside C (1102.5659 Da), impatienside A (1424.6739 Da), and holothurinoside H (1440.7091 Da).

Interestingly, structural isomers separated by corresponding retention times (r.t.) are also present in the samples. For instance, the peak observed at *m/z* 1095.5322 corresponding to $C_{53}H_{84}O_{22}$ (variegatuside C) appears in two regions in the chromatogram (Figure 2). The separation of this ion in reversed phase–UPLC may suggest a different saponin which is a structural isomer of variegatuside C.

The putative saponin ions listed in Table 1 were subjected to subsequent MS/MS analysis to further characterize and partially deduce their structures. Low-energy collision-induced dissociation (CID) of saponins resulted predominantly in sequential cleavage of all the glycosidic bonds, leaving the aglycone or triterpene backbone intact. Fragmentation of parent ions that resulted from the neutral losses of 132, 146, 162, and 176 Da correspond to losses of xylose, quinovose, glucose, and methylglucose, respectively (Khotimchenko 2018). Some peaks can also be assigned as having generated from the loss of H_2O (–18 Da), CO_2 (–44 Da), and in the case of sulfated saponins, $NaHSO_4$ (–120 Da).

To illustrate, neutral losses in the MS/MS spectra of the parent ion at *m/z* 1477.64794 were analyzed and assigned corresponding sugar residues. A loss of 638 Da was observed which corresponds to the loss of the aglycone moiety. The peaks observed from *m/z* 809.45 followed by *m/z* 647.41, 515.37, 339.13, and 177.08 correspond to the successive loss of a Glc-Xyl-MeGlc-Glc chain (Figure 3). The aglycone component product ion is larger by 86 Da, which indicated that the xylose attached to the aglycone underwent cross-ring cleavage. In summary, our MS/MS data shows that the *m/z* 1477 ion we detected in the *S. cf. horrens* extracts is stichoposide E.

Purified saponins sometimes appear as $[M + H]^+$ ions instead of sodiated ones, and seem to provide more fragmentation data. The ion at *m/z* 1477 previously described was also observed as a protonated ion at 1455 when purified *via* HPLC. The MS^2 spectra in Figure 4 show some degree of difference between the fragmentation of protonated and sodiated ions. Key neutral losses were still present, as the sugars Xyl, MeGlc, and Glc were still observed. Taken together, the MS/MS profiles of the protonated and the sodiated saponin ions help in assigning the glycan and aglycone components and verifying that the identification of the other eight previously characterized saponins is correct.

We then proceed to determine whether the other 13 compounds that we detected could be saponins by analyzing their tandem MS. For example, the ion at 1414.6971 Da shows neutral losses corresponding to a successive loss of Glc-Xyl (Figure 5). The structure of the aglycone with mass 647 Da has not been previously reported and the tandem MS data did not provide any further information to further elucidate its structure. Therefore, at this point, we report possible aglycone molecular formulas that are consistent with 647 Da (Table 2).

Similarly, the unknown ion at *m/z* 1447.6729 shows a sugar chain composing of Glc-Xyl-MeGlc-Glc from the neutral losses observed from 809.4590–647.4293–515.3790–339.1269–177.0756 (Figure 6). The mass of the aglycone at 638 Da has not been observed in other

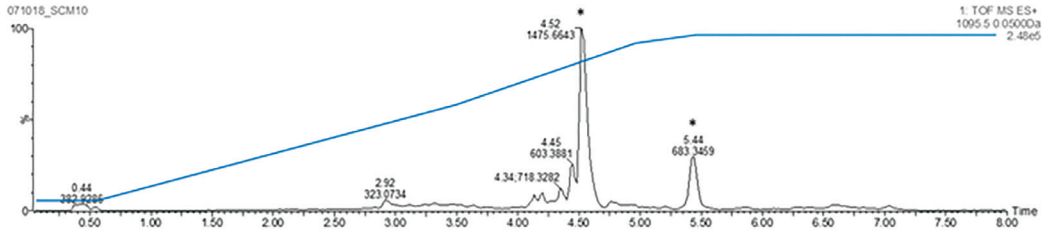

Figure 2. Extracted ion chromatogram (XIC) of the peak *m/z* 1095.5322 as $[M + Na]^+$. Presence of the ion within a tolerance of 0.05 Da is indicated by an asterisk (*). r.t. values (min) are indicated on top of each peak. The elution gradient (% ACN) is shown as a blue overlay.

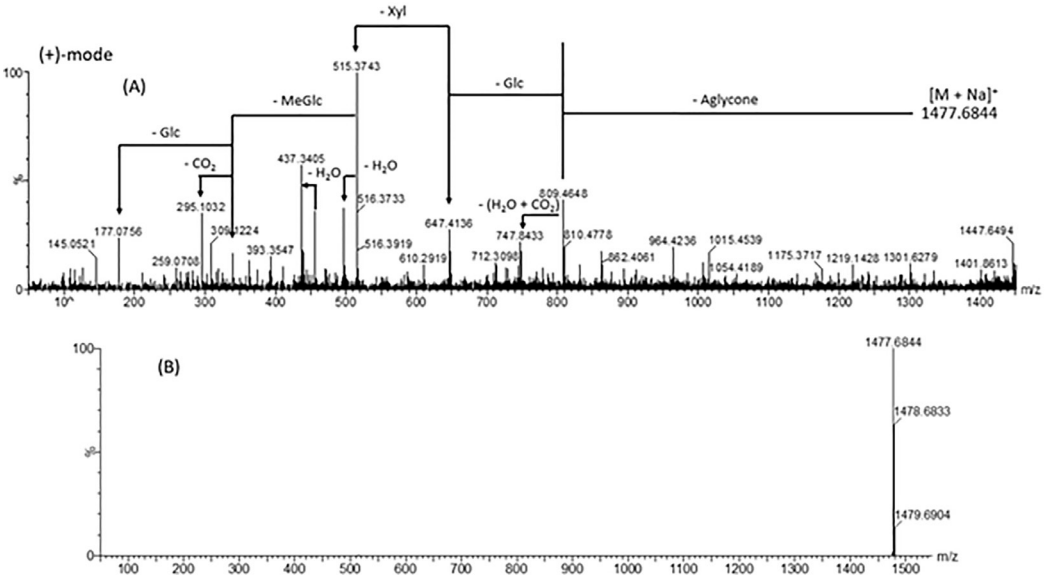

Figure 3. Tandem MS of *m/z* 1477.6844. Neutral losses of 162, 132, and 176 Da were assigned to loss of glucose, xylose, and methylglucose moiety respectively. A 10x magnification of the spectra at *m/z* range 50 to 1500 is provided in A, while the full MS/MS spectra are shown in B.

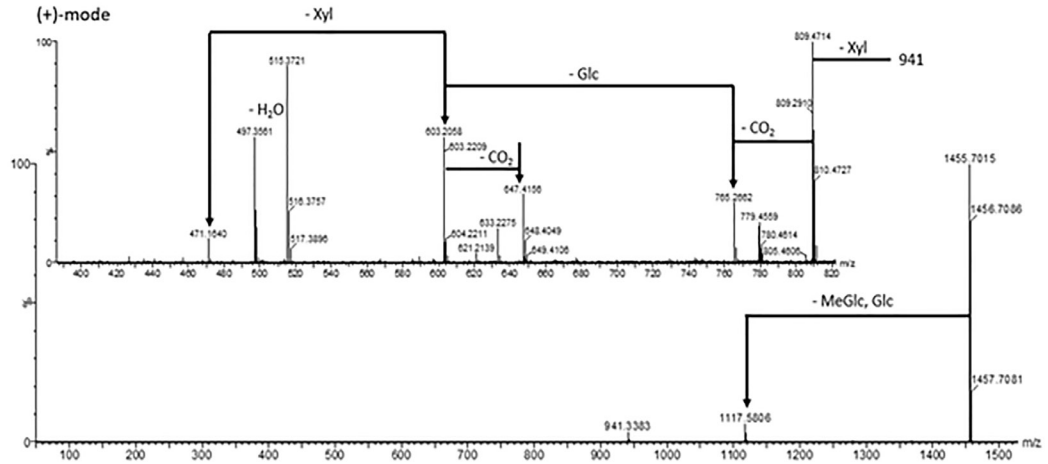

Figure 4. Tandem MS of *m/z* 1455.7015. A 10x magnification of the spectra at *m/z* range 400–800 is provided as an inset to highlight lower-intensity peaks.

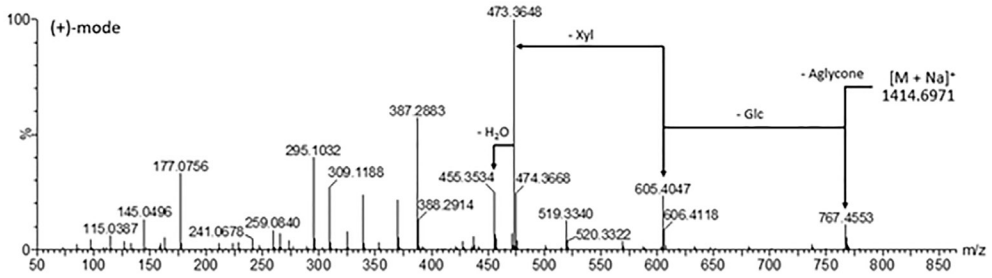

Figure 5. Analysis of the fragmentation patterns of the ion *m/z* 1414.6971 observed at r.t. 4.227 min.

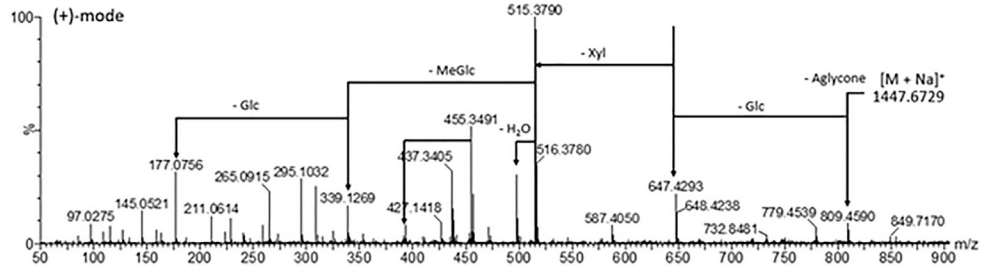

Figure 6. Analysis of the fragmentation patterns of the ion *m/z* 1447.6729 observed at r.t. 4.740 min.

Table 2. Possible glycan components and aglycone composition of the putative saponins based on tandem MS. Calculated aglycone masses after accounting for an uncleaved xylose sugar for those above 600 Da are provided inside parentheses and are used as the basis for computing possible R_1 molecular formulas.

Parent ion (*m/z*)	Glycans present	Apparent aglycone mass (neutral)	R_1	R_2	R_3
807.50	Glc, Xyl	490.40	$C_{11}H_{16}$		H
809.49	Glc, Xyl	492.41	$C_{11}H_{18}$		H
1095.59	MeGlc, Xyl	492.16	$C_{11}H_{18}$		H
1123.57	Xyl	456.16	$C_7H_{14}O$		H
1125.56	2 Xyl	469.42	$C_8H_{15}O$		H
1155.59	2 Xyl	513.47	C_8H_{16}		$C_2H_3O_2$
1167.59	2 Xyl	511.45	C_8H_{14}		$C_2H_3O_2$
1169.62	2 Xyl	513.48	C_8H_{16}		$C_2H_3O_2$
1414.70	Glc, Xyl	(515.20) 647.24	C_8H_{17}		$C_2H_3O_2$
1417.67	2 Glc, Xyl, MeGlc	(476.14) 608.18	$C_{10}H_{14}$	H	H
1433.66	Glc, Xyl, MeGlc	(536.16) 668.20	$C_{10}H_{16}O_2$		$C_2H_3O_2$
1435.67	Glc, Xyl	(536.17) 668.22	$C_{10}H_{16}O_2$		$C_2H_3O_2$
1447.67	2 Glc, Xyl, MeGlc	(506.17) 638.21	$C_{10}H_{17}$		$C_2H_3O_2$
1463.71	Glc, Xyl, MeGlc	(522.18) 654.22	$C_{11}H_{16}O_2$		H
1466.68	Glc, Xyl, 2 MeGlc	797.40			
1475.75	Glc, Xyl, MeGlc	(534.22) 666.27	$C_{10}H_{14}O_2$		$C_2H_3O_2$
1477.68	2 Glc, Xyl, MeGlc	(536.18) 668.22	$C_{10}H_{16}O_2$		$C_2H_3O_2$
1507.76	Glc, 2 Xyl, MeGlc	707.36			
1508.70	2 MeGlc, 2 Xyl	839.43			

saponins from the family *Holothuriidae* and the possible molecular formula of the aglycone is shown in Table 2.

In general, the *Stichopus* cf. *horrens* saponins do not contain sulfated saponins nor fucoidans as we did not detect any neutral loss of 120 Da in both positive and negative mode spectra. While glycan components can easily be determined from the MS^2 data, aglycone components are not fragmented as easily using low-energy CID and, thus, the tandem MS provides little information about their structures (Claeys *et al*. 1996). Hence, identification of the saponin aglycone backbones was performed by comparing the experimental MS data with the literature (Mondol *et al.* 2017). The putative molecular formulas of aglycones were suggested based on the possible R-groups of the lanostane backbone shown in Figure 7. The R_2 groups can either be -H or -OH while R_3 groups can be -H, a carbonyl (=O), or an acetate (-OAc) functional group. The R_1 moieties are often based on C_6H_{13}, which can be unsaturated with one or two double bonds, or have one or two =O, -OH, -OAc, -O-n-But, or -OEt groups replacing hydrogen (Mondol *et al*. 2017). The glycan and possible aglycone components of the saponins are listed in Table 2.

The aglycone units, which have a mass difference of more than 300 Da compared to the lanostane backbone such as those found in *m/z* 1414.6971 and 1433.6576, were presumed to have an uncleaved xylose residue (Kalinin *et al*. 2005). Aglycone masses with more than 450 Da difference compared to the lanostane backbone, such as those found in *m/z* 1466.6829 and 1508.696, were presumed to have at least two sugar residues still attached. However, except for the first xylose moiety, it is impossible to determine with certainty which sugars are still attached using only our fragmentation data (Kalinin *et al.* 2005).

Nevertheless, the R groups were assigned based on previously reported saponin structures, which have holostane backbones that predominate in sea cucumbers. Some non-holostane glycosides that lack the γ18(20)-lactone feature can also be found in holothurians. However, these are relatively rare and are only found in a few species, the majority of which are in Cucumariidae (Mondol *et al*. 2017; Silchenko *et al*. 2016, 2017). While it is possible that some of the saponins we obtained have non-holostane structures like saponins from some members of Stichopodidae, low-energy CID used in our experiments are unable to fragment the aglycone of saponins and, hence, provide little information about their true structure (Wang *et al*. 2012). Given this, multiple stages of fragmentation (MS^n) using an orbitrap or ion cyclotron resonance–MS instrument or nuclear magnetic resonance analysis need to be performed to definitively characterize these saponin structures.

CONCLUSIONS

Characterization of saponins using LC-MS methods allows us to rapidly profile and characterize potential saponins present in sea cucumbers. Rapid characterization of sea cucumber saponin extracts will enable the selection of potential sources of bioactive saponins from our local sea cucumbers, precluding the need for tedious purification methods that typically precedes structure characterization. This preliminary study shows that the Philippine *S.* cf. *horrens* sea cucumbers produce an untapped resource of known bioactive saponins. For instance, variegatuside E/F, stichloroside A2/B2, and 26-Nor-25-oxo-holotoxin A1 have been indicated to be potential antifungal compounds, while holothurinoside C/H have been shown to have antitumor activities. In addition, several saponins from the extracts we have reported have not yet been identified or published in the literature and could be potentially novel. Further purification, additional MS^n experiments or NMR studies need to be performed to validate the actual saponin

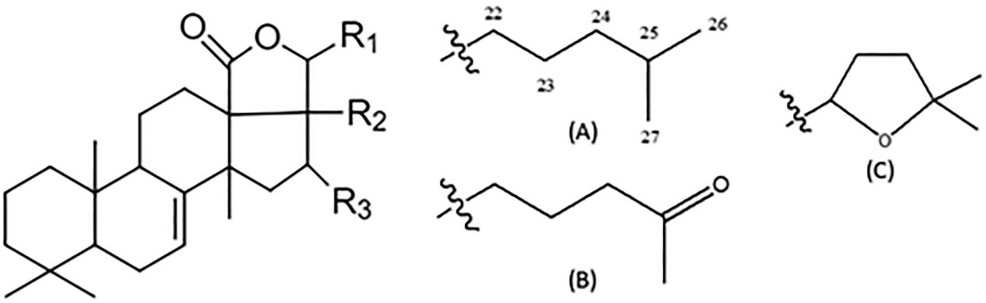

Figure 7. Structure of the lanostane backbone (left) and common R_1 motifs (A, B, C) found in sea cucumbers. The unsaturation at C-7(8) is sometimes shifted to the C-9(11) position The R_1 skeleton labeled A can be modified by adding an unsaturation or either an -OH, -OAc, -OEt, -O-n-But, or =O group.

structures. Following this, further characterization such as testing cytotoxic and hemolytic activities can also be done on the purified saponins to determine their chemical and biochemical relevance.

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