REGIONAL DIFFERENCE OF ALGINATES EXTRACTED FROM DIFFERENT BROWN SEAWEEDS

FENG TING

NATIONAL UNIVERSITY OF SINGAPORE

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Feng Ting

(*B. Sc., Sun Yat-sen University*)

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety, under the supervision of Prof. Sam Li Fong Yau, Departments of Chemistry, National University of Singapore, between 04/08/2013 and 01/08/2014.

I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Feng Ting		04/08/2014
Name	Signature	Date

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This one-year in Singapore means a lot to me. It is the first time for me to experience foreign life, the first time to learn how to do the research, how to conduct the experiments and finish a comprehensive project.

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Summary

Alginate, fucoidan and laminarin are the three main polysaccharides found in brown seaweeds. They have various bioactive functions, such as antioxidant, biocompatibility, non-toxicity and non-immunogenicity. Alginate has relatively large molecular weight with high viscosity. It is composed of α-L-guluronic acid (G) block and β-D-mannuronic acid (M) block units. The M and G blocks can form both homo-polymeric and hetero-polymeric units. Different conformations and chemical structures of M and G blocks will affect the bioactive functions of alginates. Moreover, it has been indicated that growing conditions could influence the structure formation. In order to analyze the regional difference, six brown seaweeds were collected from different locations. Subsequently, alginates were extracted by alkaline method. Saturated and unsaturated alginate oligosaccharides were also prepared by acid hydrolysis and alginate lyase degradation, respectively. Then we applied NMR and LC-MS/MS to detect both alginates and alginate oligosaccharides. The NMR testing results of M and G block concentrations were represented by F_M and F_G values: *Podina* from Malaysia belongs to high G species; Whereas *Laminaria japonica* from Shandong and Fujian belongs to high M species; *Laminaria saccharina* from U.S., *Laminaria digitata* from Iceland, and *Ascophyllum nodosum* from Indonesia are defined as intermediate alginates. Thus, *Podina* can provide brittle gel while the other five species produce elastic gels. In addition, two *Laminaria* species from China have similar M/G ratios, and *Laminaria saccharina* from U.S. and *Laminaria digitata* from

Iceland also have close M/G ratio. However, the concentrations of alginate solutions extracted from all these six brown seaweeds are not high enough compared with the alginate standard solution, thus the oligosaccharides are not successfully detected in the LC-MS/MS study. Therefore, more efficient and optimal extraction methods should be further explored to extract sufficient alginates for the analysis of the chemical structure, bioactive function as well as regional difference.

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1. Background

1.1 Introduction of seaweed

Seaweeds are gaining more and more attentions in recent years as renewable marine resource, with nearly six thousand seaweed species being identified to date [1]. They have no roots, leaves or vascular systems, and nourish themselves through osmosis [2]. Seaweeds have been traditionally consumed as sea vegetables, especially in Asian countries. In terms of output and value, seaweed cultivation is regarded to be more important than any other aquatic plants in Asia, and 87% of the total global seaweed cultivation lies in eastern Asia [3, 4]. Red seaweed, brown seaweed and green seaweed are the three main species. Red seaweeds usually grow in tropical areas while green and brown seaweeds are found in comparatively higher latitude, such as eastern China, Japan and North America [5]. Generally, cold waters are much better for more valuable brown seaweed cultivation, and the optimal water temperature is around 20 ℃ [6].

Besides the regional distribution differences, brown, green and red seaweed species also have different nutrient compositions and applications. For instance, red seaweeds contain a high level of proteins (around 40% of dry weight for most of the species), thus are generally used as additives for health care products. In contrast, green seaweeds have lower protein level (around 10-25% of dry weight), hence they are more suitable for biofuel production through bioethanol fermentation and biodiesel extraction [7].

Brown seaweeds have the lowest protein content but the highest polysaccharides composition [8]. For various brown seaweed species, the polysaccharide concentrations range from 4% to 76% by dry weight [9]. The main polysaccharides of brown seaweeds are cell wall structural and storage cells [3]. Due to the high polysaccharides concentration, brown seaweeds contain diverse biological activities in potential medicinal value, such as anti-viral, anti-coagulant, and anti-cancer [10, 11]. Despite all these valuable medicinal functions, most of the brown seaweeds are just simply used for food or animal feed. In Europe, brown seaweeds are traditionally used for producing additives or meals for animal nutrition [12]. In Asia, more than 60% of the total seaweed consumptions are brown seaweeds, while less than 5% are used for high nutritional products [13]. Moreover, in China, more than 90% of the *Laminaria japonica* are directly harvested and washed for food and feed [14].

Actually, polysaccharides derived from brown seaweeds can play a more important role in food, pharmaceuticals and other products [15]. For example, the traditional anticoagulants heparin resources are decreasing sharply these years, it has been indicated that new natural anticoagulants could be extracted from brown seaweeds for safer therapy [16]. Thus it is necessary to explore cheap and valuable alternative polysaccharides sources derived from brown seaweeds to replace the existing bioactive compounds that have limited resource. However, the specific functions and processes of extracting bioactive compounds form brown seaweeds are not clear enough. Therefore, more comprehensive and systematic research will need to be conducted to analyze and extend the potential pharmaceutical and health

applications of brown seaweed polysaccharides.

1.2 Brown seaweed polysaccharides

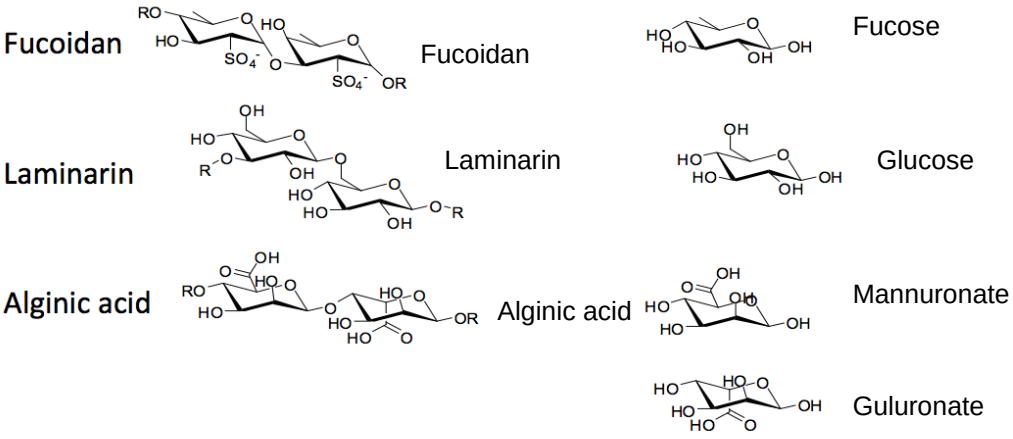

Fig. 1-1 Chemical structure of brown seaweed polysaccharides [11]

Polysaccharides are a class of macromolecules consisting of various oligosaccharides [12]. Laminarin, fucoidan and alginate are the three main polysaccharides found in brown seaweeds. The different compositions and structural activities of the three polysaccharides will affect the function of brown seaweeds.

Fucoidan, a unique class of polysaccharides solely found in brown seaweed, consist fucose, galactose, and sulfate subunits (Fig. 1-1) [11, 17]. Fucoidan is an expensive additive used in health care products for its bioactive properties such as anti-adhesive, anti-complimentary, anti-oxidant, anti-proliferative and anti-viral activities [18]. Laminarin is comprised of laminarin and glucose. Most laminarins are considered as dietary fibers for human consumption because it is resistant to hydrolysis [19].

Alginate is mainly located in brown seaweed cell wall and matrix [13]. It has relatively high molecular weight. Alginate also has various bioactive properties, such

as antioxidant, biocompatibility, non-toxicity, and biodegradability and is widely used among the most versatile biopolymers [20-22]. Alginate is highly viscous in aqueous solution, and it can form gel in the presence of divalent cations such as calcium [23, 24]. This particular physicochemical characteristic provides alginate with great potential in numerous applications such as food processing, cosmetics, and pharmaceutical industries [25]. Different grades of alginate are available for specific application: normal sodium alginates are used for industry application; high grade ones are used for pharmaceutical and food use, for instance, drug delivery excipients, wound dressings, and dental impression materials [26]. Some alginate coats of brown seaweeds, such as *P. aeruginosa,* could provide protection from immune defense system [27]. Moreover, alginate can protect against potential carcinogens and clear the digestive system. Furthermore, large molecular weight alginates can also prevent obesity, diabetes, and hypocholesterolemia[3].

In addition, alginate oligosaccharides (AOS) and their derivatives have specific bioactive functions for applications as releasing agents in pharmacy and additives in food industry [28, 29]. For instance, alginate oligosaccharides and their derivatives can promote plant growth due to the anti-tumor function. Moreover, recent studies have indicated that enzymatically depolymerized unsaturated alginate oligosaccharides can cause increases in shoot elongation after germination of *Komatsuna* [23]. In this case, more studies need to be devoted to analyze the influence of oligosaccharide sequences on the biological and physicochemical properties of alginates [30].

Alginate is an acidic linear polysaccharide composed of α-L-guluronic acid (G) block and β-D-mannuronic acid (M) block units [29, 31, 32]. The M and G blocks can join as both homopolymeric or heteropolymeric units in either one of three chains: α-L-guluronic acid (G group), β-D-mannuronic acid (M group) or alternating units of α-L-guluronic acid and β-D-mannuronic acid (MG group) [33]. The different concentrations of M and G block and M/G ratios will all affect the bioactive activities of alginate [28].

The M block forms β linkages to make linear and flexible conformation while the G block forms α linkages to provide folded and rigid structural conformations building steric hindrance around the carboxyl groups [20]. The different structure distributions of M and G block along the polymer chain and the ratio of M/G could affect the gel formation properties [25]. High-M concentration alginates form gels quicker while G-rich alginates hold calcium thus form gels slowly [33]. In order to comprehensively understand the chemical-physical properties and structure-function relationships of alginates, we aim to identify the sequence structure of alginate oligosaccharides [29, 34].

Besides the genetic factors affecting seaweed species, environmental factors (such as water temperature and salinity) and harvesting season may also influence the chemical compositions of brown seaweed polysaccharides [35]. Some research has previously focused on the seasonal difference of brown seaweed polysaccharides [36, 37]. Taking the *Laminaria* species as an example, the maximum amounts of polysaccharides are produced during summer and autumn, between June to November

[3]. Contrastingly, the winter harvests in February result in small amount or none polysaccharides production, which indicated significant seasonal variations [3]. Similarly, the best harvesting seasons for other seaweed species also differ [38].

Alginates based industry started in the late 1800s, and was enlarged to commercial scale since the 1930s [39]. At the very beginning, alginates were just been used as food and food additions, and then paper producing industries and finally in pharmaceuticals [33]. Each alginate has different functions depends on the different chemical structures [40]. Unfortunately, even till now, the specific function based on each alginate are not clear, more other underlying mechanisms of alginate bioactivities are on the list waiting for research to explore [28]. Moreover, combining with the environmental influences on alginate chemical structures, the various bioactive functions of alginates extracted from different regions also worth researching. The purpose of this study is to determine regional difference of alginates as well as evaluate their potential applications.

1.3 Alginate extraction from brown seaweed

Generally, alginates are extracted by chemical, physical or biological methods, such as acid hydrolysis, supercritical water hydrolysis, enzymatic digestion, thermal degradation, microwave extraction, ultraviolet photolysis and oxidative–reductive reactions [41-43]. Commercially available alginates are typically extracted from brown seaweeds, including *Laminaria digitata*, *Laminaria japonica*, *Ascophyllum nodosum*, and *Macrocystispyrifera* by treatment with thermolysis under alkaline or

acidic conditions [44]. It is estimated that *Ascopyllum nodosum* has the largest concentration of polysaccharide, ranging from 42% to 64% [44]. The average polysaccharide concentration of *Laminaria* is about 38%.

In order to analyze the regional difference of alginates, we selected six different brown seaweeds, two *Laminaria japonica* species, *Laminaria digitata*, *Laminaria saccharina*, *Ascopyllum nodosum* and *Podina.*

1.4 NMR and LC-MS analysis of polysaccharides

This research aims to investigate the structure compositions and characteristics of different alginate species quantitatively and qualitatively [45]. High resolution of ^{1}H and ^{13}C using Nuclear Magnetic Resonance (NMR) techniques are primary, rapid, and efficient technology for sequence determination of polysaccharides [29]. It helps to determine the composition and most of the detailed block structures of both alginates and oligosaccharides [38]. Some previous research has applied NMR to identify the M and G block compositions and M/G ratios of alginates extracted from different brown seaweeds. The molecular size, composition，charge and concentration of certain blocks can be well defined by chromatography [46].

Comparatively, less research has been conducted on quantitative determination of alginate polysaccharides [31]. Mass spectrometry (MS) detection can identify the molecular masses and fragmentation patterns of M and G block [47]. One of the most popular detection systems is LC-MS/MS performed using a triple quadrupole (QqQ) mass spectrometer combined with selected reaction monitoring (SRM) [45]. Alginates

are enzymatically or chemically degraded to small oligosaccharides before the MS detection, thus providing valuable information for the application of alginate oligosaccharides as health food ingredients or for medical purposes [23]. LC-MS/MS analysis is of high accuracy but also much more challenge than NMR analysis [48]. Purification and isolation of alginate oligosaccharide is the premise of the detection [49]. Alginate lyase have been applied to precisely break the alginate linkage into oligosaccharides [38]. Sometimes, it would be difficulty to detect alginate oligosaccharides when the sample is not concentrated enough. Because oligosaccharides in low concentration might not be applicable to allow purification to single isomers [32].

1.5 Research approach

According to the literature review, the valuable bioactive functions of alginates have not been comprehensively studied. Moreover, the regional differences of brown seaweed alginates has not well documented yet. In this research, we collected six brown seaweed species from different locations and optimized the extraction methods to obtain the largest alginates yields. Subsequently, alginates were extracted from six brown seaweeds and analyzed by NMR to calculate the sequence concentrations. Moreover, alginates were degraded by alginate lyase and acid hydrolysis respectively to get unsaturated and saturated oligosaccharides for LC-MS/MS detection. Based on these tests, we should be able to provide information of M and G block distribution, M/G ratio as well as the regional differences.

2. Materials and Methods

2.1 Materials

Alginate standards and alginate lyase were purchased from Sigma-Aldrich (Singapore). Alginate standards were stored at room temperature while alginate lyase were stored at 4 ℃ in the fridge. 75% ethanol, 0.04% hydrochloric acid, 2% $CaCl_2$ and 3% Na_2CO_3 were applied for alginate extraction.

2.2 Sample preparation

2.2.1 Brown seaweed sample preparation

In order to determine the regional differences of alginates, different brown seaweed samples were collected from different regions:

- Sample A: *Laminaria japonica* from Shandong, China;
- Sample B: *Laminaria japonica* from Fujian, China;
- Sample C: *Laminaria saccharina*, from U.S.;
- Sample D: *Laminaria digitata* from Iceland;
- Sample E: *Ascophyllum nodosum* from Indonesia;
- Sample F: *Podina* from Malaysia.

We collected fresh *Laminaria japonica* from Shandong (along the Coast of Bo Sea), China (A) and Fujian (along the cost of Nan Sea), China (B) respectively. The samples were thoroughly washed with distilled water, frozen, dried to constant weight, and milled into powder. The samples were stored in airtight plastic bottles at room

temperature until further composition analysis. Similar sample preparation procedures were applied to fresh seaweeds collected from Indonesia (eastern coast) (E) and Malaysia (eastern coast) (F). Sample C and sample D were milled from dry *Laminaria saccharina* and *Laminaria digitata* products purchased from U.S. and Iceland respectively, and subsequently, stored at room temperature.

2.2.2 Alginate extraction

Alginate extraction experiments were performed according to the designs of Rioux (2007) [50] and Nora (2003) [51] with modifications. Based on the previous studies, we gathered that the use of ethanol, hydrochloric acid, $CaCl_2$ and Na_2CO_3 consecutively to extract alginates from the different seaweeds would result in the highest alginate yields [52]. Factors such as solution concentrations, extraction durations, and extraction temperatures will all affect alginate extraction efficiency, hence we fixed the solution concentration at 75% ethanol, 0.04% hydrochloric acid, 2% $CaCl_2$ and 3% Na_2CO_3.

In order to define the optimal extraction condition, two different temperatures and durations were selected for ethanol, hydrochloric acid, and also $CaCl_2$ extraction processes and efficiencies of different temperature and duration combinations were compared. We took *Laminaria japonica* from Shandong as an example and determined the optimal temperature combinations first and then defined the best durations based on the selected temperatures for each process (Fig. 2-1). At first, milled seaweed samples were dissolved in 75% ethanol at different temperatures

(70 ℃ or 25 ℃) in a constant mechanical stirring in 500±15 rpm for 12h. Temperature was controlled using oil bath with thermal control. Then the solvent was separated from residual seaweeds by vacuum filtration using Whatman #4 filters. The residues were continually treated with $CaCl_2$ 2% (w/v) at different temperatures (70 ℃ or 25 ℃) for 6h. After centrifugation at 4℃ in 3500 rpm for 15 minutes, laminarin and fucoidan were retained in the supernatant. The remaining fucoidan and alginate in the residues could be separated with 0.04% hydrochloric acid at different temperatures (70 ℃ or 25 ℃) for 6h and then, centrifuged to extract fucoidan in the supernatant. Since alginate is alkaline soluble, they were finally extracted with 3% Na_2CO_3 (w/v) at 70 ℃ for 1.5 h and centrifuged. All polysaccharides were dialyzed (cut off 1000 Da) overnight, freeze-dried and stored at 4 ℃ for further analysis.

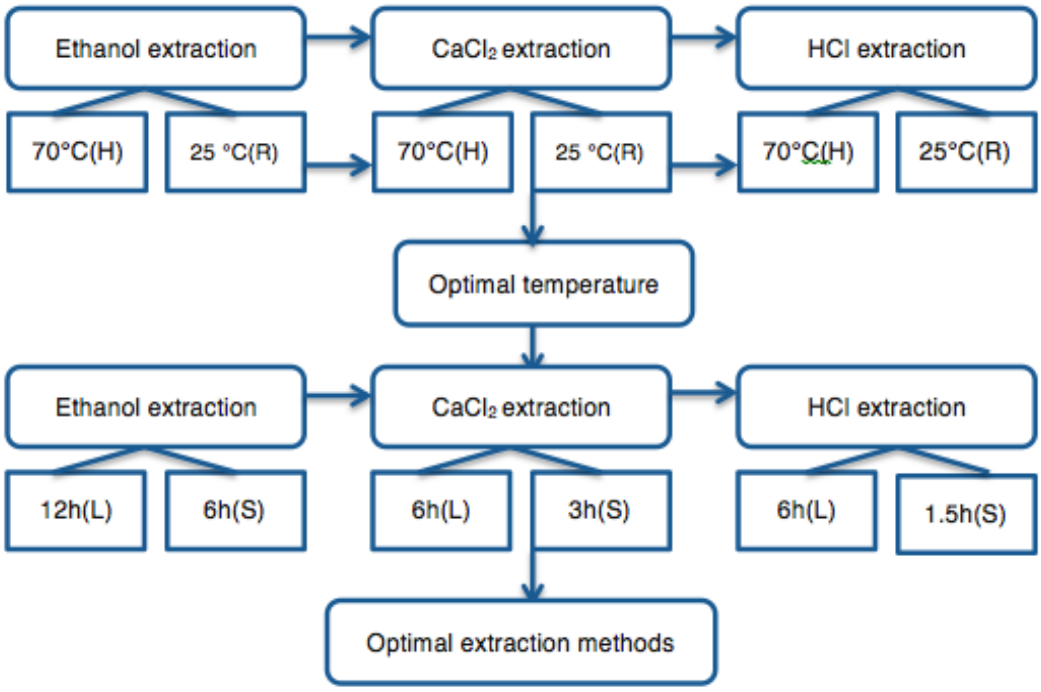

Fig. 2-1 Extraction conditionds for brown seaweed alginates

2.2.3 NMR testing tube preparation

Viscosity is one of the most important properties of alginate. However, it is much more difficulty to conduct the NMR detection of alginate due to its high viscosity. Thus pretreatment of alginate to reduce viscosity is important before the NMR detection. At first, we weight 20mg extracted alginates and mixed them with 5ml ultrapure water, and then put the alginate solution tube in 90 ℃ water bath for 1h. Then 1ml 0.1N hydrochloric acid was added into the tube and heated in 90 ℃ water bath for another 2h. Subsequently, the alginate solution sample was cooled and neutralized with 0.1N NaOH and freeze-dried overnight. Then we dissolved the dry sample with 2ml 99.9% atom D_2O and freeze dried to remove the level of water present. Finally, 10mg of the hydrolyzed alginate was dissolved in 1ml 99.9% atom D_2O with 2.9 mM *Trimethylsilyl-propionate* (TSP) added as internal standard. Then we extracted 700 μL solution into the NMR tube for the further analysis. Following the extraction methods introduced before, we extracted alginates from the six brown seaweeds and prepare the NMR testing tubes (Fig. 2-2).

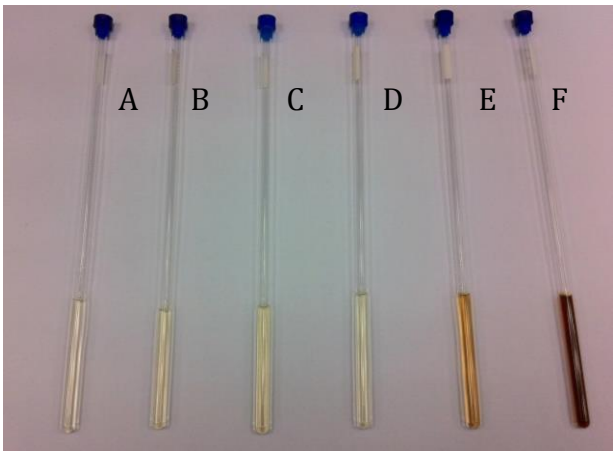

Fig. 2-2 The NMR testing tubes of alginates from six different brown seaweeds
Instruction: From left to right, they are: Sample A-*Laminaria japonica* (Shandong); Sample B-*Laminaria japonica* (Fujian); Sample C-*Laminaria saccharina* (U.S.); Sample D-*Laminaria digitata* (Iceland); Sample E-*Ascophyllum nodosum* (Indonesia); Sample F-*Podina* (Malaysia).

2.3 NMR Analysis

2.3.1 NMR testing

^{1}H NMR spectra of alginates were acquired on 600 MHz NMR equipped with OneNMR Probe (Agilent Technologies, CA, USA). The normal NMR running temperature is 25 ℃. However, because of the viscosity of alginates, the spectra are not obvious at room temperature. So we tested different temperatures, 25 ℃, 40 ℃, 55 ℃ and 70 ℃ to compare the spectra. It turned out that 70 ℃ is the optimal analysis temperature to obtain clear chemical shifts as well as not overlap with the water peak. The NMR detection of alginate was recorded with a spectral width of 600 MHz, an acquisition time of 14min, and 256 scans.

2.3.2 Data analysis

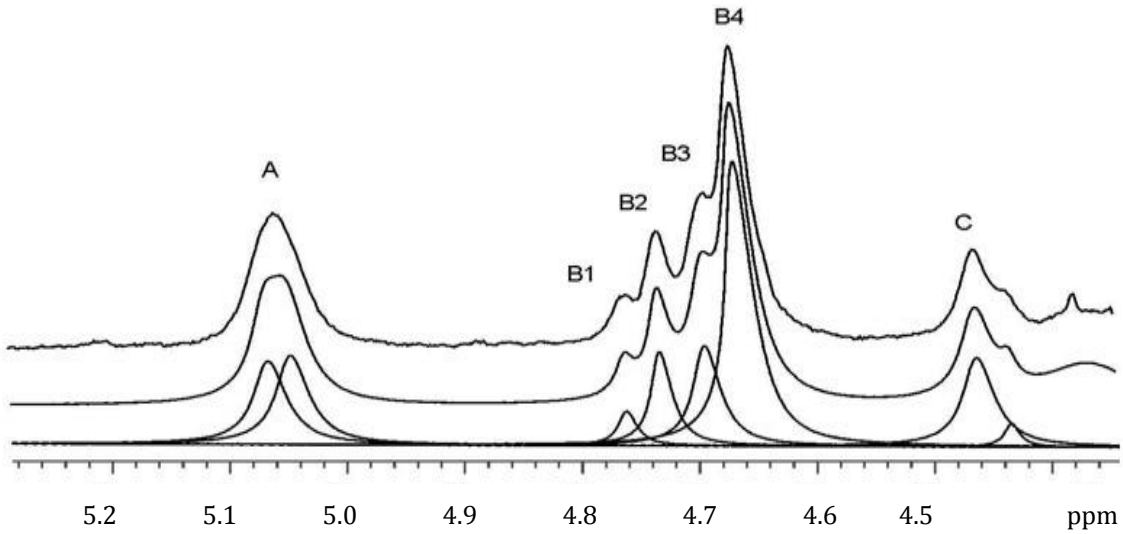

Fig. 2-3 ^{1}H NMR (600 MHz) spectra of alginate standard solutions at 70 °C[53]
Instruction: A: G-block; B_1: GGM-block; B_2: MGM-block; B_3: MG-/GM-block; B_4: MM-block; C: GG-block.

According to the previous research, alginate is an acidic linear polysaccharide consisting of G-block and M-block units in the form of MM-block, GG-block, MG-/GM-block, MGM-block, GGM-block, MMM-block or GGG-block. Fig. 2-3 shows the NMR spectrum allowing the determination of M/G composition of alginates by comparison of relevant signal areas. The A, B_1, B_2, B_3, B_4 and C peak in this spectrum represent for G-block, GGM-block, MGM-block, MG-/GM-block, MM-block and GG-block, respectively (Fig. 2-3). Based on the concentration of internal standard (TSP) and the areas of all the peaks, we can measure the concentration of each chemical shift by using Chenomx NMR suite 6.0 and VnmrJ 3.1. Then we calculated the values of F_M, F_G, F_{GG}, F_{MM}, F_{MG}/F_{GM} to establish the proportion of the different M and G sequence based on the following formulas [54]:

$F_G = 0.5(A + C + 0.5(B1+B2+B3))$

$F_M = B4+0.5(B1+B2+B3)$

$F_{GG}=0.5(A+C-0.5\ (B1+B2+B3))$

$F_{MG}=F_{GM}=0.5(B1+B2+B3)$

$F_{GG} + F_{GM=} F_G$

$F_{MM} + F_{MG=} F_M$

$F_M + F_G = 1$

2.4 LC-MS/MS analysis

2.4.1 Alginate oligosaccharide preparation

Unsaturated alginate oligosaccharide

Alginates should be degraded to oligosaccharides before the LC-MS/MS detection. For the unsaturated alginate oligosaccharides, alginates were degraded by alginate lyase. 25 mg alginate was dissolved in 5 ml ultrapure water and adjusted to pH 7.0 ± 0.2. Alginate lyase was added into the 100 mM Tris buffer to make 500 μg/ml enzyme solution and the pH was adjusted to 7.2. 5 mL alginate solution was treated with 50 μL alginate lyase solution and then incubated at 40 ℃ for 24 h. And then the solution was heated by boiling water (about 100 ℃) for 10 min to inactive the enzyme reaction.

Saturated alginate oligosaccharide

5mg alginate was dissolved in 1ml ultrapure water and the pH was adjusted to 7.0

± 0.2. Then acid solutions (hydrochloric acid, acetic acid, and formic acid) were added into the alginate solution respectively and adjusted to pH 4.0 ± 0.2. Subsequently, the mixed solution was hydrolyze at 120 ℃ for 3 h.

2.4.2 LC-MS/MS analysis

After the degradation, alginate oligosaccharides were analyzed on Dionex Ultmate 3000 HPLC coupled to AB sciex QTRAP 5500. 5 mg/mL unsaturated or saturated alginate oligosaccharide solutions were diluted to 1 ppm by adding an equal amount of solvent A (10 mM ammonium formate + 0.1% formic acid in ultrapure water) and solvent B (10 mM ammonium formate in 95:5 acetonitrile: ultrapure water) and then injected into the column at the flow rate of 20 μL/min.

The MS detection was operated in negative ion electrospray mode to acquire source-induced fragments of oligosaccharides. The basic MS collection parameters were set based on the previous research, with the scan range of m/z 100-1000[55]. We tested different parameters to determine the ambient declustering potential, entrance potential, collision energy and collision exit potential for both saturated and unsaturated alginate oligosaccharides (AOS).

For the LC separation, we tried the Phenomenex Luna HILIC column (4.6 mm x 150 mm) at first, but did not achieve separation. Then we found that Tosoh TSK gel Amide column 3um (2.0 mm * 150 mm) turns out to work for unsaturated AOS separation. However, for the saturated AOS, the target ions were not detectable, so we tried different AOS concentrations (1ppm, 5ppm to 10ppm), LC flow rates (10

μL/min and 20 μL/min), different reaction temperatures (30 ℃, 35 ℃ and 40 ℃) and injection volumes (5μL and 10μL). Moreover, we neutralized solvent A and B with ammonium hydroxide and also changed the acetonitrile concentration of solvent B in initial LC condition to be 80% or 90%.

Meanwhile, different gradients were also tested for a better separation. At first, we applied a faster gradient, obtaining narrow peaks. The gradient started at initial 95% solvent B for the first 2 min, and linearly decreased to 45% solvent B in 14 min, and finally proceed back to 95% solvent B. Then another linear gradient with much longer duration was used to elute the polysaccharides with the initial solvent composition at 70% solvent B and decreased to 45% solvent B in 40 min and then progressed to 70% solvent B at last.

3. Results and Discussion

3.1 NMR analysis results

3.1.1 Alginate extraction condition

In order to determine the most suitable alginate extraction condition, we tested the effects of different temperatures and durations. Fig. 3-1 shows the NMR spectra of alginates extracted with different temperatures at the same duration, where "R" and "H" means that room temperature or 70 ℃ was applied during respective processes.

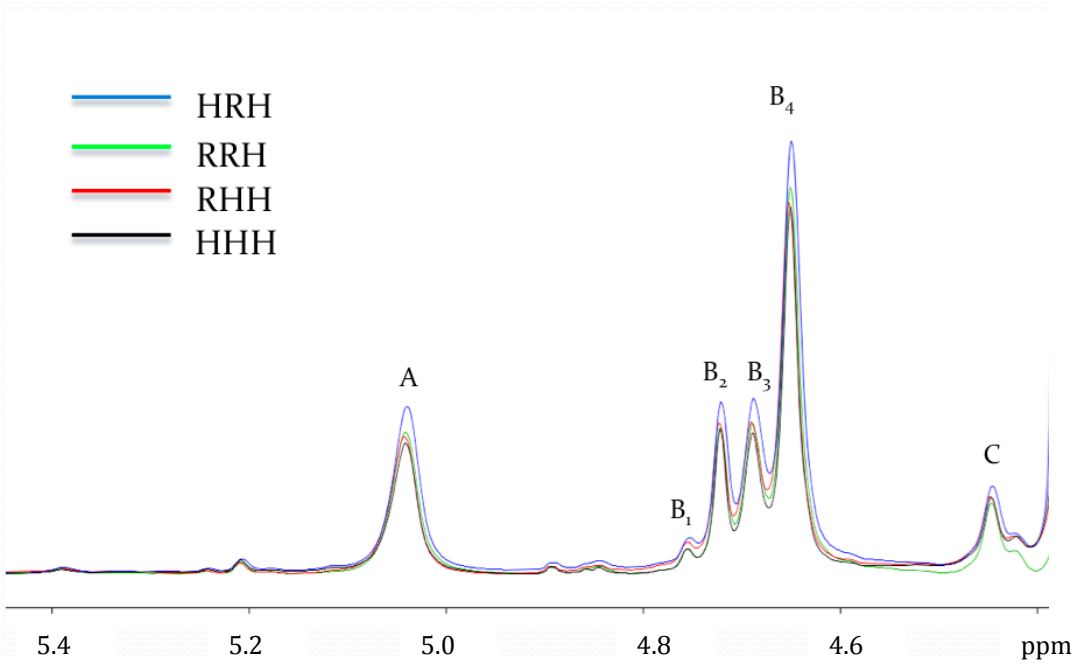

Fig. 3-1 ^{1}H NMR (600 MHz) spectra of alginate standard solutions (10 mg*mL^{-1}) in D_2O with different extraction temperatures
Instruction: A: G-block; B_1: GGM-block; B_2: MGM-block; B_3: MG-/GM-block; B_4: MM-block; C: GG-block.

Although we are able to observe clear regions in the NMR spectra which represented G-block (A), GGM-block (B1), MGM-block (B2), MG-/GM-block (B3), MM-block (B4) and GG-block (C) respectively, visual inspection is unable to pick up

slight variations in peak intensity influenced by temperature. So we applied Chenomx NMR suite 6.0 and VnmrJ to integrate the peaks, and subsequently tabulated the intensities in Table 3-1. According to the peak intensities, "HRH" turns out to obtain the block with the highest intensity. So we fixed the extraction temperatures to be "HRH" and then analyzed the efficiencies of different durations.

Table 3-1 ^{1}H NMR chemical shifts of *Laminaria japonica* alginate with different extraction temperatures

SAMPLE	A	B1	B2	B3	B4	C
HHH	1.442	0.174	0.810	1.023	2.557	0.560
HRH	**1.679**	**0.214**	**0.935**	**1.167**	**3.079**	**0.704**
RRH	1.630	0.207	0.944	1.133	2.879	0.679
RHH	1.652	0.196	0.922	1.103	2.924	0.636

The spectra of alginate extracted by different durations using the "HRH" temperature condition are shown in Fig. 3-2, "S" means short duration was applied in this process while "L" means long duration was applied in this process.

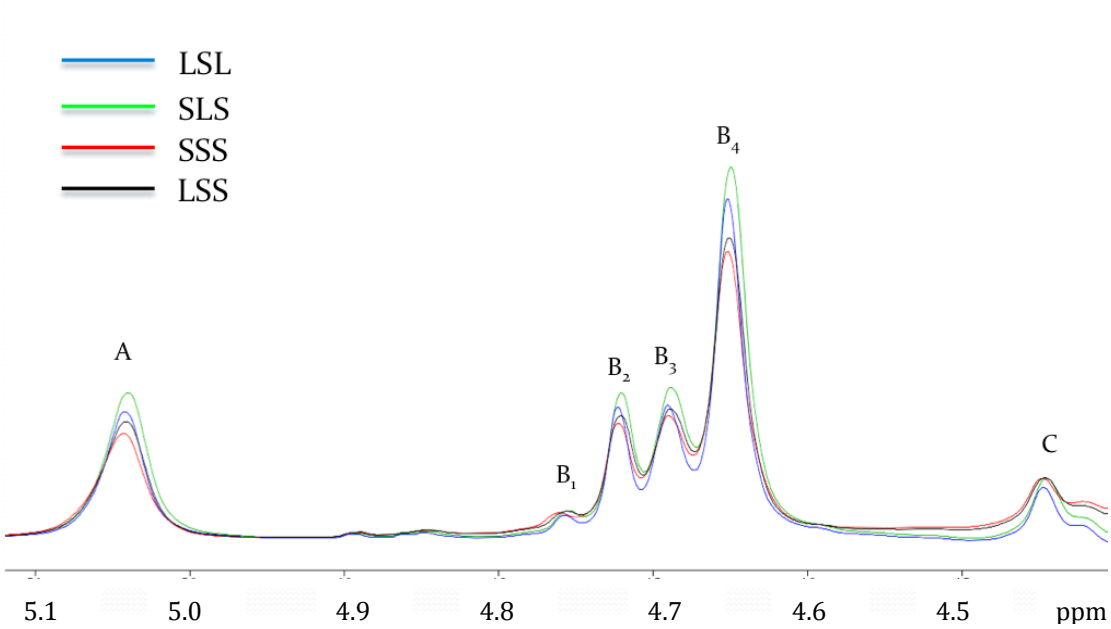

Fig. 3-2 ^{1}H NMR (600 MHz) spectra of alginate standard solutions (10 mg*mL^{-1}) in D_20 at different extraction durations

Instruction: A: G-block; B_1: GGM-block; B_2: MGM-block; B_3: MG-/GM-block; B_4: MM-block; C: GG-block.

From the figure, it seems that "SLS" is the best extraction condition for alginate. From the spectra concentration calculated by Chenomx NMR suite 6.0 listed in Table 3-2, we finally determined to choose "SLS" as the extraction duration.

Table 3-2 ^{1}H NMR chemical shifts of *Laminaria japonica* alginate with different extraction durations

SAMPLE	A	B1	B2	B3	B4	C
LLL	1.623	0.220	0.867	1.065	2.807	0.868
SSS	1.615	0.205	0.878	1.069	2.961	0.592
LSS	1.686	0.205	0.978	1.177	2.955	0.611
SLL	1.512	0.192	0.831	1.028	2.585	0.719
SSL	1.562	0.211	0.832	1.086	2.740	0.760
LLS	1.553	0.196	0.894	1.076	2.800	0.572
LSL	1.780	0.215	0.975	1.212	3.148	0.808
SLS	**1.777**	**0.222**	**1.030**	**1.214**	**3.109**	**0.652**

Based on the data analysis, the combination of ethanol extraction at 70 ℃ for 6h, $CaCl_2$ extraction at room temperature (around 25 ℃) for 3h and hydrochloric acid extraction at 70 ℃ for 1h can obtain the largest amount of alginates. So we determined the final protocol for alginate extraction from brown seaweed (Fig. 3-3).

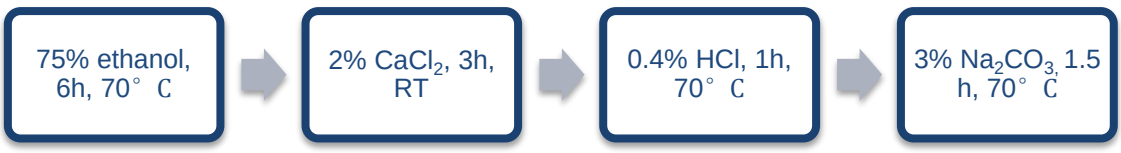

Fig. 3-3 The selected process of extraction alginate from brown seaweeds

3.1.2 NMR testing condition

Fig. 3-4 shows the alginates spectra of *Laminaria japonica* under different NMR running temperatures. The chemical shifts of alginates only shows slight perturbation

at different temperatures, but the water peak moves obviously in various temperatures (Fig. 3-4). Considering the spectrum presentation as well as the equipment toleration, we defined 70 ℃ as the optimal temperature for analysis.

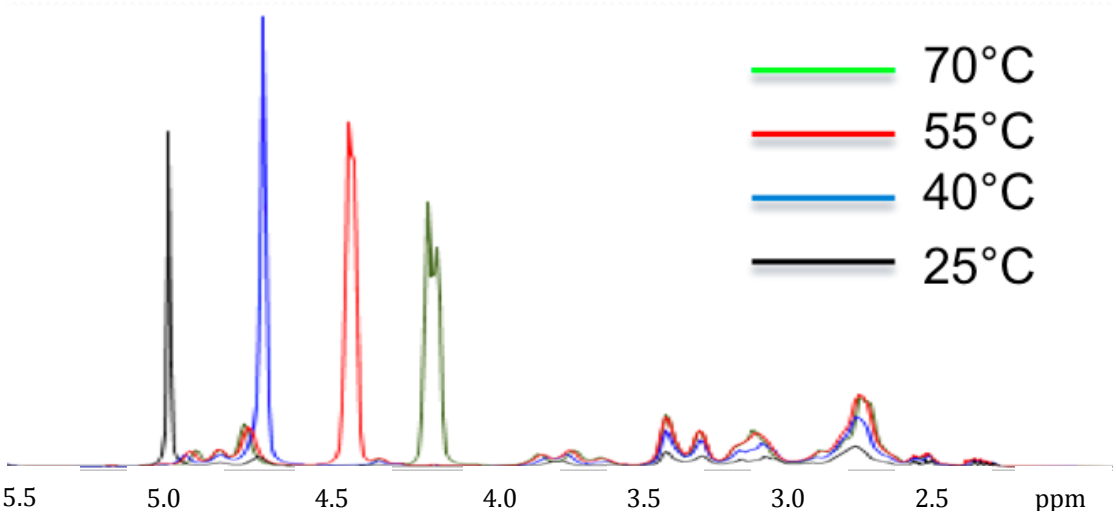

Fig. 3-4 ^{1}H NMR (600 MHz) spectra of alginate standard solutions (10 $mg*mL^{-1}$) in D_20 at different NMR testing temperatures

3.1.3 NMR testing

The NMR spectra of alginates extracted from the six brown seaweed samples with the selected extraction method and testing parameters are shown in Fig. 3-5. Compared with the spectra of protocol testing (Fig. 2-2), the alginate spectra of these six brown seaweeds are similar.

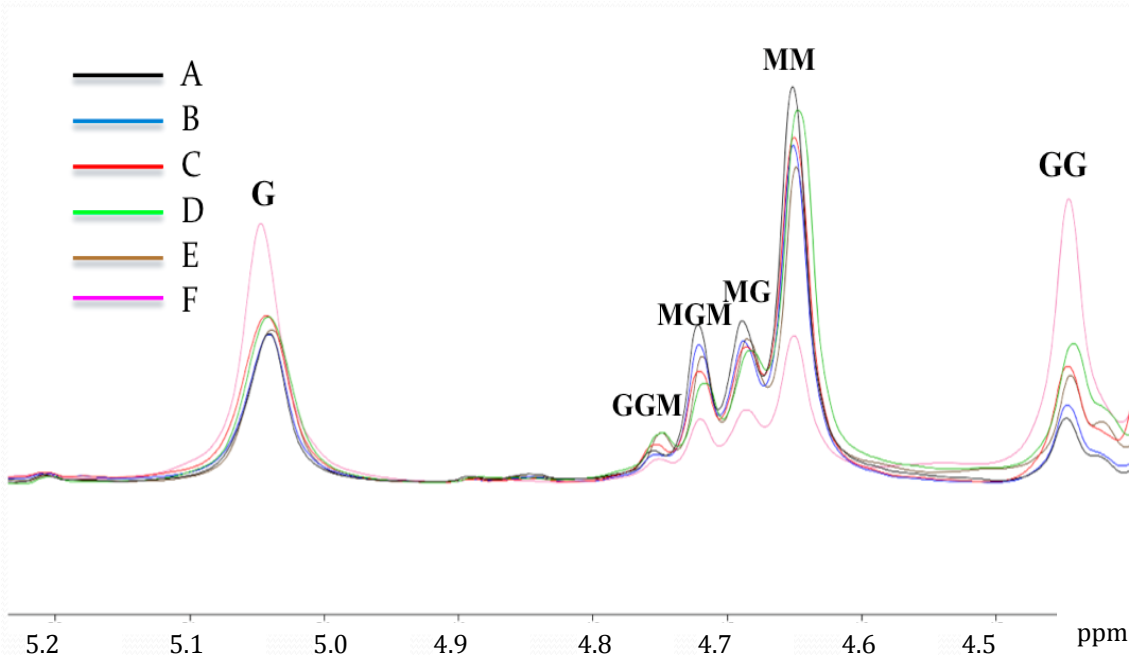

Fig. 3-5 ^{1}H NMR (600 MHz) spectra of poly-mannuronate and poly-guluronate blocks of six different alginate solutions (10 mg*mL^{-1}) in D_20 by acid hydrolysis
Instruction: Sample A-*Laminaria japonica* (Shandong); Sample B-*Laminaria japonica* (Fujian); Sample C-*Laminaria saccharina* (U.S.); Sample D-*Laminaria digitata* (Iceland); Sample E-*Ascophyllum nodosum* (Indonesia); Sample F-*Podina* (Malaysia).

Fig. 3-5 only depicts a simple relationship among the six samples. According to the figure, sample F seems to be quite different from the other five seaweed samples. In order to precisely present the regional differences, we calculated the values of sequences based on formula described before and listed in Table 3-3.

Table 3-3 ^{1}H NMR chemical shifts of six brown seaweed alginates under the same extraction methods

Sample	F_{GG}	F_{MM}	F_{GM}/ F_{MG}	F_{GGG}	F_{MGM}	F_{GGM}	F_G	F_M	N_G	$N_{G>1}$	M/G	N_M
Shandong	0.093	0.511	0.198	0.023	0.161	0.037	0.291	0.709	1.472	3.535	2.436	3.579
Fujian	0.134	0.480	0.193	0.035	0.154	0.039	0.327	0.673	1.695	4.479	2.058	3.486
US A	0.185	0.458	0.179	0.060	0.127	0.052	0.363	0.637	2.035	4.570	1.755	3.563
US B	0.183	0.498	0.159	0.064	0.101	0.058	0.342	0.658	2.148	4.165	1.924	4.127
Ascophyllum	0.145	0.471	0.192	0.048	0.138	0.054	0.337	0.663	1.756	3.693	1.967	3.459
Podina	0.515	0.255	0.115	0.087	0.081	0.034	0.630	0.370	5.483	15.999	0.587	3.223

The polyguluronic acid blocks content can largely determine the gel-forming capacity of alginates. Table 3-3 shows that the six alginates have a range of compositions varying from the relatively high G-containing alginate (F_G = 0. 630>0.6) extracted from *Podina* to the very high M-block content alginate (F_M = 0.709>0.6) extracted from *Laminaria japonica* from Shandong, the *Laminaria japonica* from Fujian also contains high M-block content. Since the F_M values of the other four brown seaweeds are between high M and high G species, so they are defined as intermediate or 'MG' alginates. The M- and G-block length are highly related with the content of M and G monomers for high G or high M species. While the correlation is not good enough for the intermediate alginates, they were found to be much shorter than the other block types[34]. The evaluation of the M/G ratio also provides fundamental information. High M/G ratio species will give elastic gels, such as *Laminaria japonica* from Shandong (2.436) and Fujian (2.058), *Laminaria saccharina* from U.S. (1.755) and *Laminaria digitata* from Iceland (1.924); whereas low M/G ratio will provide brittle gels, such as *Podina* from Malaysia.

However, compared with the alginate standard solution (10 mg/mL), the concentration of alginates extracted from the seaweed sample A is much lower, hence further extraction optimization could be carried out to improve the alginate yield (Fig. 3-6). For instance, we can change the concentration of the solvents we used for extraction to compare the efficiency. Moreover, we can try different extraction solvents, such as using NaCl instead of Na_2CO_3.

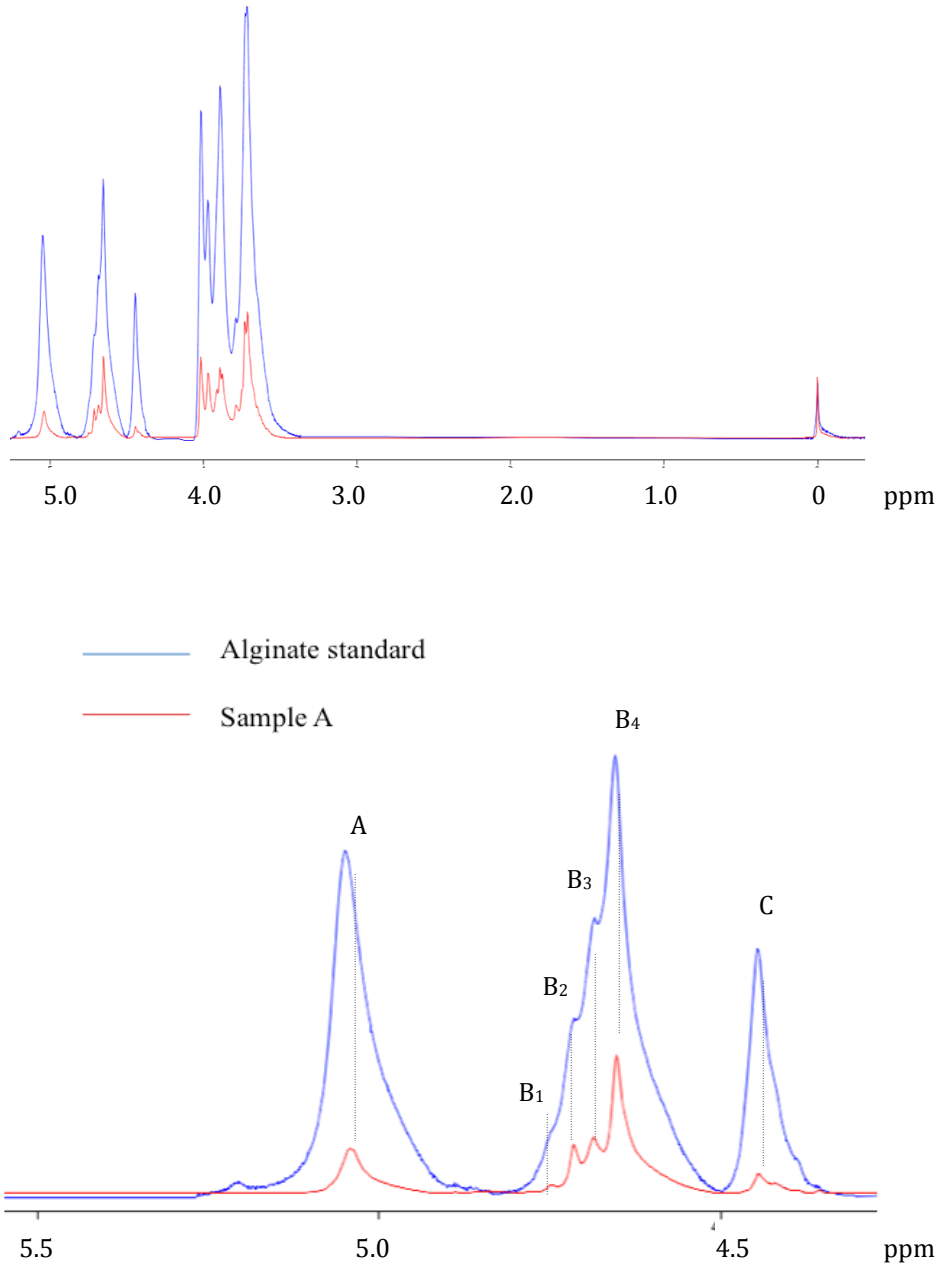

Fig. 3-6 Comparison of ^{1}H NMR (600 MHz) spectra of alginates solutions (10 mg*mL^{-1}) in D_20 extracted from *Laminaria japonica* (Sample A) and alginate standards
Instruction: A: G-block; B_1: GGM-block; B_2: MGM-block; B_3: MG-/GM-block; B_4: MM-block; C: GG-block.

3.2 LC-MS/MS analysis results

3.2.1 MS Analysis

Alginate oligosaccharides were prepared by mild acid hydrolysis and alginate

lyase degradation of alginate standards. The quality analysis of the parent alginate was performed by ^{1}H NMR spectroscopy. Negative mode LC-ESI-MS/MS was initially used to assess precursor ions for optimal sequence information.

Unsaturated alginate

According to the research of Zhang et. al [27], the major precursor-ion spectra of unsaturated AOS are listed in Table 3-4: the major precursor ions for unsaturated AOS are *m/z* 351, 527, and 703 representing dimer, trimer and tetramer respectively. Moreover, the dominant product-ions for unsaturated AOS include *m/z* 157, 175, 291, 333, 351, 467, 509, 527 and 643. The proposed fragmentation of unsaturated AOS (ΔMMM) is shown in Fig. 3-7. Mannuronate (M) and guluronate (G) are structural isomers, which produce similar fragmentation patterns, hence the differentiation between the two residues may be challenging. While the nonreducing terminus of unsaturated AOS is fixed as unsaturated hexuronic acid, residues at internal or reducing position can be identified through careful comparison of weak fragment ions. For example, an internal M residue produces a unique ion at m/z 307, whereas internal G does not, hence the availability of m/z 307 is used to indicate the identity of unsaturated AOS internal residues. The identity of reducing terminal can be revealed by comparing the intensity ratio of two weak reducing terminal ions, $[^{2,5}A_r]/[^{0,4}A_r]$, where M has a higher ratio as compared to G. Based on the fragmentation patterns established by Zhang et al., we proceed to determine the sequence of unsaturated

AOS in alginate standard, followed by the alginate extracts from seaweed samples collected at 6 different locations [27].

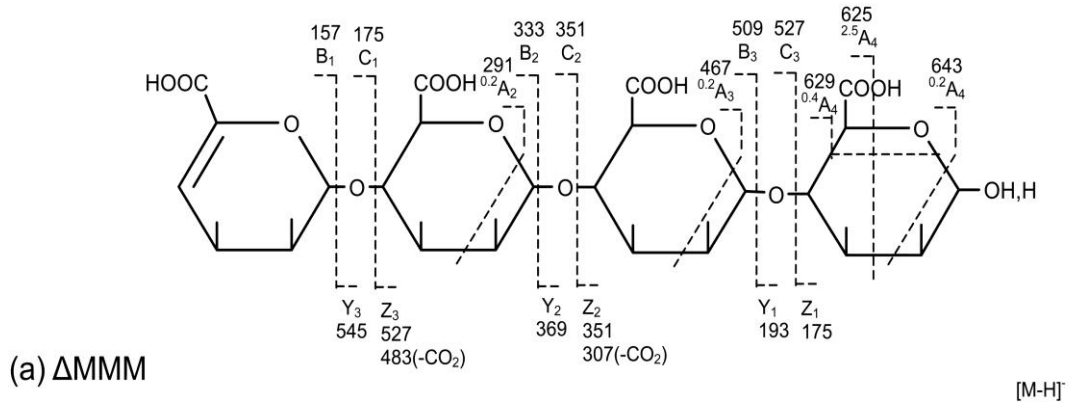

Fig. 3-7 Proposed fragmentation of unsaturated alginate oligosaccharides[27]

Table 3-4 Fragment ions observed in the product-ion spectra of unsaturated alginate oligosaccharides[27]

	9M-H]$^-$	B_1	C_1	$^{0.2}A_2$	B_2	C_2	$^{0.2}A_3$	B_3	C_3	$^{0.2}A_4$	$^{2.5}A_r$	$^{0.4}A_r$
ΔG	351	157	175	307							273	277
ΔGG	527	157	175	291	333	351	467				449	453
ΔMG	527	157	175	291	333	351	467				449	453
ΔMM	527	157	175	291	333	351	467				449	453
ΔGGG	703	157	175	291	333	351	467	509	527	643	625	629
ΔMGG	703	157	175	291	333	351	467	509	527	643	625	629
ΔGMG	703	157	175	291	333	351	467	509	527	643	625	629
ΔMMM	703	157	175	291	333	351	467	509	527	643	625	629

First of all, we hydrolyzed alginate standard by alginate lyase and prepared 1 ppm unsaturated AOS solution by diluting in equal volumn of solvent A (10 mM ammonium formate + 0.1% formic acid in ultrapure water) and solvent B (10 mM ammonium formate in 95:5 acetonitrile: ultrapure water). The unsaturated AOS from alginate standard was directly infused into MS, and a MS scan over *m/z* range from 250 to 1100 was acquired. Fig. 3-8 shows the MS scan of 1ppm unsaturated AOS from alginate standard. Similar to previous study, we were able to determine the

unsaturated AOS dimer, trimer, tetramer, pentamer and hexamer with precursor ions of *m/z* 351, 527, 703, 879, 1055 respectively.

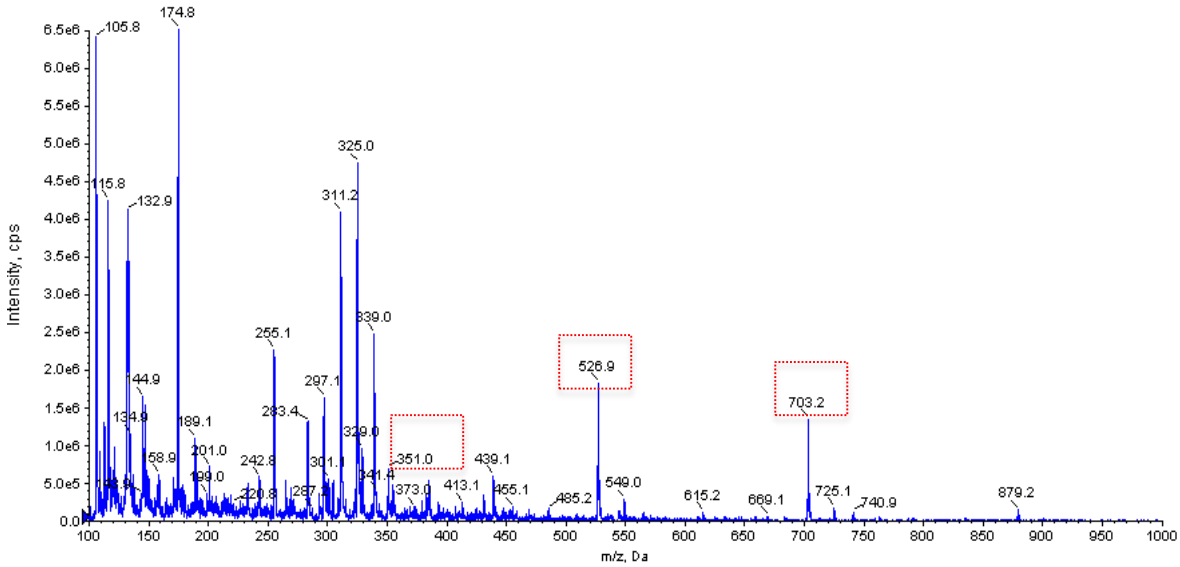

Fig. 3-8 Precursor-ion spectra of 1ppm unsaturated AOS of alginate standard in 10 mM Ammonium formate with 0.1% Formic acid (50% A: 50% B)

Subsequently, we determined the optimal declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP) for the respectively polymer. The product-ions detected and the optimized detection parameters are listed in Table 3-5. The absence of m/z 307 indicates that there are no M internal residues. However, we are unable to identify the reducing residue as the signals for reducing terminal ions, $^{2,5}A_r$ and $^{0,4}A_r$ are undetectable.

Table 3-5 Optimizing conditions for MS scan of brown seaweed alginates

	Precusor Ion	Product Ions	Dwell Time	Declustering Potential	entrance potential	collision energy	collision exit potential
Dimer	351.3	157.3	100	-64.83	-6.42	-21.22	-3.76
	351.3	175.1	100	-64.83	-6.42	-17.16	-10.44
	351.3	193	100	-64.83	-6.42	-18.87	-10.62
Trimer	527.1	157.2	100	-85.59	-5.93	-34.38	-8.85
	527.1	175.1	100	-85.59	-5.93	-29.64	-9.08
	527.1	193.2	100	-85.59	-5.93	-33.15	-5.74
	527.1	291.1	100	-85.59	-5.93	-29.11	-6.35

	527.1	333.1	100	-85.59	-5.93	-30.71	-7.19
	527.1	351.3	100	-85.59	-5.93	-24.5	-7.21
	527.1	369.2	100	-85.59	-5.93	-26.07	-4.89
	527.1	466.7	100	-85.59	-5.93	-23.06	-10.28
Tetramer	703.1	156.9	100	-88.52	-5.52	-45.92	-8.77
	703.1	175.3	100	-88.52	-5.52	-40.12	-4.24
	703.1	193.2	100	-88.52	-5.52	-42.14	-4.76
	703.1	291.2	100	-88.52	-5.52	-41.6	-6.16
	703.1	333	100	-88.52	-5.52	-41.13	-7.47
	703.1	351	100	-88.52	-5.52	-34.66	-7.89
	703.1	369.1	100	-88.52	-5.52	-37.43	-8.14
	703.1	466.8	100	-88.52	-5.52	-38.38	-10.35
	703.1	508.9	100	-88.52	-5.52	-37.14	-6.27
	703.1	526.9	100	-88.52	-5.52	-32.02	-6.46
	703.1	643.2	100	-88.52	-5.52	-32.72	-7.33
Pentamer	879	175	100	-128.73	-7.03	-50.12	-9.73
	879	193.2	100	-128.73	-7.03	-51.45	-11.7
	879	351.1	100	-128.73	-7.03	-48.67	-7.35
	879	369	100	-128.73	-7.03	-48.13	-9.83
	879	467.1	100	-128.73	-7.03	-46.79	-9.97
	879	527.3	100	-128.73	-7.03	-42.58	-6.18
	879	703.4	100	-128.73	-7.03	-39.28	-4.97
	879	791	100	-128.73	-7.03	-36.07	-9.48
	879	861.3	100	-128.73	-7.03	-43.05	-6.14
Hexamer	1055.4	175.2	100	-131.44	-8.01	-61.57	-10.54
	1055.4	350.9	100	-131.44	-8.01	-57.85	-7.78
	1055.4	527.1	100	-131.44	-8.01	-48.2	-6.81
	1055.4	703.2	100	-131.44	-8.01	-47.24	-6.73
	1055.4	879.3	100	-131.44	-8.01	-48	-10.68

According to the optimizing parameters provided in Table 3-5, we conducted the MS scan for 1ppm alginate AOS extract from *Laminaria japonica* (sample A)(Fig. 3-9). Unfortunately, none of the precursor ions of *m/z* 351, 527 and 703 are detected. This indicates that the alginate extract from seaweed sample is not pure as compared to the alginate standard, which is in line with our NMR analysis showing drastic differences in the peak intensity of standard against sample.

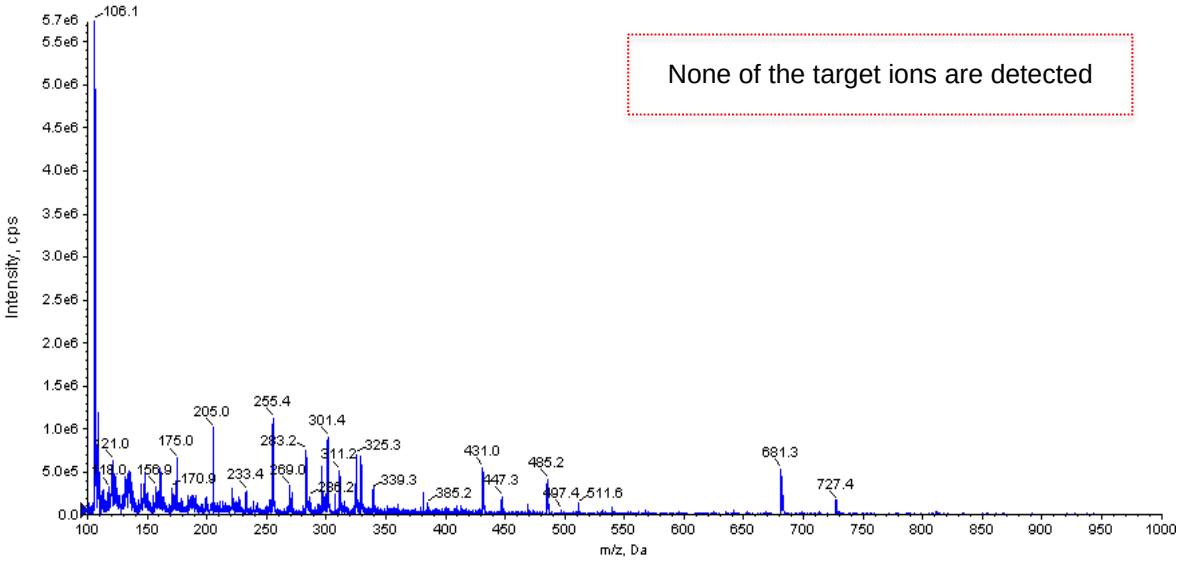

Fig. 3-9 Precursor-ion spectra of 1ppm unsaturated AOS of Laminarin japonica (sample A) in 10 mM Ammonium formate with 0.1% Formic acid (50% A: 50% B)

Considering the relatively low concentration of alginates extracted from seaweed samples than the alginate standards (Fig. 3-6), the missing precursor ions might due to the low concentration of alginate oligosaccharides hydrolyzed from alginates. So we increased the alginate oligosaccharide concentrations to 5ppm (Fig. 3-10) and 10ppm (Fig. 3-11). However, the target precursor ions are still invisible.

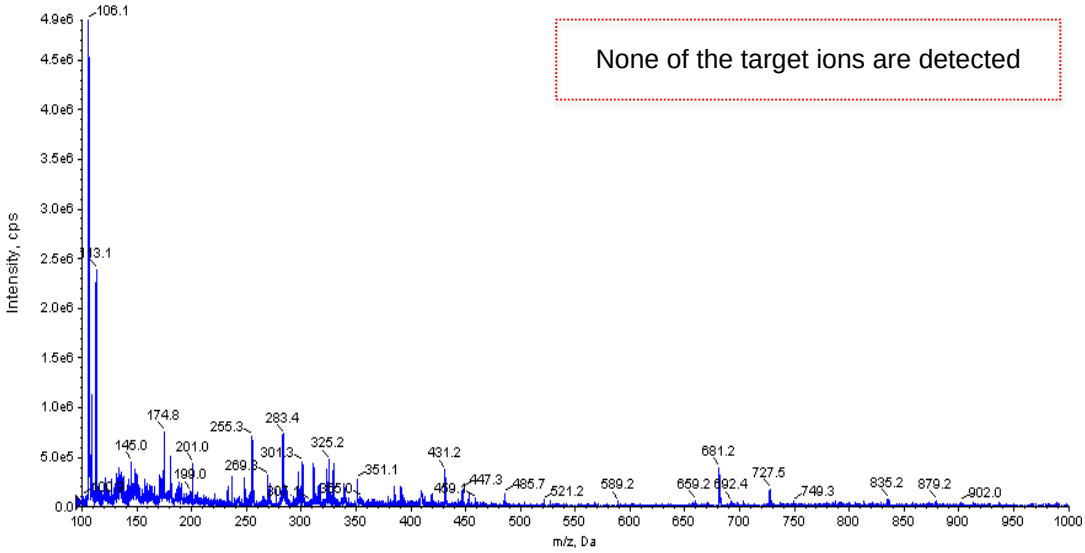

Fig. 3-10 Precursor-ion spectra of 5ppm unsaturated AOS of Laminarin japonica (sample A) in 10 mM Ammonium formate with 0.1% Formic acid (50% A: 50% B)

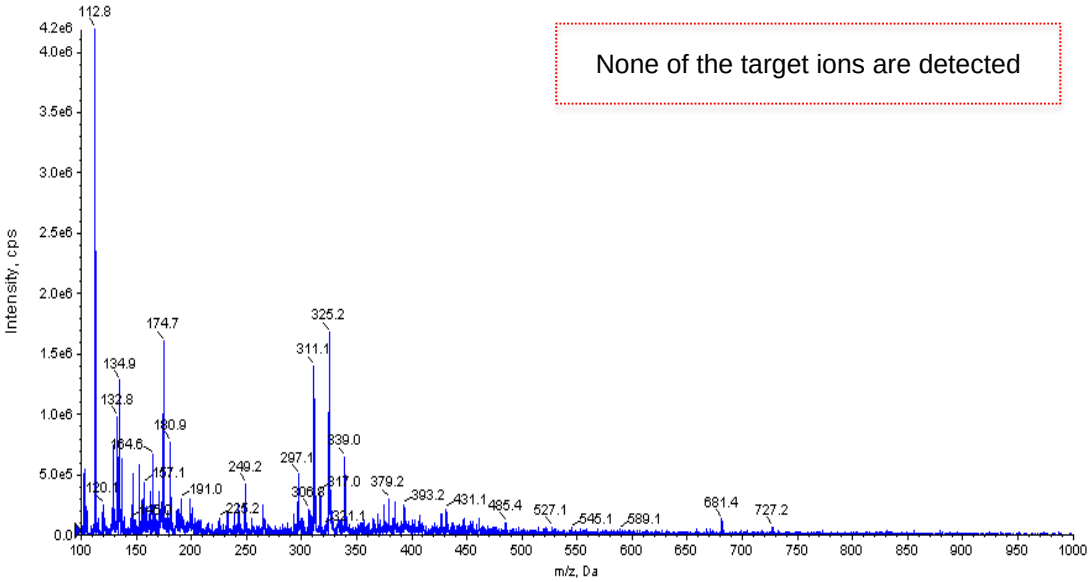

Fig. 3-11 Precursor-ion spectra of 10ppm unsaturated AOS of Laminarin japonica (sample A) in 10 mM Ammonium formate with 0.1% Formic acid (50% A: 50% B)

To improve the ionization of unsaturated AOS, we changed the LC buffer from 10 mM ammonium formate to ammonium acetate, and the LC solvent from acetonitrile to methanol. However, such changes do not improve the ionizability of unsaturated AOS, and the precursor ions were not detected (check appendix Fig. A1 and Fig. A2).

Saturated alginate

Zhang et. al [27] determined the major features of saturated AOS, which produced similar ions as those in unsaturated AOS except for a 18Da mass shift due to the non-reducing terminal. The major precursor ions for saturated AOS disaccharides, trisaccharides, tetrasaccharides and pentasaccharides are *m/z* 369, 545, 721 and 897, respectively, and the product-ions include *m/z* 175, 193, 309, 351, 369, 485, 527, 545 and 703 (Table 3-6).

Table 3-6 Fragment ions observed in the product-ion spectra of saturated alginate oligosaccharides[27]

	$M-H]^-$	B_1	C_1	$^{0.2}A_2$	B_2	C_2	$^{0.2}A_3$	B_3	C_3	$^{0.2}A_4$	B_4	C_4	$^{0.2}A_4$	$^{2.5}A_r$	$^{0.4}A_r$
GG	369	175	193	309										291	295
GGG	545	175	193	309	351	369	485							467	471
GGGG	721	175	193	309	351	369	485	527	545	661				643	647
GGGGG	897	175	193	309	351	369	485	527	545	661	703	721	837	819	823
MM	369	175	193	309										291	295
MMM	545	175	193	309	351	369	485							467	471
MMMM	721	175	193	309	351	369	485	527	545	661				643	647
MMMMM	897	175	193	309	351	369	485	527	545	661	703	721	837	819	823

Then we prepared 1ppm saturated alginate oligosaccharides hydrolyzed by HCl and conducted the MS detection. Unlike unsaturated AOS, the precursor ions for saturated AOS of alginate standard at *m/z* 369, 545, 721 and 897 could not be found in the MS scan (Fig. 3-12). Despite increasing the concentration of saturated AOS from 1 ppm to 10 ppm, the product ions are still not detected (Fig. 3-13). This indicates that saturated AOS is less ionizable as compared to unsaturated AOS. As direct infusion of a high concentration of sample could compromise the sensitivity of the MS, we could not increase the standard concentration further, but instead we attempt to improve compound ionization by varying the buffer and solvent.

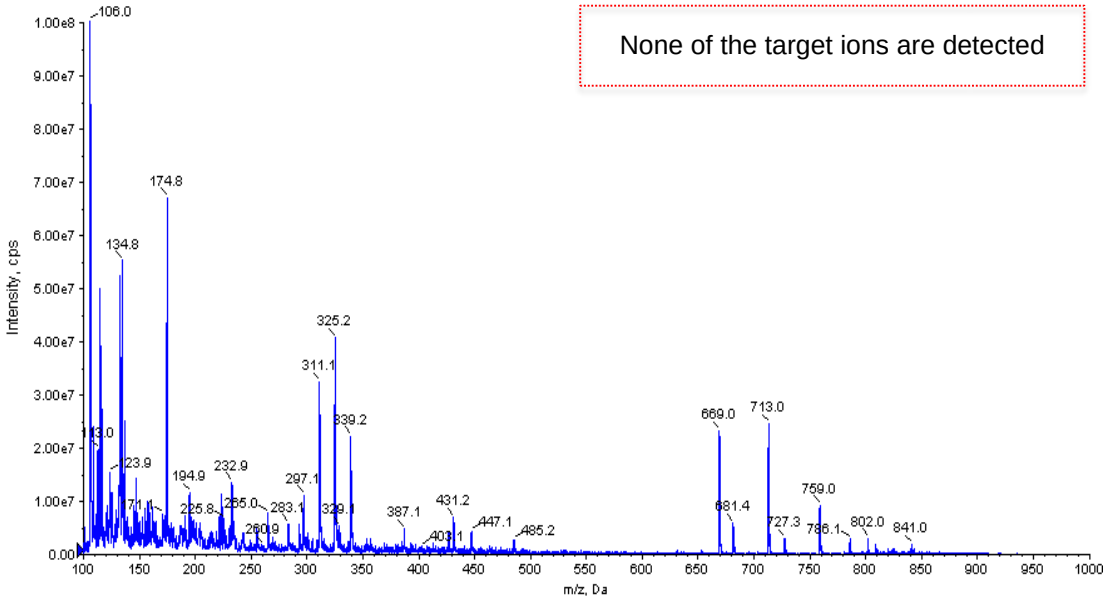

Fig. 3-12 Precursor-ion spectra of 1ppm saturated AOS diluted in 10 mM Ammonium formate with 0.1% Formic acid (50% A: 50% B); alginates were hydrolyzed with HCl (pH=2) for 180 min at 100 °C

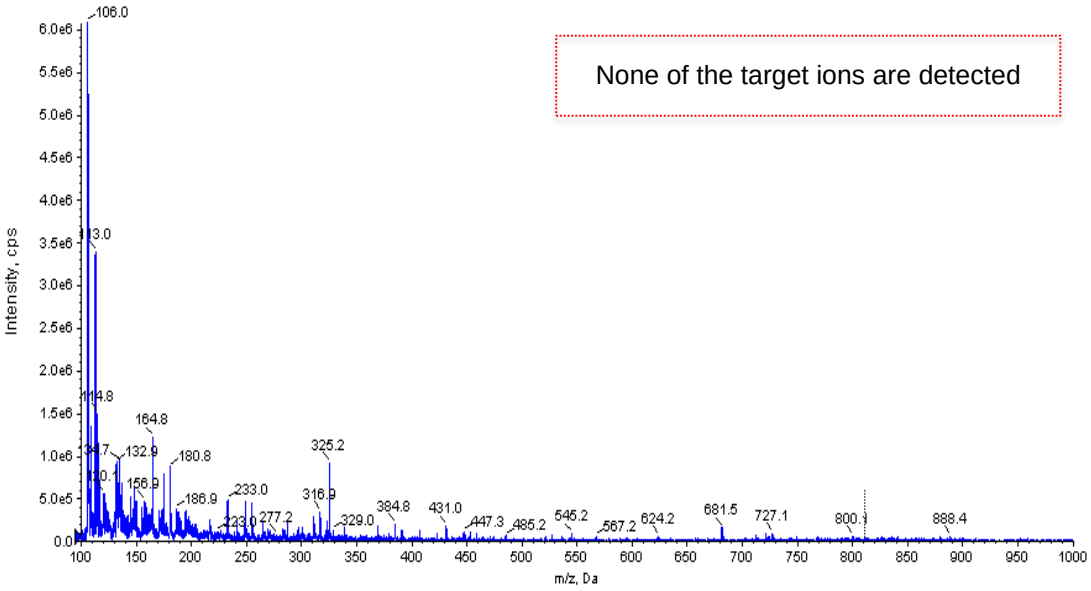

Fig. 3-13 Precursor-ion spectra of 10ppm saturated AOS diluted in 10 mM Ammonium formate with 0.1% Formic acid (50% A: 50% B); alginates were hydrolyzed with HCl (pH=2) for 180 min at 100 °C

Then we tried ammonium acetate instead of ammonium formate to prepare the solution A and diluted alginate samples into 1pm. However, the target ions are not detectable (Fig. 3-14). As the alginate extract from seaweed sample is expected to

have much lower concentration than alginate standard, we did not attempt to analyze alginate extract via direct infusion.

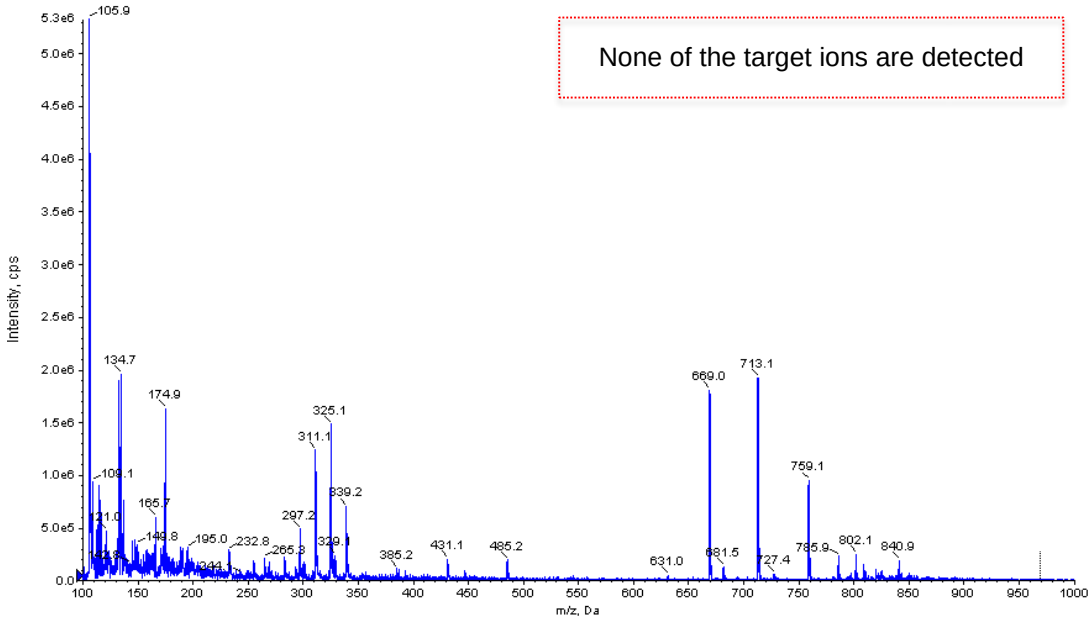

Fig. 3-14 Precursor-ion spectra of 1ppm saturated AOS diluted in 10 mM Ammonium acetate with 0.1% Formic acid (50% A: 50% B); alginates were hydrolyzed with HCl (pH=2) for 180 min at 100 °C

As pH of the sample affects ionization, where negative mode ionization favors higher pH, we decided to adjust the pH of saturated AOS in an attempt to improve ionization. The initial acidic condition (pH 2) of saturated AOS as alginate was subjected to HCl hydrolysis for 3h, was neutralized to pH 7 using ammonium bicarbonate and later stored at room temperature. According to Fig. 3-15, the spectrum of the corresponding ions are more concentrated than pH=2, but the precursor ions of *m/z* 369, 545, 721 and 897 of alginate oligosaccharides still could not be detected.

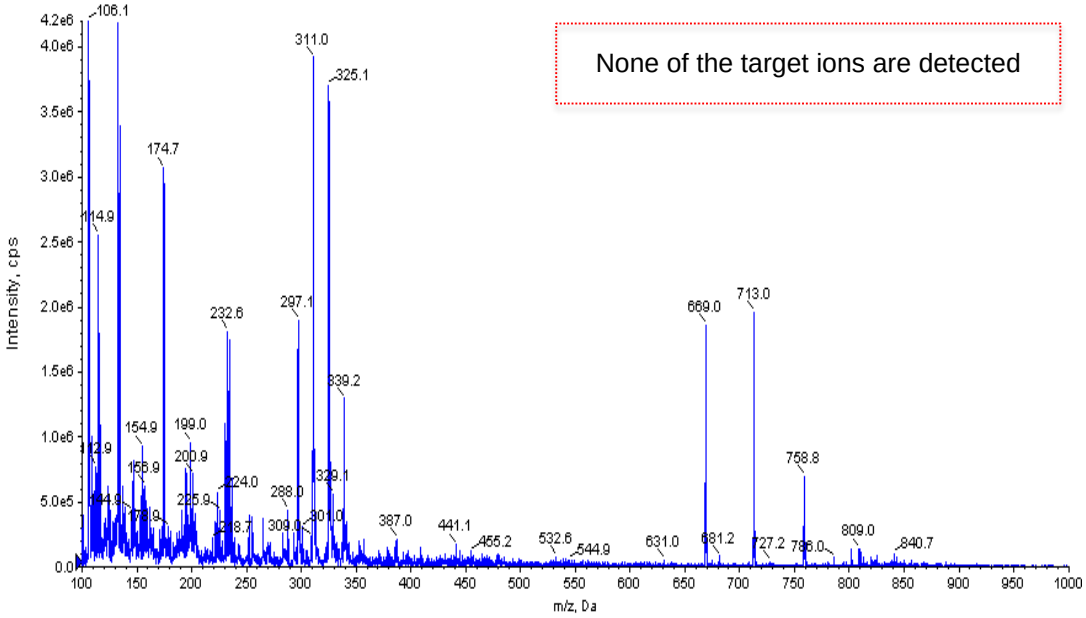

Fig. 3-15 Precursor-ion spectra of 1ppm saturated AOS diluted in 10 mM Ammonium acetate with 0.1% Formic acid (50% A: 50% B); alginates were hydrolyzed with HCl (pH=7) for 180 min at 100 °C

Considering that the use of HCl may also affect ionization, we replaced HCl with acetic acid and formic acid for alginate acid hydrolysis, and subsequently prepared 1 ppm saturated AOS in 10 mM ammonium acetate with 0.1% FA (50% A: 50% B) for direct infusion. However, the peaks are still not detectable.

3.2.2 LC Separation

Unsaturated oligosaccharide of alginate standard

Through compound optimization by direct infusion, we reveal that unsaturated AOS are not only available in different chain sizes but also have different sequences. Hence, 1ppm unsaturated alginate oligosaccharides were ran on LC in an attempt to separate the different alginate fractions. Fig. 3-16 demonstrates the LC separation of unsaturated alginate oligosaccharide fractions degraded by alginate lyase using

Phenomenex Luna HILIC (4.6 mm x 150 mm) column, the flow rate is 0.5 ml/min with 5ul as injection value at 35℃. However, the different fragments were not separated in this condition.

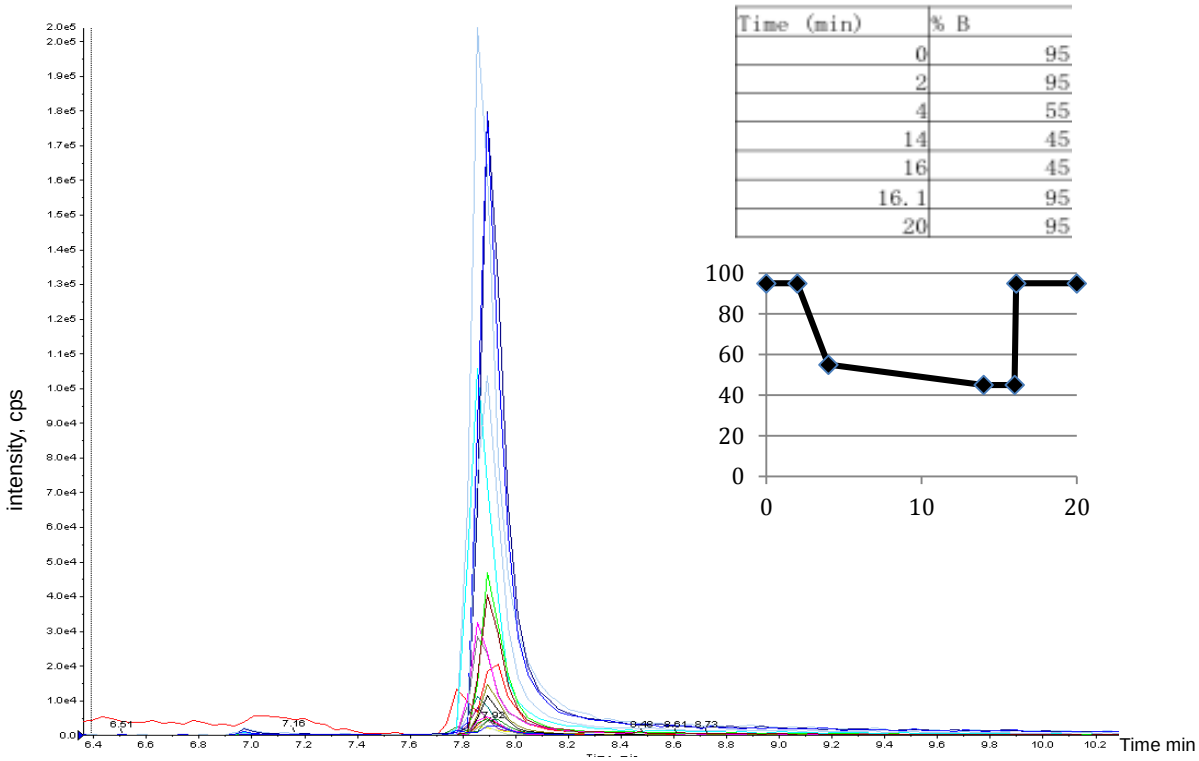

Fig. 3-16 LC separation of 1ppm unsaturated alginate oligosaccharide using Phenomenex Luna HILIC (4.6 mm x 150 mm) column at 0.5 ml/min flow rate, 5ul injection value at 35°C

Then we tried Tosoh TSK gel Amide 3um (2.0 mm x 150 mm) column and modified the elution gradient to obtain a better separation between alginate oligosaccharide fragments. Clusters of peaks with similar m/z values are isomers of alginate oligosaccharide with different sequence. Fig. 3-17 shows the LC separation of 1ppm unsaturated alginate oligosaccharide using Tosoh TSK gel Amide 3um (2.0 mm x 150 mm) column at 0.3 ml/min flow rate, 5ul injection value at 35℃. The dimer, trimer, and tetramer were separated successfully in this condition.

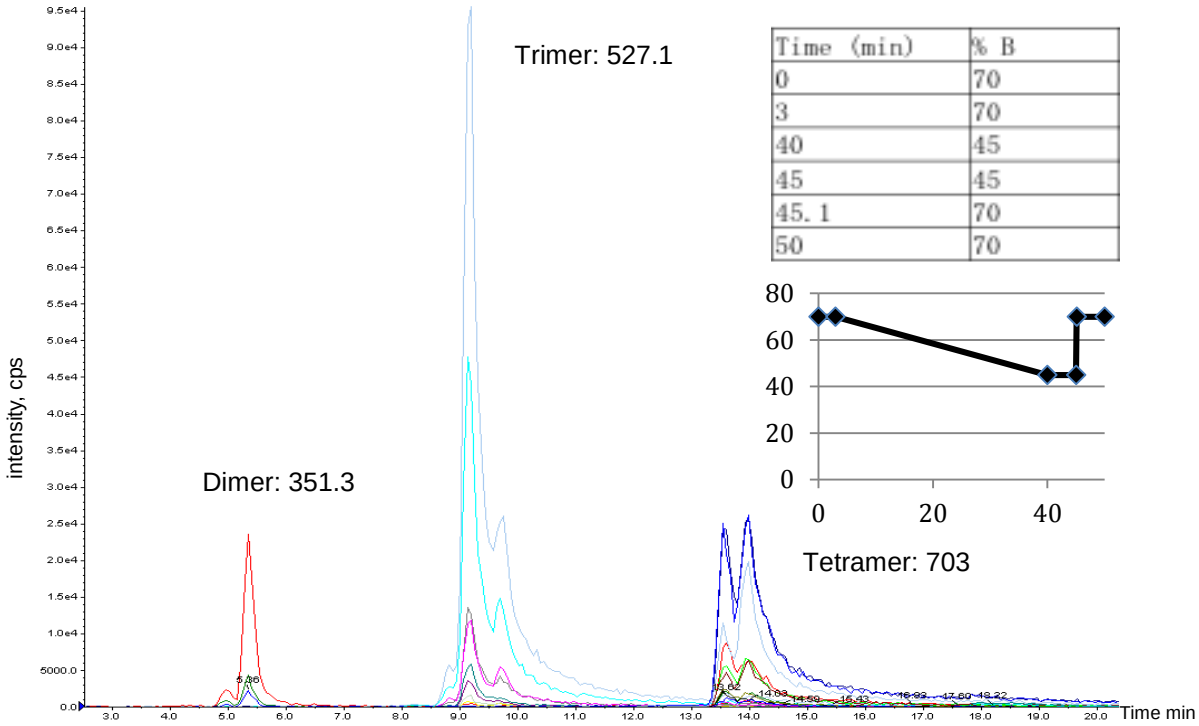

Fig. 3-17 LC separation of 1 ppm unsaturated alginate oligosaccharide using Tosoh TSK gel Amide 3um (2.0 mm x 150 mm) column at 0.3 ml/min flow rate, 5ul injection value at 35°C

When increasing the temperature from 35 ℃ to 40 ℃ under the same condition, pentameter and hexamer can also be detected (Fig. 3-18). The LC scans of dimer, trimer, tetramer, pentameter, and hexamer are also presented in appendix Fig. A3-A7 respectively.

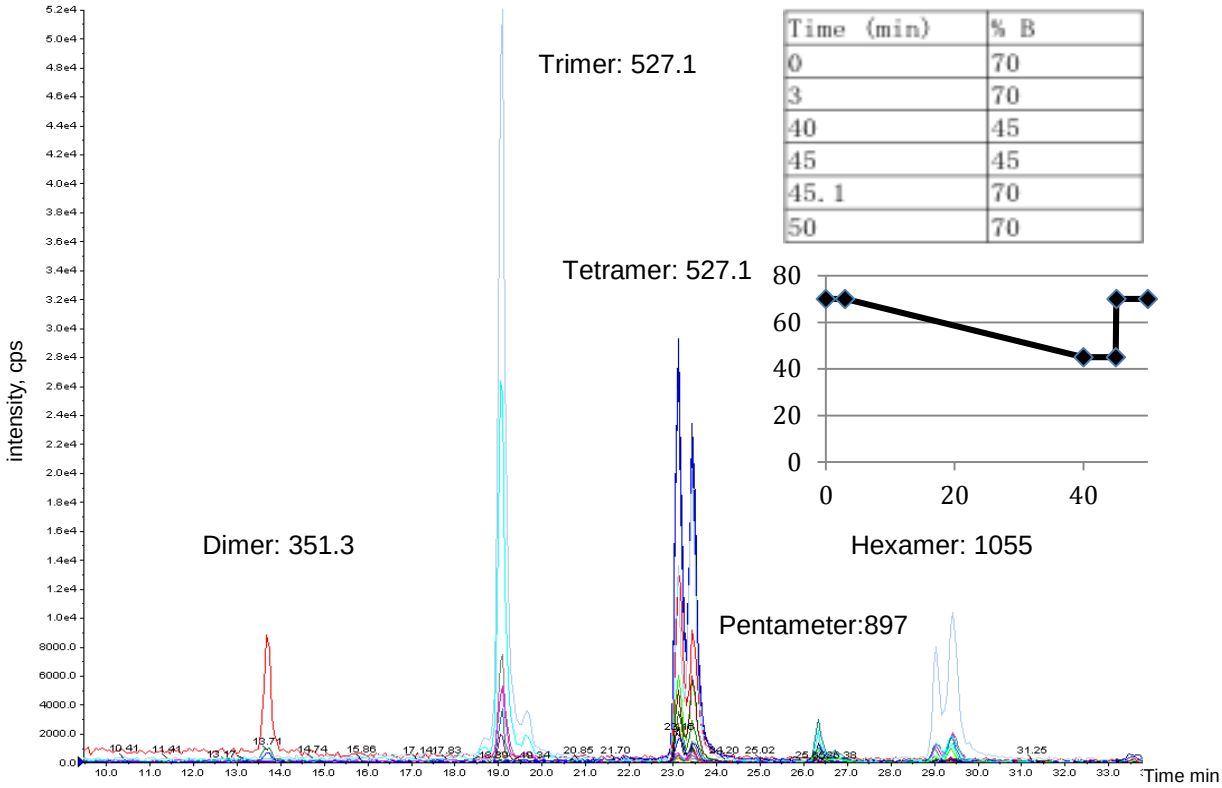

Fig. 3-18 LC separation of 1 ppm unsaturated alginate oligosaccharide using Tosoh TSK gel Amide 3um (2.0 mm x 150 mm) column at 0.3 ml/min flow rate, 5ul injection value at 40°C

Saturated oligosaccharide of alginate standard

According to the previous studies on the MS detection of saturated alginate oligosaccharide, successful ionizations are not obtained. Nevertheless, we still conducted the LC separation of saturated alginate oligosaccharide at 1ppm, 5ppm, and 10 ppm using Tosoh TSK gel Amide 3um (2.0 mm x 150 mm) column at 0.3 ml/min flow rate, 5ul or 10ul injection values under different testing temperatures (check appendix Fig. A3-A6). The alginates were hydrolyzed by both acetic acid and ammonium formate solution at pH 7.0 (check appendix Fig. A8-A11). However, none of these testing achieved clear fragment separation.

Up to now, there is still no comprehensive detection of alginate oligosaccharides extracted from brown seaweed using LC-MS. In the base peak chromatogram of unsaturated alginate, a number of oligosaccharide has already been recognized. However, the detection of saturated alginate oligosaccharide failed. One main reason is because of the low concentration of alginates extracted from brown seaweed. According to the literature, some previous LC-MS detections prepared the alginate oligosaccharides solutions of 100 ppm, so that they can successfully obtained good ionization and separation. However, the Dionex Ultmate 3000 HPLC coupled to AB sciex QTRAP 5500 we applied in this study can only afford the maximum concentration of 10 ppm for analysis, otherwise it might compromise the performance of the instrument.

4. Conclusion

In this research, we extracted alginates from six different brown seaweeds collected from various locations and conducted NMR and LC-MS/MS to study the regional difference. Based on the NMR detection results, the six alginates have a wide range of compositions, varying from the relatively high G-containing alginates to the very high M-containing ones. *Podina* collected from Malaysia belongs to the high G species; Whereas *Laminaria japonica* from Shandong and Fujian are similar, belonging to high M species; *Laminaria saccharina* from U.S., *Laminaria digitata* from Iceland, and *Ascophyllum nodosum* from Indonesia have the F_M values between high M and high G species, so they are defined as intermediate or 'MG' alginates. In addition, the M/G ratios of the two *Laminaria* species from China are similar, and *Laminaria saccharina* from U.S. and *Laminaria digitata* from Iceland also have close M/G ratio. Therefore, these four brown seaweeds can provide elastic gels. On other hand, *Podina* from Malaysia shows substantial difference from the other five species with relatively low M/G ratio thus can provide brittle gels.

However, compared with the concentration of alginates extracted from alginate standard, the alginate yields of all these six brown seaweeds are not high enough. That is the main reason for the failed LC-MS/MS detection of the targeted precursor ions of alginate oligosaccharide extracted from the six selected brown seaweed samples. On the other hand, the unsaturated oligosaccharide hydrolyzed from alginate standards could be successfully detected. The ability to identify the oligosaccharides

in the brown seaweed alginates is essential to the assessment of potential bioactive functions of different seaweed species, hence further experiments including improvement of alginate extraction efficiency and optimization of saturated and unsaturated AOS ionization are required to lend insights into the sequence variation in alginate produced from different seaweed species.

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Appendix

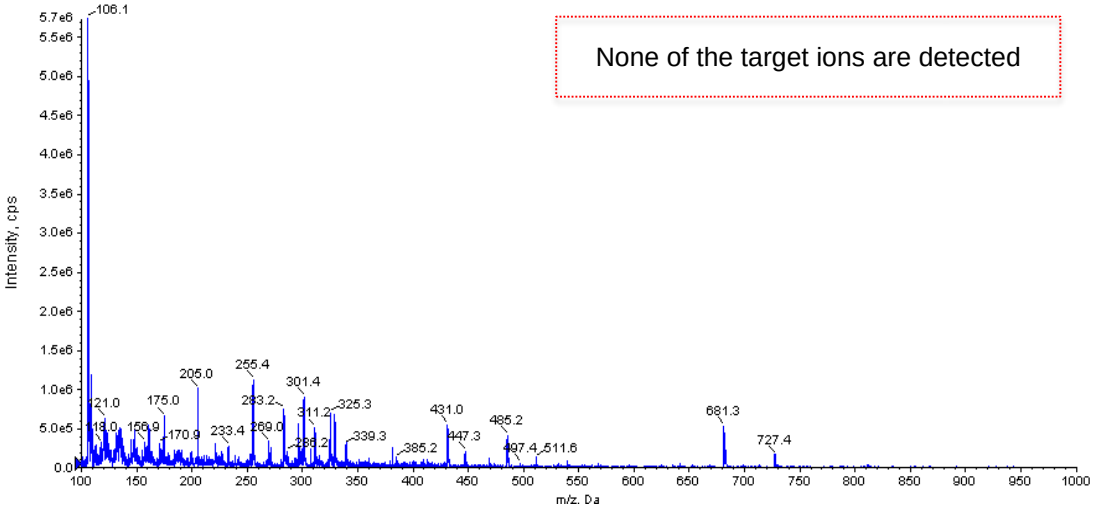

Fig. A1 Precursor-ion spectra of 10 ppm unsaturated AOS of Laminarin japonica (sample A) in 10 mM Ammonium Acetate with 0.1% FA (50% A: 50% B-acetonitrile)
solvent A (10 mM ammonium formate + 0.1% formic acid in ultrapure water)
solvent B (10 mM ammonium formate in 95:5 acetonitrile: ultrapure water)

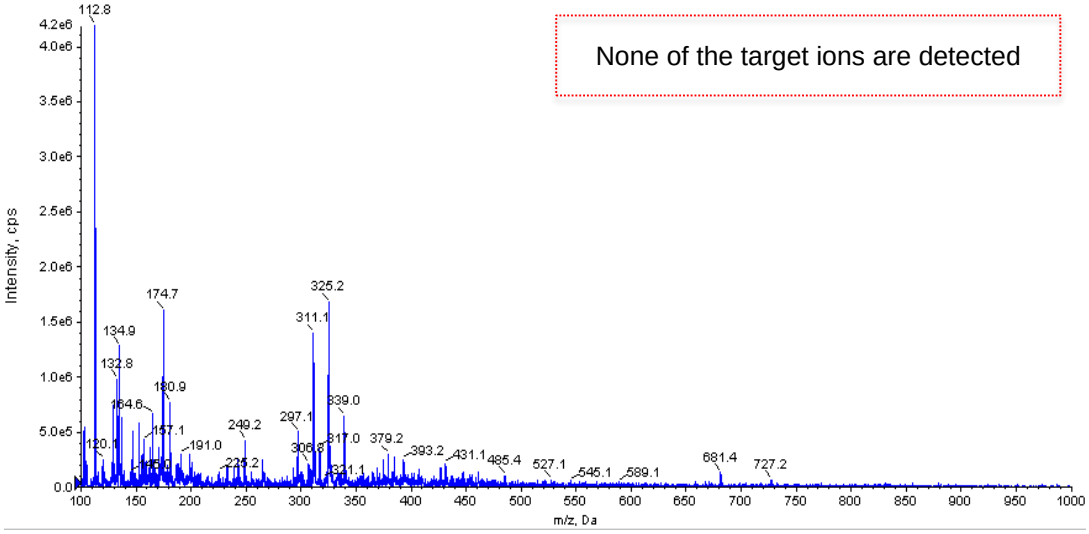

Fig. A2 Precursor-ion spectra of 10 ppm unsaturated AOS of Laminarin japonica (sample A) in 10 mM Ammonium Acetate with 0.1% FA (50% A: 50% B-methanol)
solvent A (10 mM ammonium formate + 0.1% formic acid in ultrapure water)
solvent B (10 mM ammonium formate in 95:5 methanol: ultrapure water)

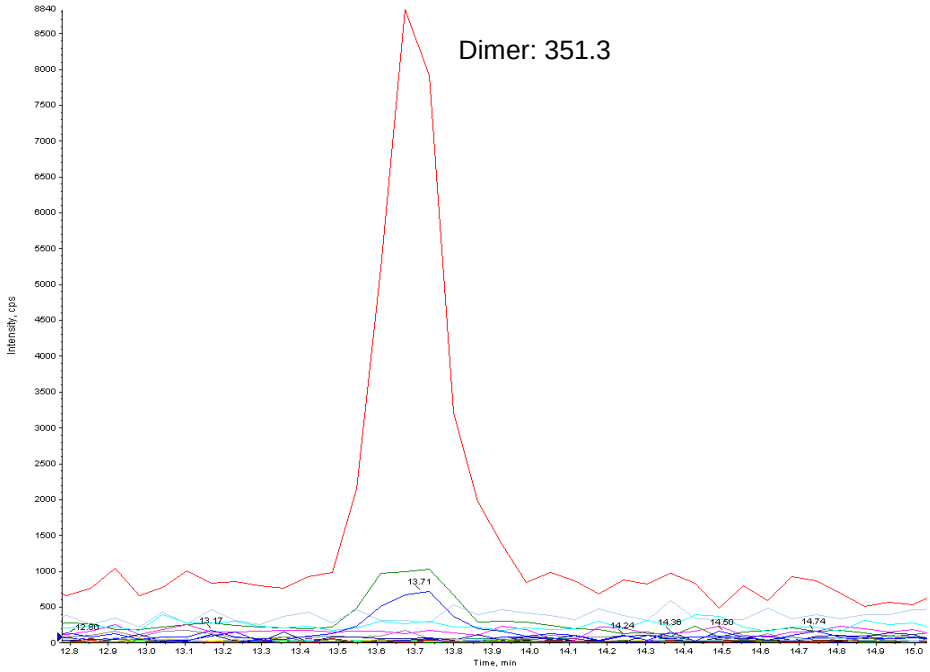

Fig. A3 the LC separation of alginate oligosaccharide dimer (m/z=351.3)

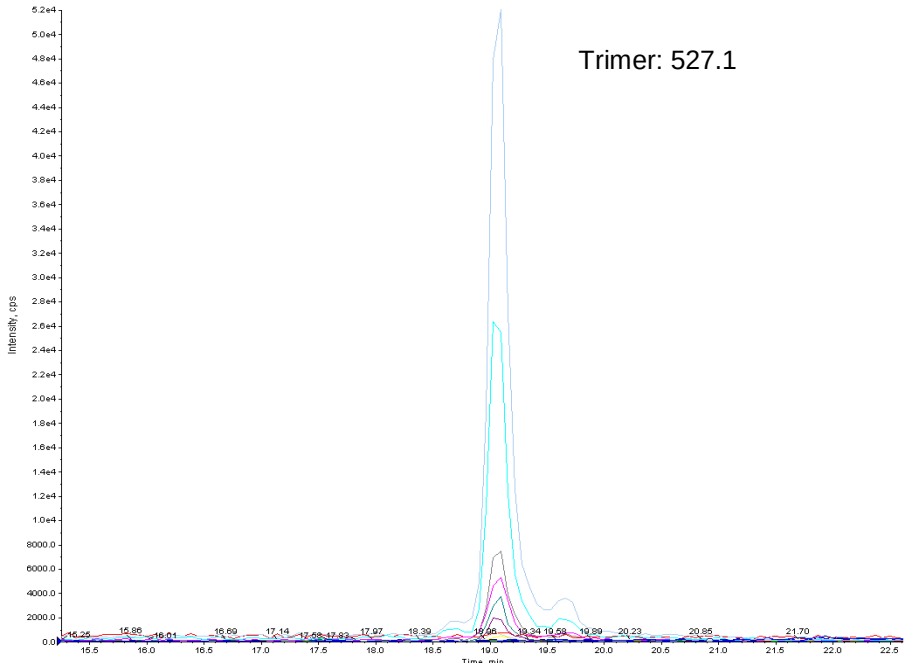

Fig. A4 the LC separation of alginate oligosaccharide trimer (m/z=527.1)

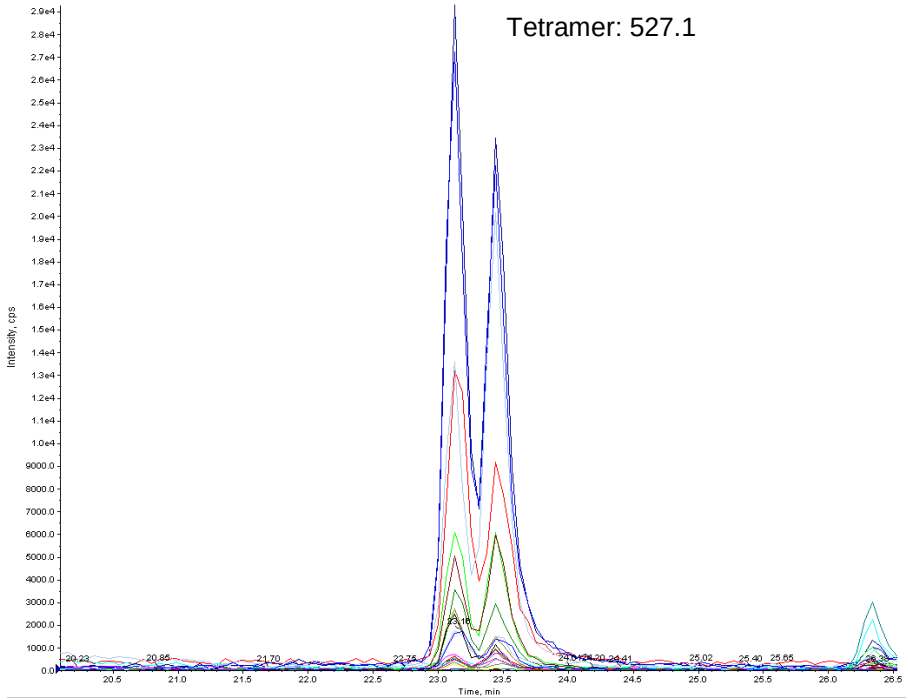

Fig. A5 the LC separation of alginate oligosaccharide tetramer (m/z=703)

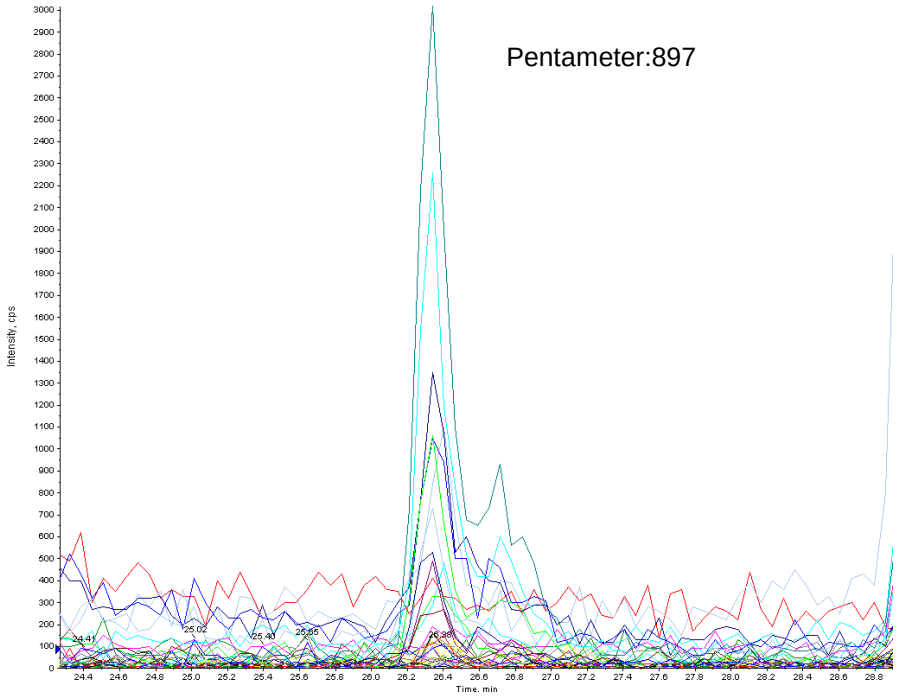

Fig. A6 the LC separation of alginate oligosaccharide pentameter (m/z=897)

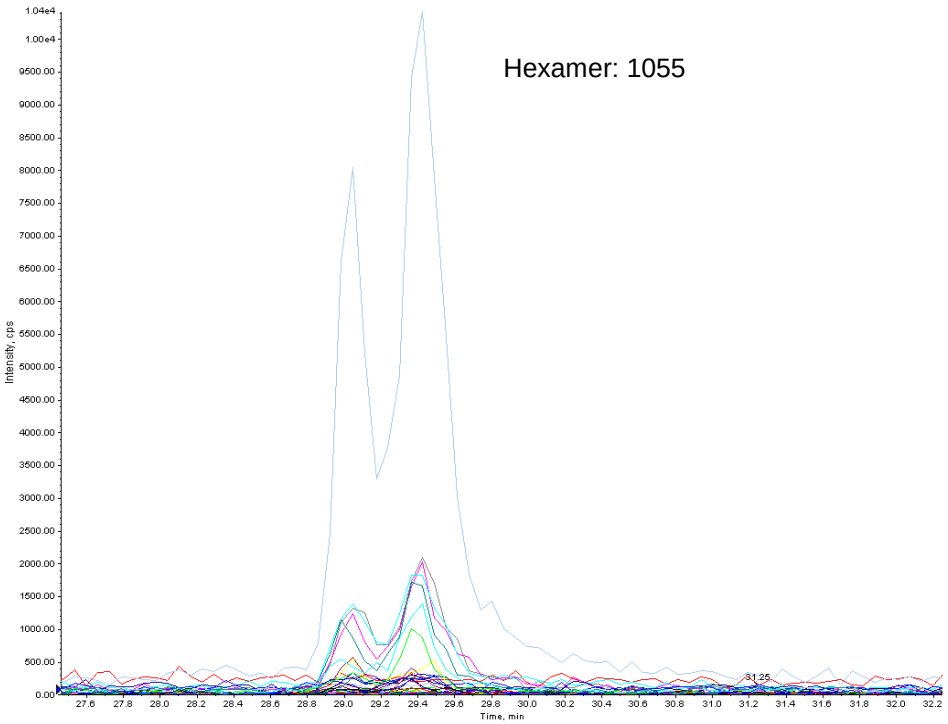

Fig. A7 the LC separation of alginate oligosaccharide hexamer (m/z=1055)

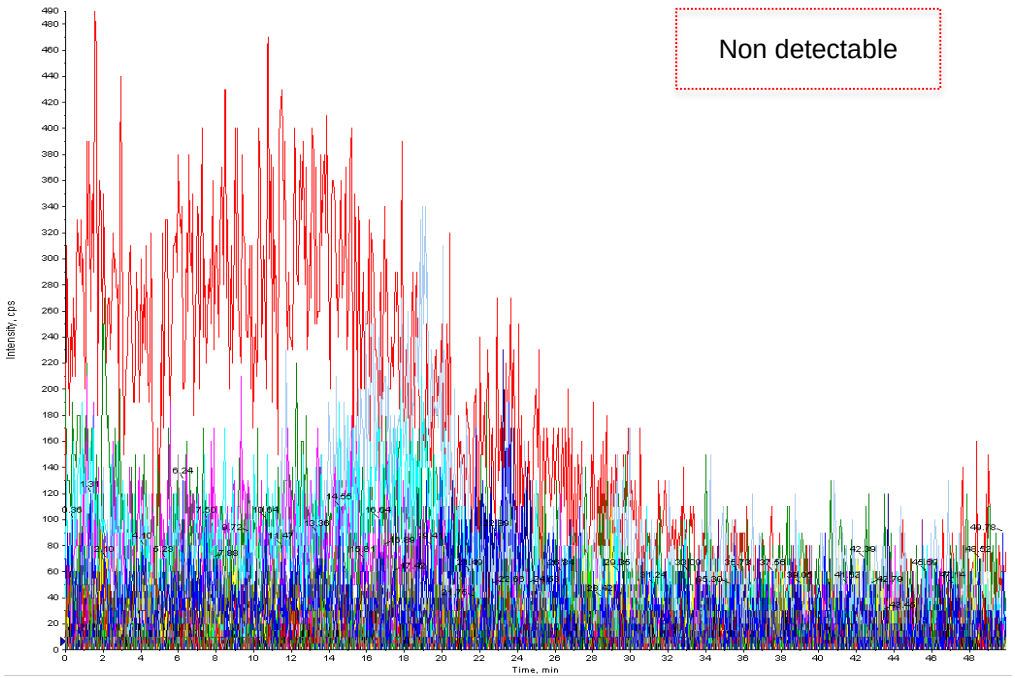

Fig. A8 LC separation of 1 ppm saturated alginate oligosaccharide using Tosoh TSK gel Amide 3um (2.0 mm x 150 mm) column at 0.3 ml/min flow rate, 5ul injection value at 30°C

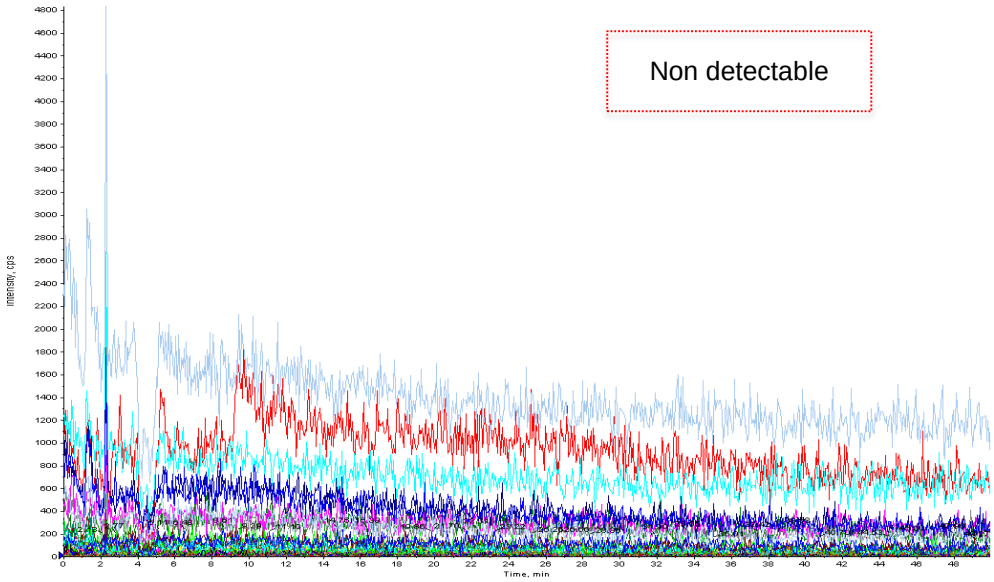

Fig. A9 LC separation of 10 ppm saturated alginate oligosaccharide using Tosoh TSK gel Amide 3um (2.0 mm x 150 mm) column at 0.3 ml/min flow rate, 5ul injection value at 30°C

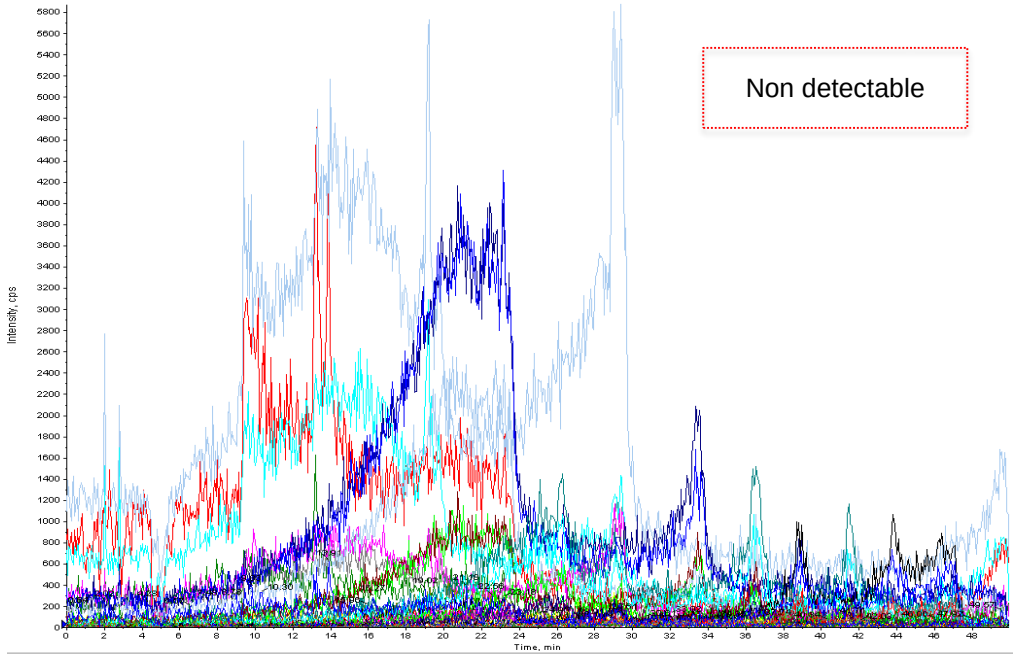

Fig. A10 LC separation of 10 ppm saturated alginate oligosaccharide using Tosoh TSK gel Amide 3um (2.0 mm x 150 mm) column at 0.3 ml/min flow rate, 5ul injection value at 35°C

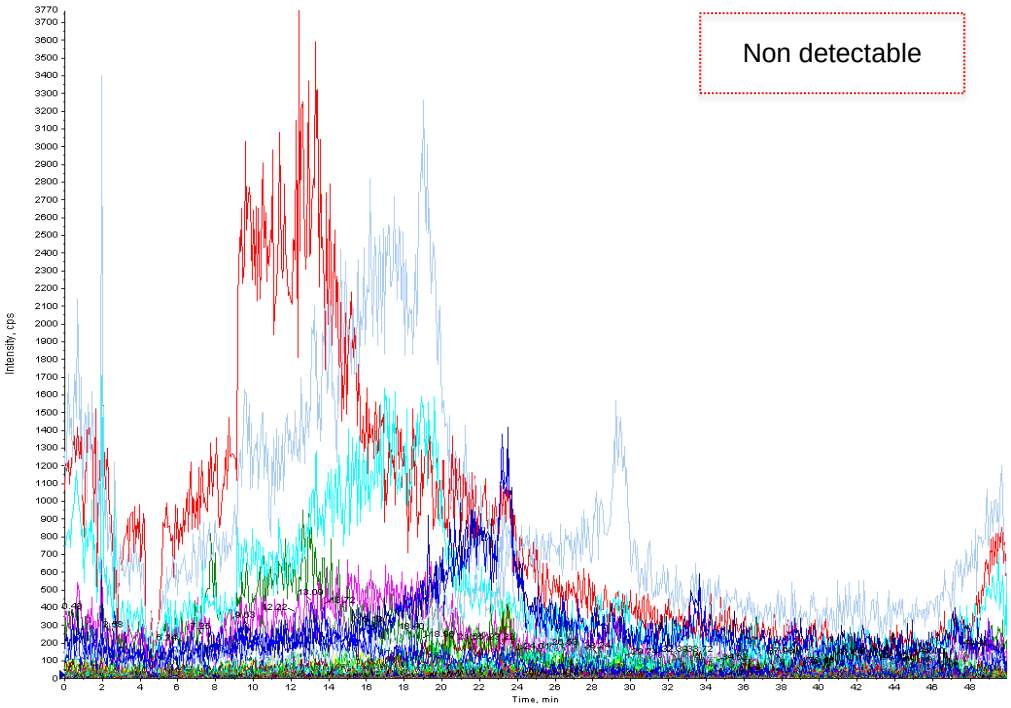

Fig. A11 LC separation of 10 ppm saturated alginate oligosaccharide using Tosoh TSK gel Amide 3um (2.0 mm x 150 mm) column at 0.3 ml/min flow rate, 5ul injection value at 40°C