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The seasonal variation of fucoidan within three species of brown macroalgae

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ABSTRACT

Fucoidan is comprised of a fucose backbone with sulphate groups, whose variation is important to the functionality of the polysaccharide. The structure of fucoidan has been reported to vary according to species, season, location and maturity; however there is currently little published data to support this. Understanding the seasonal variation of fucoidan is important for industrial applications to identify optimum harvesting times and ensure consistent product composition. This study explores the seasonal variation of three species of brown macroalgae, *Fucus serratus* (FS), *Fucus vesiculosus* (FV) and *Ascophyllum nodosum* (AN), harvested monthly off the coast of Aberystwyth, UK. Average fucoidan content is 6.0, 9.8 and 8.0 wt% respectively for FS, FV and AN, with highest quantities extracted in autumn and lowest in spring. Fucose content, varied between 18 and 28, 26–39 and 35–46 wt% and sulphate content between 30 and 40, 9–35 and 6–22 wt% for FS, FV and AN respectively, with both fluctuating inversely to the total fucoidan content. Size exclusion chromatography (SEC) has provided insight into the structural differences between the species. Based on the molecular weight (MW) distribution, and in line with previous research, it is hypothesised that fucoidan in FS has a more complex structure, with a higher degree of associated sulphate ions than in FV and AN which have a simpler, linear structure with less associated sulphate ions.

1. Introduction

Fucoidan is a sulphated polysaccharide found in brown macroalgae. Its structure is dependent on the species, season, harvest location and maturity of the plant. Its basic structure is comprised of a sulphated fucose backbone, with the most common configuration shown in Fig. 1, but which also contains small quantities of other sugars, such as xylose, uronic acids and galactose. Branched side chains are also common in some species. Reported molecular weight (MW) varies widely, with Rioux et al. quoting 43 kDa [1], and Gupta et al. 1600 kDa [2], a difference of over 1550 kDa. It was originally identified by Kylin in 1913 [3], who named it "fucoidin" and reported an extraction mainly containing fucose. Since then, fucoidan has been widely researched, with advances in both knowledge of its structure and potential properties.

The extraction of fucoidan from macroalgae has been performed by several authors in the published literature [4–9]. In general this consists of four main steps: an initial purification to remove pigments and lipids, often using an alcohol; an extraction step, often repeated several times to ensure full extraction of fucoidan and most commonly using calcium chloride, dilute hydrochloric acid (HCl) or water; further purification of the extract to remove alginate and other impurities before fucoidan is finally precipitated using ethanol [4–9]. A comparison of the three extraction solvents was carried out by Ponce et al. (2003) [10]. The results indicated that distilled water and HCl extraction gave the highest and comparable yields of 10.8 and 9.6 wt% respectively, with the structure of each extract being very similar. Zhang and Row (2015) further developed this work by identifying the best conditions for fucoidan extraction from *Laminaria japonica* [11]. Their findings suggest an extraction time of 4 h at 80 °C and 0.1 M HCl yielding the best results, giving 17 wt% fucoidan. The fucoidan extraction method used in this paper is based on these findings, making it a well-documented and reliable process.

Current research focuses primarily on fucoidan's use in the pharmaceutical industry, with the most extensively studied properties being anticoagulant, anti-thrombotic, immunomodulation, anti-cancer and anti-proliferative [12], although nutraceutical, functional food and cosmetic properties [13] have also been identified. Cho et al. [14] and Senthilkumat et al. [15] have both reported on the anticancer properties of fucoidan, showing inhibition in growth and migration of, as well as being cytotoxic to, cancer cells. Anti-inflammatory properties have been presented by Park et al. [16], who suggest that the properties seen could offer potential for the treatment of neurodegenerative diseases. Ponce at al. [10] demonstrate the antiviral properties of fucoidan

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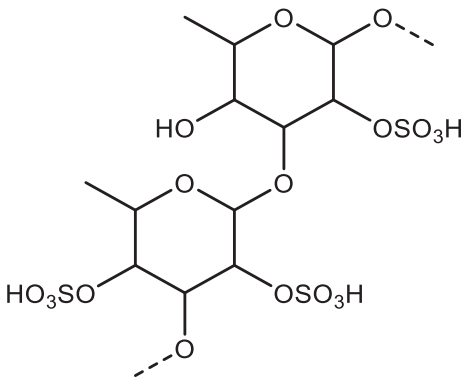

Fig. 1. Simple structure of fucoidan backbone.

fraction from *Adenocystis utricularis*, which showed high inhibition against herpes simplex virus 1 and 2. These properties have been shown by several authors to depend on the molecular weight (MW), the degree of sulphation and sulphation pattern of the fucoidans [17, 18]. Therefore knowledge about how these properties are affected by seasonal variation is vital.

The seasonal variation of fucoidan is often mentioned in the literature, although there is very little published data on the subject at present and the few references cover only a few months of the year. Rioux et al. have investigated the bioactive polysaccharides of 4 samples of *Saccharina longicruris*, from March, April, November 2005 and June 2006 [19]. The galactofucans (a type of fucoidan containing roughly equal proportions of fucose and galactose) extracted were seen to have an increase in sulphate content of 1.6% between March and November 2005, while decreasing by 7.2% between November 2005 and June 2006. A similar study by Mak et al. investigated the variation in fucoidan between July and October for *Undaria pinnatifida* [20]. They found that the fucoidan content almost quadrupled between July and September (3.6–13.7 wt%) and only dropped slightly in October. A similar trend was observed in the sulphate content of the fucoidan. The fucose content decreased significantly between July and September.

Other than the two studies mentioned [19,20], the authors were unable to find other published literature on the seasonal variation of fucoidan. Considering the change in biomedical properties due to the varying composition of fucoidan, understanding the seasonal variation of the chemical content of fucoidan is very important. The work presented here attempts to characterise fucoidan extracted from 3 species of brown macroalgae over a calendar year. Samples of *Fucus serratus* (FS), *Fucus vesiculosus* (FV) and *Ascophyllum nodosum* (AN) were collected monthly over a 12 month period between April 2010 and March 2011, fucoidan was extracted from these samples and analysed for elemental composition, fucose and sulphate content. Furthermore, size exclusion chromatography (SEC) and liquid chromatography-mass spectrometry (LC-MS) has been performed in order to gain insight into the MW of the samples and structural differences.

2. Methods

2.1. Materials

Samples of *Fucus serratus* (FS), *Fucus vesiculosus* (FV) and *Ascophyllum nodosum* (AN) were collected monthly between April 2010–March 2011 off the coast of Aberystwyth (Latitude: 52.41°N, Longitude: −4.08°W) at low tide. The samples were freeze-dried and ground using a Fritsch pulverisette 14 rotor mill through a 500 μm sieve. Whole plants were collected, frozen within two hours of collection and subsequently freeze dried for one week to ensure full lyophilisation. Dried samples were stored in sealed containers for further analysis. Standard fucoidan (F5631) was supplied by Sigma Aldrich.

2.2. Fucoidan extraction

0.5 g of ground, dried macroalgae was weighed into a 50 ml centrifuge tube and 10 ml of 85% ethanol was added and stirred overnight at room temperature. This was centrifuged and the supernatant removed. The pellet was washed once with 10 ml ethanol followed by 10 ml acetone and allowed to dry to a constant weight at room temperature. 0.3 g of the washed seaweed was weighed into a new 50 ml centrifuge tube with 7.5 ml 0.1 M HCl and stirred at 80 °C for 4 h before cooling, centrifuging and decanting the supernatant into a clean 15 ml centrifuge tube. The pH of the supernatant was determined and neutralised to pH 5–7 using a pH meter (HQ40d, Hach) if required using 1 M $Ca(OH)_2$. 1 volume (~6 ml) of 1% $CaCl_2$ was added and stored at 4 °C overnight to precipitate alginate present. The tube was centrifuged and the supernatant transferred to another clean tube, where ethanol was added to give a final concentration of 40% v/v ethanol. This was left for at least 4 h at 4 °C to precipitate the laminarin. The solution was centrifuged, the supernatant decanted into a clean tube and ethanol added again to give a final concentration of 70% v/v ethanol. It was left to precipitate fucoidan for at least 4 h at 4 °C, before being centrifuged for a final time. The extracted fucoidan was allowed to air dry to a constant weight, around 24 h.

2.3. Ultimate analysis

Analysis of the C, H, N and S content of the extracted fucoidan was carried out using a CE Instruments Flash 1112 Series analyser. Samples were prepared by weighing 2.5 ± 0.5 mg of dry fucoidan into 8 × 5 mm tin capsules, along with ~5 mg of vanadium pentoxide, required to combust the sulphur.

2.4. Analysis of fucose and sulphate content

Aqueous samples containing 2.5 wt% of the extracted fucoidan were prepared from a set of fucose standards, at 30–150 mg l^{-1}, and relevant blanks. 1 ml of each solution (either calibration, water or sample) were placed into a 15 ml Pyrex tube with 4.5 ml of 6:1 v/v H_2SO_4 (98% purity). The tube was capped, inverted several times to mix and left at room temperature for 5 min. Each tube was then placed in a boiling water bath for exactly 10 min, removed and cooled under tap water to quench the reaction. 0.1 ml of 3% aqueous cysteine hydrochloride 10 was added to each tube, inverted several times and left for 30 min before measuring at 396 and 427 nm in a UV/VIS spectrophotometer (Multiskan GO, Thermo Scientific).

Sulphate analysis was performed on the same 2.5 wt% solutions of fucoidan using the sulphate testing kit (LCK353) supplied by Hach-Lange.

2.5. Size exclusion chromatography (SEC)

SEC was performed on the fucoidan extracts using a Dionex ultimate-3000 system, fitted with a Waters 500 Ultrahydrogel column and guard column (Dionex). Aqueous, 2.5 wt% fucoidan samples were prepared and filtered through 0.2 μm syringe filters into HPLC vials. Samples were set to run with distilled water as the mobile phase, as described by Zhang et al. [11] to give the best separation for seaweed extracts, with an oven temperature of 30 °C and a flow rate of 0.5 ml min^{-1}. The high performance liquid chromatography (HPLC) software (Chromeleon v.6.8 with extension pack v.2.0) was calibrated with a set of polyethylene glycol/polyethylene glycol standards (MW 200 to 1,015,000 Da) (Fluka) and the MW of the extracted fucoidan was determined using Chromeleon integrated software.

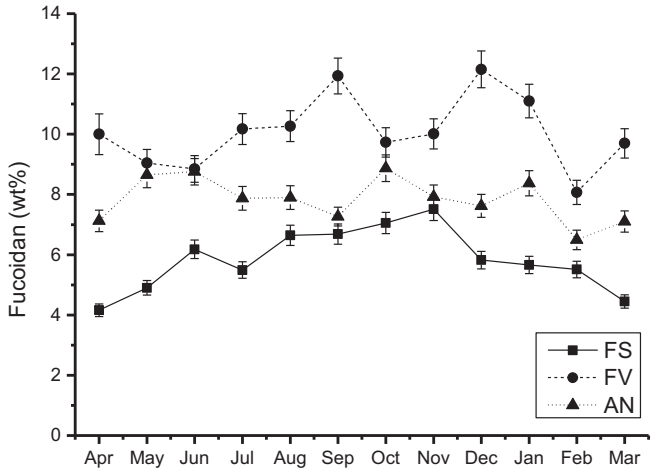

Fig. 2. Seasonal variation of fucoidan content for FS, FV and AN.

2.6. High performance liquid chromatography-mass spectrometry (HPLC-MS)

HPLC-MS was performed on the fucoidan extracts using an Aligent 1200 series HPLC and Brucker HCTultra MS. The HPLC was fitted with a Waters 500 Ultrahydrogel using identical chromatographic conditions to that used in Section 2.5, oven temperature of 30 °C and flowrate of 0.5 ml/min. The MS was operated in negative ion mode with a mass scan between 100 and 1300 *m/z*. Samples of extracted Fucoidan were compared to the Fucoidan standard (F5631) supplied by Sigma Aldrich.

2.7. Experimental replication and statistical treatment

All analyses, including fucoidan extraction, sulphate and fucose analysis and ultimate analysis, have been performed in duplicate. The average values are reported along with the standard deviation in all tables and figures. For colourimetric analysis, absorbance readings for each sample are taken in duplicate, of which the average is taken forward for further calculation.

3. Results and discussion

The fucoidan content of the 3 species varies throughout the year, as shown in Fig. 2. The trend suggests lower fucoidan content in spring, rising to its maximum in late autumn, before decreasing over the winter, although for AN, the levels are more uniform throughout the year. FV has the highest content throughout the year, reaching a maximum of 12.2 wt% in December. This is followed by AN, with a maximum of 8.9 wt% in October, with FS reaching a high of 7.5 wt% in November. Corresponding minimums are 8.1 wt% in February, 6.5 wt% in February and 4.2 wt% in April for FV, AN and FS respectively. This would suggest that the best time to harvest for maximum fucoidan content would be late autumn/early winter. However, the difference from maximum to minimum is 5.7 wt%, 2.4 wt% and 3.3 wt% respectively for FV, AN and FS; a relatively small fluctuation suggesting a good yield could be obtained at any time of the year. This is particularly advantageous for industrial applications, removing the potential need for drying and storage. Fresh seaweed typically have a water content of 80 wt% [21] and will

Table 1
Ultimate analysis and MW of fucoidan extracts.

		Ultimate analysis				Atomic ratio		Ave MW of SEC peaks (kDa)		
	Month	C	H	S	Other	C:H	C:S	Main	1st	2nd
Fucus serratus	Apr	21.8 ± 1.0	4.0 ± 0.0	6.9 ± 0.2	66.8 ± 0.8	2.2	1.15	1505	200	73
	May	23.6 ± 1.1	4.3 ± 0.0	6.0 ± 0.4	65.8 ± 0.8	2.2	1.04	1724	253	94
	Jun	24.2 ± 0.6	4.3 ± 0.1	5.7 ± 0.3	65.5 ± 0.6	2.1	1.01	1671	297	117
	Jul	24.8 ± 0.1	4.3 ± 0.1	5.4 ± 0.1	65.3 ± 0.4	2.1	0.99	1566	288	114
	Aug	25.5 ± 0.3	4.5 ± 0.4	5.7 ± 0.6	64.1 ± 1.3	2.1	0.94	1590	286	115
	Sep	26.2 ± 0.3	4.4 ± 0.3	5.3 ± 0.6	64.1 ± 0.7	2.0	0.92	1711	351	137
	Oct	23.4 ± 0.5	4.2 ± 0.3	6.7 ± 0.3	65.6 ± 1.0	2.1	1.05	1539	302	125
	Nov	26.0 ± 1.6	4.8 ± 0.3	6.1 ± 0.6	63.2 ± 1.2	2.2	0.91	1438	309	128
	Dec	24.2 ± 0.1	4.6 ± 0.1	7.4 ± 0.5	63.7 ± 0.5	2.3	0.99	1509	280	112
	Jan	23.4 ± 0.7	4.6 ± 0.2	7.2 ± 1.2	64.6 ± 0.3	2.4	1.03	1410	297	119
	Feb	23.5 ± 0.4	4.3 ± 0.3	6.6 ± 0.1	65.2 ± 0.6	2.2	1.04	2024	234	98
	Mar	27.1 ± 1.8	4.9 ± 0.3	6.6 ± 0.3	61.0 ± 2.4	2.2	0.84	1336	206	75
	Apr	25.4 ± 3.1	4.7 ± 0.7	9.1 ± 0.2	60.2 ± 3.7	2.2	0.89	1653	373	117
Fucus vesiculosus	May	22.4 ± 0.9	4.2 ± 0.1	9.7 ± 0.0	63.6 ± 1.0	2.2	1.06	1336	370	196
	Jun	24.0 ± 0.0	4.3 ± 0.0	10.1 ± 0.1	61.5 ± 0.1	2.1	0.96	1296	355	221
	Jul	23.9 ± 0.5	4.5 ± 0.1	9.0 ± 0.39	61.4 ± 0.2	2.2	0.96	1184	345	199
	Aug	25.0 ± 1.2	4.5 ± 0.3	8.1 ± 0.1	62.2 ± 1.4	2.2	0.93	1373	388	204
	Sep	27.9 ± 1.6	5.2 ± 0.4	6.4 ± 0.5	60.2 ± 1.5	2.2	0.81	1430	438	221
	Oct	23.6 ± 0.9	4.2 ± 0.1	9.4 ± 0.4	62.4 ± 0.7	2.1	0.99	1440	365	176
	Nov	22.9 ± 0.8	4.3 ± 0.1	8.4 ± 0.1	64.2 ± 0.7	2.3	1.05	1406	262	129
	Dec	26.6 ± 1.0	4.8 ± 0.0	7.7 ± 0.1	60.7 ± 1.0	2.2	0.86	1789	457	119
	Jan	23.0 ± 0.4	4.2 ± 0.1	9.4 ± 0.2	63.2 ± 0.7	2.2	1.03	1405	269	148
	Feb	24.4 ± 0.2	4.4 ± 0.1	10.1 ± 0.1	60.9 ± 0.4	2.2	0.93	1399	378	221
	Mar	22.7 ± 0.5	4.3 ± 0.0	9.9 ± 0.6	62.9 ± 0.0	2.3	1.04	1369	298	153
Ascophyllum nodosum	Apr	20.3 ± 0.2	4.2 ± 0.0	12.1 ± 0.2	63.1 ± 0.4	2.5	1.16	1396	186	59
	May	22.9 ± 1.1	4.2 ± 0.1	10.00.1	62.7 ± 1.3	2.2	1.03	1376	321	190
	Jun	25.5 ± 0.5	4.7 ± 0.0	7.6 ± 0.5	61.8 ± 0.2	2.2	0.91	1469	272	154
	Jul	22.5 ± 1.1	4.3 ± 0.3	8.8 ± 0.0	64.3 ± 1.5	2.3	1.07	1465	357	213
	Aug	23.8 ± 0.1	4.6 ± 0.0	8.7 ± 0.1	62.7 ± 0.1	2.3	0.99	1420	321	187
	Sep	27.2 ± 0.5	4.9 ± 0.2	6.8 ± 0.2	61.0 ± 0.5	2.1	0.84	1274	261	144
	Oct	23.2 ± 0.2	4.4 ± 0.0	8.9 ± 0.3	63.3 ± 0.0	2.3	1.02	1364	353	185
	Nov	24.0 ± 0.4	4.6 ± 0.1	8.2 ± 0.1	63.0 ± 0.4	2.3	0.98	1408	263	152
	Dec	22.7 ± 0.1	4.4 ± 0.1	9.2 ± 0.3	63.5 ± 0.1	2.4	1.05	1334	342	186
	Jan	22.2 ± 1.2	4.2 ± 0.3	9.5 ± 0.6	63.8 ± 2.0	2.3	1.08	1326	296	163
	Feb	22.5 ± 0.0	4.3 ± 0.0	10.4 ± 0.0	62.5 ± 0.0	2.3	1.04	1308	307	171
	Mar	23.7 ± 0.2	4.6 ± 0.0	9.9 ± 0.3	61.5 ± 0.4	2.4	0.97	1486	228	75

decompose rapidly in a short period of time. If seaweed were only collected once a year, drying would be necessary in order to store and produce the pure fucoidan. Assuming functionality is prevalent throughout the year, the seaweeds could be processed wet throughout the year reducing the energy consumption associated with drying.

Ultimate analysis of the fucoidan extract is displayed in Table 1. The average atomic ratio of C:H:S:O are very similar for the three species, being 1:2.2:0.1:2.0; 1:2.2:0.2:2.0 and 1:2.3:0.1:2.0 for FS, FV and AN respectively. The nitrogen values are negligible. While the variation in the C:H values remain fairly constant over the year, the C:S values show a negative parabolic trend for all species. The variation of C is very similar for all species, with a minimum in April, rising to a maximum in September, decreasing again through the autumn and winter months. This same trend is seen for H, and the reverse is seen for S and Others, which are at a maximum in the winter months and low in the summer. These trends suggest that the extracted fucoidan contains a higher proportion of sulphate in the winter compared to the summer. As functionality is dependent on the degree of sulphation [17,18], it is likely that the functionality of fucoidan varies over the year.

The large "other" value in the CHNS results, average 64.8, 62.9 and 62.9 wt% respectively for FS, FV and AN, suggests a high oxygen content in the extracted F2 fucoidan fraction. As the extraction process ensures a relatively pure product, many of the other components such as salts will have been removed. Furthermore, high oxygen content would be expected due to the high sulphate content, where 4 oxygen atoms are associated with each sulphur atom and the high fucose content, which contains up to 5 oxygen atoms per monomer unit, depending on the degree of sulphation.

Table 2
Review of analysis of extracted fucoidan found in the literature.

Paper	Species	Month	MW	Fucose	Sulphate
Current	*Fucus serratus*	Year average	1608 kDa	24 ± 3.1%	34 ± 3.7%
[23]	*Fucus serratus*[a]	Aug	–	46.6%	31.8%[b]
[24]	*Fucus serratus*	–	–	24.8%	29.2%[b]
Current	*Fucus vesiculosus*	Year average	1364 kDa	35 ± 4.4%	19 ± 7.7%
[31]	*Fucus vesiculosus*[a]	Sept	–	48.1%	25.4%
[32]	*Fucus vesiculosus*	Commercial	–	33.3%	23.0%
[24]	*Fucus vesiculosus*	–	–	26.1%	23.6%[b]
[25]	*Fucus vesiculosus*	Commercial		13.8%	34.6%
Current	*Ascophyllum nodosum*	Year average	1374 kDa	40 ± 3.7%	15 ± 4.5%
[31]	*Ascophyllum nodosum*[a]	Sept	–	33.0%	20.9%
[24]	*Ascophyllum nodosum*	–	–	26.6%	24.4%[b]
[26]	*Ascophyllum nodosum*	Sept	420/47 kDa	52.1%	19.0%
[33]	*Ascophyllum nodosum*	Commercial	6.2 kDa	25.0%	21.7%
[34]	*Ascophyllum nodosum*	–	–	66 mol%	31 mol%

[a] Values from the most abundant fucoidan fraction stated.
[b] Sulphate content quoted as NaSO3.

Fucus and sulphate content in fucoidan, shown in Fig. 3, is seen to vary within all three species. Although there is no clear trend between the species, the fucose and sulphate levels vary proportionally to each other and inversely proportional to the total fucoidan content. The fucose content for FS, FV and AN range between 18 and 28, 26–39 and 35–46 wt% respectively, while the sulphate content varies between 30 and 40, 9–35 and 6–22 wt% respectively. Within each species there are distinguishable trend lines for fucose and sulphate: FS decreases in May and June, but is fairly constant over the rest of the year; FV increases throughout the year from a low point in April, reaching a maximum in November, before decreasing again and AN is low in September to October, but is again fairly constant over the rest of the year. Another notable point is that in FS, the fucose is lower than the sulphate content, however in FV and AN the reverse is true, with the sulphate content being higher. This indicated a higher degree of sulphation for each

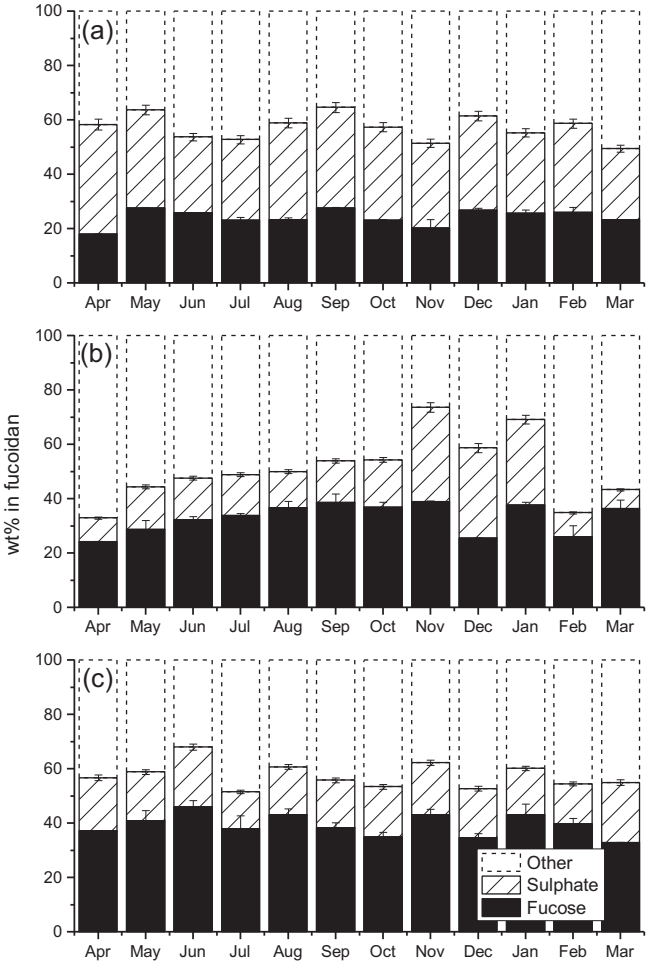

Fig. 3. Fucose and sulphate content of extracted fucoidan, where (a) is FS, (b) is FV and (c) is AN.

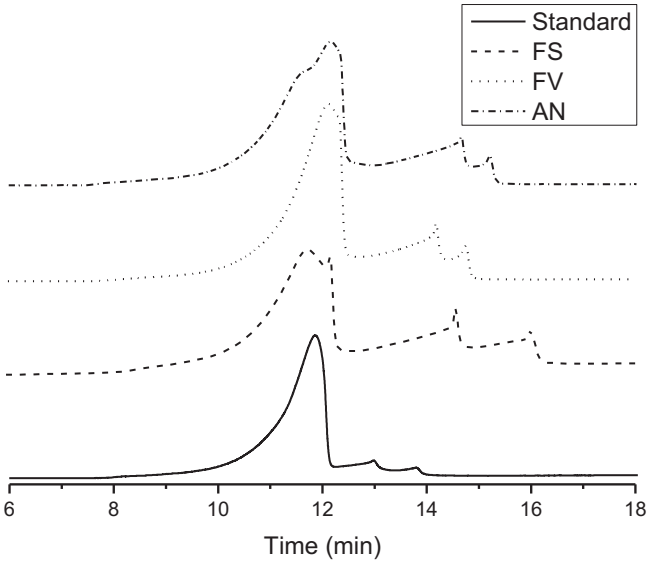

Fig. 4. SEC chromatograms for fucoidan for June, comparing the standard, FS, FV and AN extracts.

fucose in FS than for FV and AN. The variation in the sulphate content is especially important, as it has been reported that less than a 20% sulphate content leads to a complete loss of anti-proliferative and anticoagulant activity [22]. As the sulphate in FV and AN fall below this quantity during the summer months, it is an important consideration when harvesting these species for fucoidan extraction.

Previous studies have shown FS to have a sulphate to fucose ratio of between 0.9 and 1.5 [23] [24], while the average for this study is 0.73, which is comparable. The average ratios for FV and AN respectively are 2.0 and 2.7; significantly higher than for FS, but is comparable with literature values of 1.1 to 2.5 [24,25] and 1.1 to 2.7 [24] [26] for FV and AN respectively.

Table 2 lists the fucose and sulphate content reported in the literature for extracted fucoidan from the fucoids studied in this investigation. The quoted fucose and sulphate content of extracted fucoidan samples varies dramatically, with AN showing the widest range for fucose content; between 52.1 and 25 wt%. The results presented in this paper correspond well with the range of values quoted previously in other research papers, with an average fucose ± one standard deviation of 24 ± 3.1%, 35 ± 4.4% and 40 ± 3.7% and average sulphate of 34 ± 3.7%, 19 ± 7.7% and 15 ± 4.5% for FS, FV and AN respectively.

The range of literature values quoted, as well as the variation in the presented results, shows clearly the need for a thorough understanding of the way in which fucoidan content varies in order to be able to make

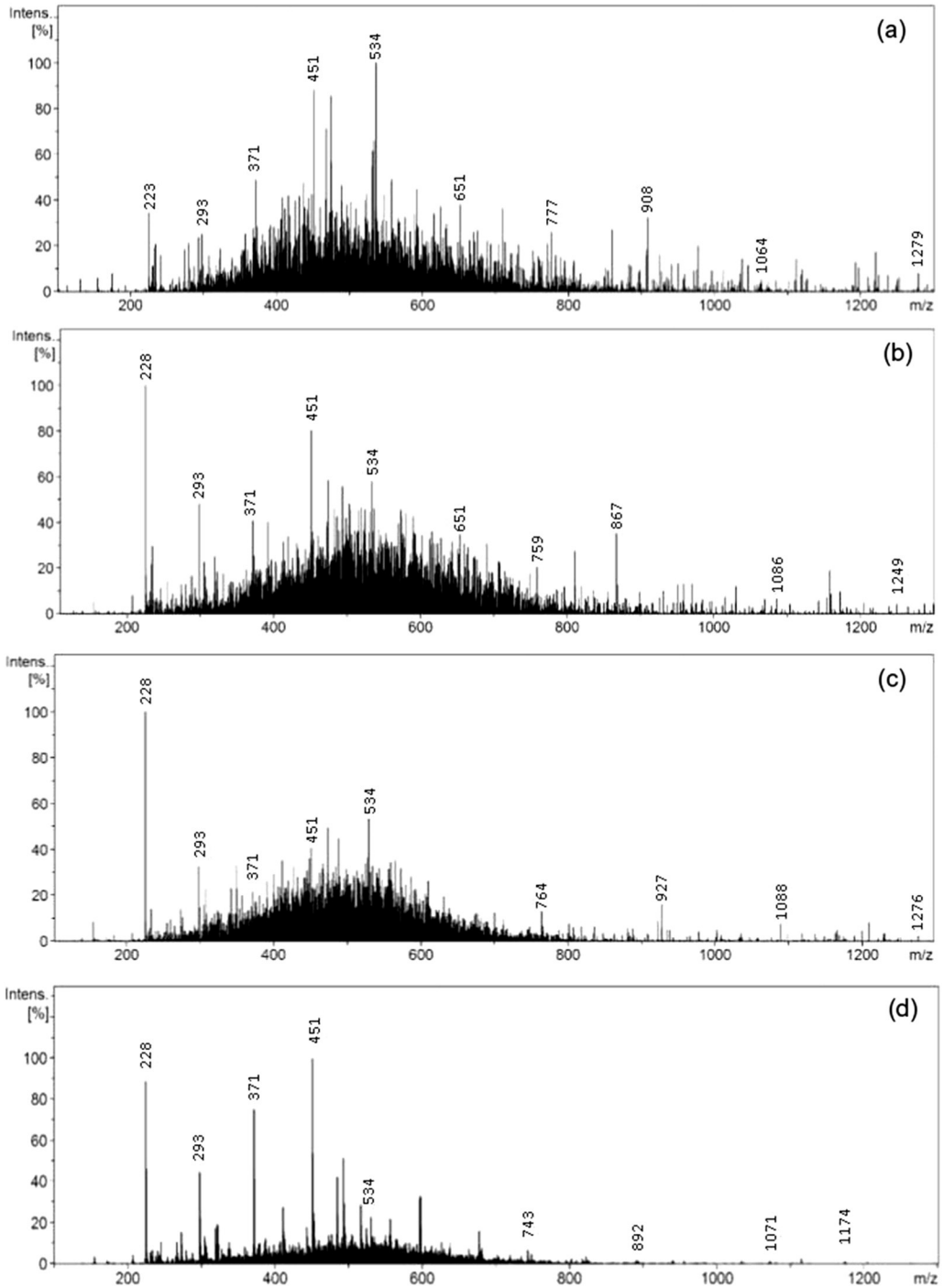

Fig. 5. LC-MS data for the main peak of fucoidan for (a) FS, (b) FV, (c) AN, from representative samples from May, June and May respectively, and (d) standard fucoidan.

full use of the resource. As many papers have shown, the potential uses for fucoidan in pharmaceuticals are vast [27–30]; however, each of these properties will be associated with a particular fucoidan, harvested in a particular place at a particular time of year. Without clear knowledge of all of these facts, the likelihood of being able to replicate the extracted fucoidan is reduced. This is also important from an industrial extraction standpoint, where economic viability will be based on being able to produce a sufficient quantity of an identical product with the desired properties.

The SEC curves of extracted fucoidan show differences between harvesting time and species. For comparison, chromatograms from extracted fucoidan harvested in June have been shown in Fig. 4, chosen due to the most pronounced variation between species. The MW of the fucoidan extracts, given in Table 1, remain fairly constant over the year for all species. The average MW for FS, FV and AN respectively are 1608, 1364 and 1374 kDa. The peaks for FS are a doublet which become less pronounced during the summer months. There is also some evidence of this for both FV and AN, although it is much less distinct. The FV samples show similar peaks to that of the standard which is expected due to standard also being extracted from FV. AN shows increasingly broader peaks through the spring, reaching a maximum in summer and begin to narrow in autumn. The most significant differences between the three extracts are the two smaller, secondary peaks after the main fucoidan peak (retention time 14–16 min) which vary in size and width between species. For FS, these are broader and further apart, while for FV they are sharper and closer together. AN has a broad first peak, with a second sharp peak. These peaks represent an average MW of 282/112, 347/187 and 309/175 kDa for the first/second secondary peak for FS, FV and AN respectively. It is likely, as the LC-MS data presented in Fig. 6 shows, that these secondary peaks are much smaller molecules than this but have been "pulled through" the column due to their association with the larger fucoidan macromolecule.

The main, double tipped peaks seen in the SEC chromatograms, especially evident in the FS samples, could be a sign of a more complex fucoidan structure. It is well known that FV gives the most simple form of fucoidan, with a linear chain of fucose [35]; this has also been shown for AN. FS, however, has been shown to have a more complex, branched structure [23]. The differences in the peaks shape and width suggests this more complex structure and variation in the chain length over the year period; a broad peak denotes high variation in MW of the macromolecule, while a double tipped peak indicates an increased abundance of two MW's. Although the MW ranges found for the extracted fucoidan are quite high, they are in line with others in the literature for similar, crude extracts [2].

LC-MS analysis on the fucoidan extracts was undertaken in order to gain more understanding of the structural differences between the fucoidan samples and the secondary peaks identified in the SEC chromatogram. A comparison of the MS chromatogram for the main peaks of the three species and standard is shown in Fig. 5. The overall shape of the peaks differ between species, indicating a difference in the structure of the polymer. FS has a roughly normally distributed curve, coming to a peak at around 200 *m/z* and spread between ~200 to 800 *m/z*, with a tail of high MW peaks above this. FV and AN have a somewhat negatively skewed distribution, with more higher MW fragments. There are also some notable differences between the most abundant peaks. The peak at 228 *m/z*, denoting a fucose monomer with 1 sulphate group (see Fig. 7), is significantly larger for FV and AN than for FS, while the peaks at 451 *m/z* (a dimer with a sulphate group removed) is larger for FS and FV than for AN. The proportion of these two peaks is correlated to the amount of sulphate in each species. As shown in Figs. 7, 228 *m/z* is likely to be a monomer with 1 sulphate group and 293 *m/z* is a monomer with 2 sulphate groups. The higher quantity of sulphate to fucose seen in FS is shown by a high quantity of 293 *m/z*, which is more sulphate rich and a smaller quantity 228 *m/z*, which is less sulphate rich. For FV and AN, where the sulphate content is less than fucose, the 228 *m/z* peak, with only one associated sulphate per fucose monomer, is significantly more abundant that the 293 *m/z* monomer. The biggest peaks for larger fragments differ in MW between species; another indicator for differing structures. Main peaks for a 4-chain of sulphated fucose (which would be expected at 1083 *m/z* for an ideal structure as in Fig. 1), occur at 1064 *m/z*, 1086 *m/z* and 1088 *m/z* for FS, FV and AN respectively, while a 3 chain, expected at 777/857 *m/z* dependant on sulphation, show at 777/908 *m/z*, 759/867 *m/z* and 764/927 *m/z* respectively.

LC-MS of standard fucoidan, shown in Fig. 5(d) shows the same somewhat negative skewed distribution between ~200 and 800 *m/z*

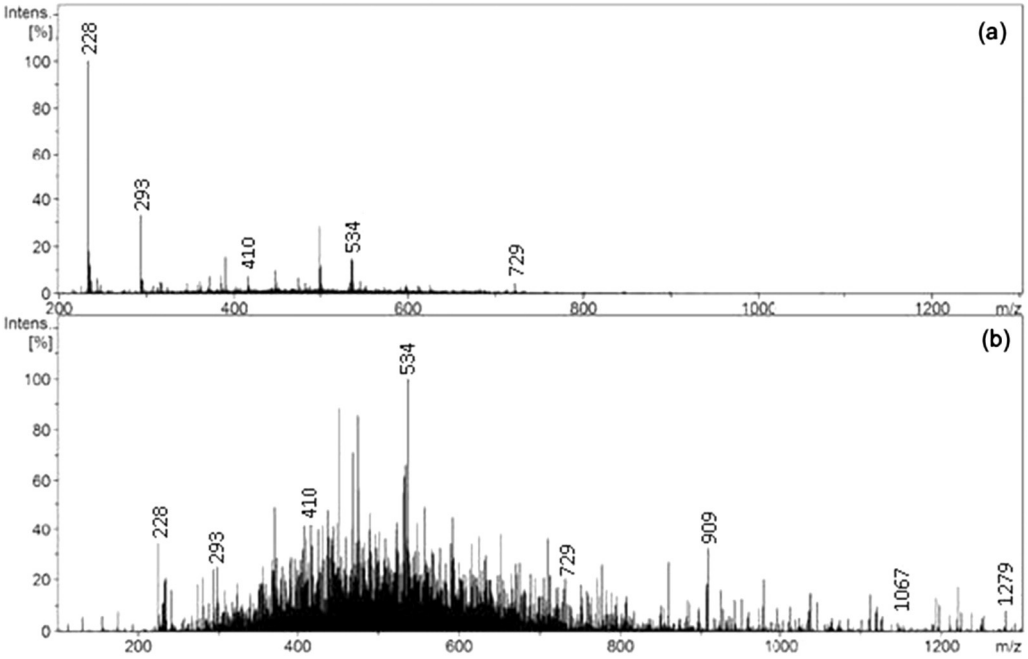

Fig. 6. LC-MS chromatogram for the May fucoidan extract of FS where (a) corresponds to 12.4 to 17 min from SEC (two small, secondary peaks) and (b) to 10–12.4 min (large main peak).

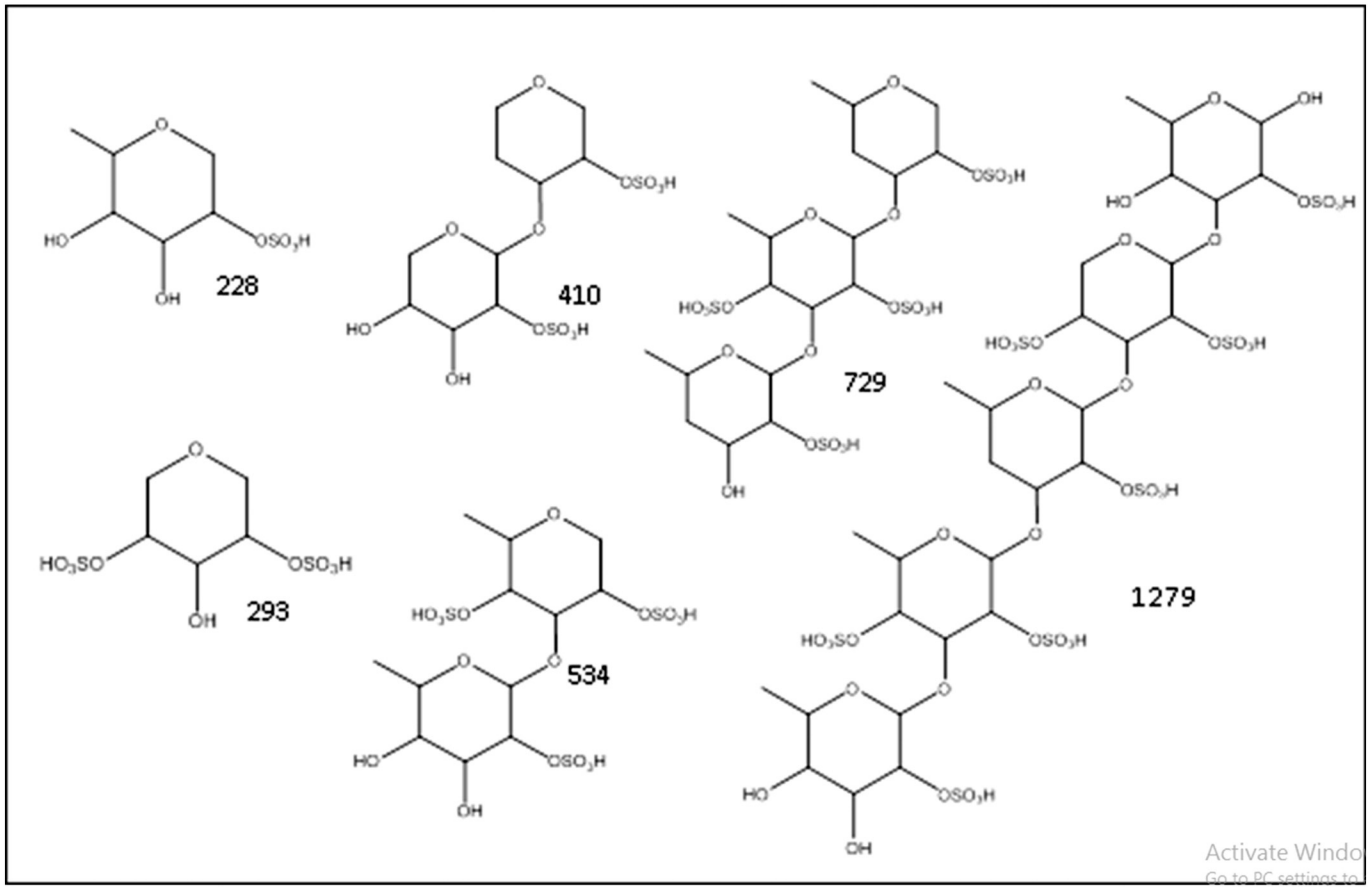

Fig. 7. Potential molecular structures for fragments found in LC-MS.

as seen for FV and AN. However, there are a few more pronounced peaks than seen for any of the extracted samples. These occur at 228, 293, 371, 451 and 534; fragments which are common and often giving the highest peaks across all samples. Differences between these fragments can be accounted for by a combination of a loss of hydroxide, methyl, sulphate or monomer units.

Fig. 6 shows the chromatogram for the May sample of FS, chosen as a representative sample, and lists the most common mass fragments for the two main peaks observed. Other than the most common five fragment ions identified in Fig. 7, each species gave slightly different fragments, evidencing the difference in structures between species, although these fragments were common for each species. In general, the difference between fragments can be attributed to the loss of a hydroxide, methyl or sulphate group, a monomer unit or a combination of these.

Comparing the LC-MS chromatogram for the main and secondary peaks, it is obvious that the main peak contains a far wider range of fragments of increasing mass. The maximum *m/z* possible with this instrument is 1300 *m/z*, but it is likely that there are fragments significantly larger than this. The two smaller peaks appear to either be fragments which have been created during the extraction process or oligomers, which have been pulled through the column by association with the larger fucoidan macromolecules. For either case, the presence of the fragments in both the main and secondary peaks infers they are from the same group of compounds and also associated with each other. The largest of these is 729 Da for FS, corresponding to a 3-fucose chain. For FV, the largest fraction in the secondary peaks is 1245; a 5-fucose chain and for AN, 829 Da; a 4-fucose chain. Possible structures for the most common fragments are given in Fig. 7. These clearly show the loss of hydroxyl, methyl and sulphate groups due to fragmentation in the mass spectrometer.

4. Conclusion

The seasonal variation of fucoidan from three species of brown macroalgae, harvested monthly from the coast of Aberystwyth in Wales, has been studied. The results show distinct differences in quantity and structure of the extracted fucoidans, both between species and for different months. SEC analysis gives an insight into the structural variation showing a more complex structure for FS along with a higher degree of sulphation. The variation of sulphate and fucose content has been studied in more depth: these are seen to vary in line with each other and follow in inverse trend to the total fucoidan content. Fucoidan content has been shown to vary over the year, with the highest content in the autumn.

While this study begins to understand the seasonal variation of the 3 species, there is still more research required to fully understand the complexity of variation. This would include similar studies on the same species harvested from different locations and the effect of the maturity of the species.

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