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Statistical analysis of the influence of extraction parameters on the extraction yields, extract and polysaccharide compositions and prebiotic activities of seaweed extracts from *Ascophyllum nodosum*

A thesis presented to the National University of Ireland for the degree of Doctor of Philosophy

by

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September 2013
"La science n'a pas de patrie, car la connaissance appartient à l'humanité, c'est un flambeau qui illumine le monde"

“Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world.”

Louis Pasteur, 14th of November 1888
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<tr>
<td>$</td>
<td>American dollar</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>μg/mL</td>
<td>Microgram per millilitre</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>1D</td>
<td>1-Dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>2-Dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>3-Dimensional</td>
</tr>
<tr>
<td>A. nodosum</td>
<td>Ascophyllum nodosum</td>
</tr>
<tr>
<td>AN</td>
<td>Ascophyllum nodosum</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>C. costata</td>
<td>Costaria costata</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>cfu/mL</td>
<td>Colony forming unit per millilitre</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>C-PAGE</td>
<td>Carbohydrate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>DB</td>
<td>Degree of branching</td>
</tr>
<tr>
<td>DM</td>
<td>Dry matter</td>
</tr>
<tr>
<td>DP</td>
<td>Degree of polymerisation</td>
</tr>
<tr>
<td>DW</td>
<td>Dry weight</td>
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<td>E.coli</td>
<td>Escherichia coli</td>
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<td>F. vesiculosus</td>
<td>Fucus vesiculosus</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organisation</td>
</tr>
<tr>
<td>Fe</td>
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<td>FOS</td>
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<td>Fucus vesiculosus</td>
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<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GOS</td>
<td>Galacto oligosaccharides</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
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<tr>
<td>H. elongata</td>
<td>Himantalia elongata</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HPAEC</td>
<td>High performance anion exchange chromatography</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>L. digitata</td>
<td>Laminaria digitata</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>Lactobacillus plantarum</td>
</tr>
<tr>
<td>LD</td>
<td>Laminaria digitata</td>
</tr>
<tr>
<td>M</td>
<td>Mole per litre</td>
</tr>
</tbody>
</table>
m  Meter
Mg  Magnesium
mg  Milligram
mg/mL  Milligram per millilitre
mg/mL/hr  Milligram per millilitre per hour
min  Minute
mL  Millilitre
mL/min  Millilitre per minute
mM  Millimole per liter
mm  millimetre
Mn  Manganese
mS/cm  Millisiemens per centimeter
MWCO  Molecular weight cut off
Na  Sodium
NaCO₃  Sodium carbonate
NaH₂PO₄  Sodium phosphate
NaOH  Sodium hydroxyde
nC*min  Nanocoulomb minute
Ni  Nickel
nm  Nanometer
NMR  Nuclear Magnetic Resonance
OD  Optical density
OGT  Oilean Glas Teoranta
P. pavonica  Padina pavonica
P. aeruginosa  Pseudomonas aeruginosa
PAD  Pulse amperometric detection
Pb  Lead
pm  Picometer
psi  Pounds per square inch
rpm  Revolutions per minute
RT  Room temperature
S. latissima  Saccharina latissima
Sc  Supernatant combined
sp  Species
t  Ton
TFA  Trifluoroacetic acid
UF  Ultrafiltration
UV  Ultra violet
V  Volume
w/v  Weight per volume
Zn  Zinc
α  Alpha
β  Beta
Δ  Delta
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Publications

Book chapters


Journal publications planned


2. Brebion J, Connan S, Hennequart F, Tuohy M.G (in progress) Experimental plan to assess the influence of key parameters in the extraction of phenolic compounds from Ascophyllum nodosum: yields and size partition.


Abstracts or Poster presentations

International meetings


National meetings


Abstract

Brown seaweeds are found in abundance along the Irish coastline and investigations of purification of specific compounds including polysaccharides are still increasing to develop new markets. A preliminary investigation of the differences obtained on the polysaccharide extraction from three brown species (*Fucus vesiculosus*, *Laminaria digitata* and *Ascophyllum nodosum*) had been initiated and the algae *A. nodosum* had been identified as the most suitable biomass for an industrial point of view. A full factorial experimental design was performed to define the influence of extraction parameters (time, temperature and the volume of extractant per gram of biomass: liquid/biomass ratio) on the main water soluble compounds extractable from industrially processed *A. nodosum*. Additionally, seasonality was also investigated by applying the experimental design on two seaweed batches harvested at different time points of the year. This statistical approach aimed to figure out the yields and the composition variations of one step aqueous extracts, to optimise the protocol to extract specific compounds (polysaccharides, phenolics…) and mathematical models were proposed to anticipate the extracted products. Moreover, extraction protocols had been shown to statistically affect the composition and size of the polysaccharide extracted (fucoidans, laminarans and alginates). Finally, the different extracts prepared were tested for their potential prebiotic activity. A preliminary screening of the ability of potentially pathogenic bacterial strains and probiotic strains to grow on seaweed extracts was achieved. Results on bioactivity were then correlated to the protocol used and, by extension to the extract composition (relative proportion of each component of the extract and composition of the polysaccharides). The possibility of scaling up the extraction was investigated in order to anticipate the production of the most promising extract to run a human clinical trial.
Chapter 1: General introduction
Chapter 1

1.1 General Overview: The context and objectives of this research

Ocean vegetation is dominated by the algae which are generally regarded as evolutionarily primitive plants. Seaweeds, also called kelps, are marine macroalgae that produce organic matter using the combination of solar energy, carbon dioxide and sea nutrients. Always macroscopic, the size of these algae ranges from few cm to over 60 m for the giant kelps. Seaweeds play a crucial role in coastal areas by maintaining the fragile physico-chemical equilibrium as well as providing shelter for many marine species; alive or cast ashore, seaweeds are also a source of food for many species of marine life (Lobban & Harrison 1994). The use of seaweeds by human kind is long-standing and quite wide and varied. The oldest records of an application for seaweeds dates back to 14,000 B.C, where seaweeds remains were excavated from a Shaman hut in Monte Verde in Chile, implying that it was used back then as food, or potentially even as a form of medicine (Dillehay et al. 2008).

Seaweeds have always been of economic importance. Human kind has used and exploited seaweed since time immemorial for purposes that range from use as a soil conditioner to use as a food source. For centuries certain cultures have used various types of seaweeds as natural remedies/medicines for a host of different illnesses. The nutritional value of marine macroalgae has long been recognized in the Orient, but in the western world the use of seaweeds for human consumption is limited; they exist as additives in so-called health foods. Seaweeds are low in fat and represent very complete sources of a range of macronutrients and key micronutrients, including vitamins and minerals (Wilkinson 1992). They also contain a large proportion of characteristic mucilaginous polysaccharides such as carrageenans, alginates, agars, laminarans, galactan sulphates and fucoidans (Popper et al. 2011). In scientific research the various pharmacological properties of algae have been well documented for a number of years (Stengel et al. 2011). It is only in the last few decades that the actual mechanisms of actions have come under scrutiny.

The west coast of Ireland is influenced by the Gulf Stream current, which sustains an abundance of macroalgal species (more than 500 species), in particular brown macro-algae (147 species) such as Ascophyllum nodosum, Fucus sp. and Laminaria sp. Traditional hand harvesting of many species of marine algae has been carried out on this coast for centuries. The main commercially harvested species is A. nodosum, which is dried and sold as a meal product or for alginate extraction, or the production of products for soil improvement and animal feeds. These primary products only sell for commodity prices in the range of $500-$800 per tonne. At present, there is little high-value processing of algae in Ireland even though, in the last ten years, a few companies (around 10 companies on the west part of
Ireland but the origin of the used is not always mentioned) have initiated production and commercialization of higher value products. Scientific estimates indicate that a sustainable annual harvest for *A. nodosum* of 75,000 tonnes is possible, which is more than double the current annual harvest of 35,000 tonnes (Morrissey et al. 2001). Hence, according to this rather old study, a doubling of the existing harvest could be accommodated without interfering with the sustainability of this resource.

Why are seaweeds good for us? What is the scientific basis for this declaration? From the work that will be presented in this thesis, scientific data documenting levels of one type of polysaccharide, fucoidan, within a seaweed species of commercial value, as well as its compositional analysis, will highlight the potential health benefits of an algal-rich diet. The research has also highlighted the value of *A. nodosum* as a source of extracts enriched in fucoidan and other polysaccharides (alginites, laminarans) and non-carbohydrate biomolecules (including phenolic substances) with potential prebiotic and selective antimicrobial properties.

“Let food be thy medicine, let medicine be thy food”, said Hippocrates. While this principle remains true and relevant, it has been complicated by the intransigence of dietary habits in Western society, but facilitated by technological advances permitting incorporation of an enriched form of the ‘medicine’, into food product(s). This approach to integrate a ‘medicine’ into food is more convenient in terms of preparation, consistency of supply, palatability, acceptability, benefit, shelf-life and profit (Sako et al. 1999). In contemporary society, Hippocrates’ dogma is described by ‘functional food’. Functional foods have a variety of physiologically beneficial effects (Gibson & Roberfroid 1995). One such benefit involves the use of prebiotic compounds that are a natural constituent of, or a pre-formulated additive to, staple dietary foodstuffs.

The impetus behind this project was to investigate the composition and function of extracts from a brown algal source of commercial importance in Ireland, in order to have scientific evidence to support claims for potential human health benefits. The working hypothesis for the research was that these brown algae could be a source of novel aqueous extracts and enriched polysaccharide fractions with potential to modulate positively the growth of beneficial gut bacteria (i.e. have prebiotic potential). To test this working hypothesis, the first experimental approaches consisted of selecting the most relevant seaweed species for polysaccharide extraction and investigating different polysaccharide purification techniques (Chapter 3). Once selected, the effect of extraction parameters was
investigated on one species (*A. nodosum*) to establish their effects on extraction yields (important scientifically and for sustainability at industrial scale), extract composition and the yields and composition of specific polysaccharides (Chapters 4 and 5). The findings also revealed new aspects on the real seasonal abundance of fucoidan and laminaran (and alginates) in *A. nodosum* which highlights the value and sustainability of using this seaweed as a source of these polysaccharides. Finally, screening of different extracts to investigate potential prebiotic activity was performed (Chapter 6). The value of the bioactivity tests was underpinned by correlating activity to composition and extraction method in this study (Chapter 6). A novel, reproducible and scalable aqueous-based extraction method was developed based on the results of laboratory research (in Chapters 3 to 5), which was subsequently used to produce sufficient quantities of the best candidate ‘prebiotic’ extract, identified from research presented in Chapter 6, for an on-going clinical trial (dietary intervention study).

1.2 Seaweeds and Macroalgae

1.2.1 Types and classification of Seaweeds

Three main categories of seaweeds are generally described, classified by the predominant colour of the category, as green, red and brown seaweeds (Dring 1991). In the classical five-kingdom classification system (*Figure 1.1*), macro-algae have been assigned to the kingdom Protista. In this kingdom, algae have been grouped for convenience into three divisions (phyla), i.e. Rhodophyta (red seaweeds), Phaeophyta (brown seaweeds), Chlorophyta (green seaweeds), largely on the basis of colour, although they mainly differ with regard to their reserve and cell wall polysaccharides. In an earlier classification of algae

![Classification of macroalgae under the kingdom Protista, and classification of Ascophyllum nodosum, Fucus vesiculosus (fucales) and Laminaria digitata (laminariiales) (Descombes 2006)](image)

*Figure 1.1* Classification of macroalgae under the kingdom Protista, and classification of Ascophyllum nodosum, Fucus vesiculosus (fucales) and Laminaria digitata (laminariiales) (Descombes 2006)
a fourth phylum existed, the Cyanobacteria (blue-green algae), which has been subsequently re-classified as cyanobacteria (Kingdom Monera). The brown macro-algal investigated as a source of bioactive polysaccharides, i.e. *Ascophyllum nodosum* and *Fucus vesiculosus* belong to the other order Fucales, family *Fucaceae*, whereas *Laminaria digitata* belongs to the order Laminariales, family *Laminariaceae*.

### 1.2.2 Division Phaeophyta (Brown Algae)

The division Phaeophyta constitutes an important assemblage of marine algae, classified into about 265 genera and consisting of over 1500 species (Bold et al. 1978). Although originally regarded as plants, more recent research has re-classified the brown algae as Strameophiles, a distinct evolutionary lineage from land plants. The Phaeophyta occur mainly in the marine environment, where they are a conspicuous and predominant intertidal component, extending from the upper littoral zone into the sublittoral zone. The Phaeophyta appear to flourish in temperate to subpolar regions where they exhibit the greatest diversity with regard to species and morphological expression (Davis et al. 2003). Many forms, in particular members of the order Fucales, withstand long periods of daily immersion during high tide.

The cell and cell wall composition are also biological features used to define seaweed classification. Indeed, the divisions are defined according to the colour resulting from the pigment composition. Moreover, cell wall polysaccharides are specific for each division as well as storage polysaccharides. The main seaweed compounds, by category, are summarized in *Table 1.1*. All seaweeds contain chlorophyll but in different proportions (higher content in green algae); the brown colour of the Phaeophyta is due to a high content of carotenoids (fucoxanthin).

*Table 1.1 Cell wall and structural carbohydrates and pigments in the macroalgal divisions* (Davis et al. 2003)
1.2.3 Fucaceae

In the Fucaceae family, only 24 species have been identified to date, including 15 species under the genus *Fucus* (for example *Fucus vesiculosus*) and only one in the genus *Ascophyllum* (*Ascophyllum nodosum*). The growth rate of the *Fucaceae* is very low with many variations according to the localisation. Along the French coast, Lemoine (1913) recorded a growth-rate for *F. vesiculosus* of between 2.5 and 3.3 cm per month and a growth rate of 1.6 cm per month was recorded in England (Knight & Parke 1950). The same study showed an effect of water salinity and sun exposure on the growth rate inducing a lower growth rate during winter (Knight & Parke 1950). Moreover, the natural habitat of the *Fucaceae* is located in the intertidal zone. Consequently, the seaweeds are exposed to two different environments twice a day: air and water. Thus, the *Fucaceae* have generally a higher content of antioxidants (like phenolic substances and pigments) that are thought to help these seaweeds withstand abiotic stresses such as UV radiation and air oxidation (Stengel et al. 2011). *Ascophyllum nodosum* (main species studied in this work) forms a single bladder centrally in long, strap-like fronds. The fronds hang downwards, gently draping sheltered intertidal rocks. A number of fronds grow from each basal holdfast, and the plant generally regenerates new fronds from the base when one of the larger fronds are damaged. Reproduction takes place in beautiful yellow receptacles in spring. These develop in response to short days in the autumn, mature during the winter, and are at their most prolific in spring. Eggs and sperm are released into the water, and the eggs release a low molecular weight sperm attractant known as Finnavarene, named for the village of Finavarra (“wood by the sea”) in north Co. Clare in Ireland.

![Figure 1.2 A) Fucus vesiculosus and B) Ascophyllum nodosum (this research)](image-url)
1.2.4 Laminariaceae

The *Laminariaceae* family contains more species than the *Fucaceae* with 61 species including 26 in the *Laminaria* genus (for example *Laminaria digitata*) and 21 in the *Saccharina* genus (e.g. *Saccharina latissima*). In contrast to the *Fucaceae*, the *Laminariaceae* tend to live in deeper water and are rarely exposed to the air environment thus reducing the exposure to light, especially during winter. Nevertheless, growth of *Laminariaceae* is much faster than *Fucaceae*, especially during winter and spring. During those seasons, the seaweeds can grow up to 1.5 cm per day, although in the winter and autumn periods the growth is much lower (around 0.5 cm per day) (Parke 1948). Consequently, although the same types of compounds are found in the two families, the relative proportions of each are different.

![Figure 1.3 Laminaria digitata at low tide (this research)](image)

A recent study shows a worrying fact in relation to the *Laminaria digitata* biomass along the French coast, in that it has decreased significantly due to the increase in coastal water temperatures; this change is likely to have an important impact on the ecosystem (Raybaud et al. 2013).

1.3 Main compounds in brown algae

1.3.1 Low molecular weight compounds

Without considering the metabolic products in seaweeds, low molecular weight carbohydrates (focusing on free monosaccharides) are present and specific to each main group of seaweed. While some of these materials account for a considerable proportion of
the dry weight of the algae, others are present only in trace quantities. In brown seaweeds, D-mannitol is universally present and can reach 25% of the dry matter of some *Laminaria* species in autumn. Another seven carbon sugar alcohol, Volemitol, has been identified in *Pelvetia canaliculata* (Pfetzing et al. 2000). Furthermore, mannitol-glucopyranosides have been found in all brown seaweeds and methylinositol and laminitol were identified previously in *Laminaria hyperborean* (Percival & McDowell 1968). Finally, trace quantities of sucrose, and free galactose and mannose have been also reported (Percival & McDowell 1968).

Due to their living environment, salty water and growth on rocks, brown seaweeds are particularly rich in different micronutrients and especially different divalent cations (Fe$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, Pb$^{2+}$, Ni$^{2+}$, etc.). Furthermore, the habitat close to the coastline is also exposed to heavy metal contamination by the disposal of industrial waste into the sea. The natural metal distribution in seaweed harvested from a clean site (heavy metal in sea water below the limit of detection) revealed mainly an intracellular location of the salts (Ryan et al. 2012). Moreover, divalent cations can bind anionic structures such as alginates and fucoidans. Indeed, this binding of cations has been shown to depend on the type of polysaccharide (Giusti 2001).

### 1.3.2 Alginates

Alginates still remain the principle components derived from commercial seaweed extracts, and are used as texture agents (thickener) and stabilizers in the food industry. In 2009, 35 000 tons of alginates were produced worldwide with the largest production based in Asia at 15 500 t (10 000 t in Europe). Different seaweed species are used for the extraction of this hydrocolloid, but the main species globally are the *Laminariales*, which are abundant in Asia (account for 81% of the total production). The use of *A. nodosum* for alginate extraction was, in 2009, only 2% of the worldwide production (against 16% in 1999) (Bixler & Porse 2010).

In terms of seaweed composition, alginates are the main component of the cell walls and may constitute up to 50% of the dry weight. They are localized both in the cell wall as well as in the intercellular spaces. Alginates play important roles both structurally and for ion-exchange purposes *in vivo*, as well as functioning in retarding desiccation when the algae are exposed to air (Kloareg & Quatrano 1988). Alginate is actually a linear co-polymer composed of two monomeric C6 acidic sugars, which are C5 epimers of each other: β-D-mannuronate (M) and α-L-guluronate (G) with interconnecting (α-1, 4) linkage (*Figure 1.4*). The orientation of the carboxyl group is above the plane of the ring in the M unit and below the plane in the G unit (Wong et al. 2000). Three different structures have been identified in
the alginates according to the monomer sequence: homopolymeric M blocks, homopolymeric G blocks and heteropolymeric M/G blocks (randomly arranged M and G sequence) (Haug et al. 1967). The molecular weight of alginate ranges generally between 100 and 200 kDa. Its solubility is influenced by factors such as pH, concentration, ions in solution and the presence of divalent ions (Rioux et al. 2007).

1.3.3 Sulphated fucans and fucoidan

Sulphated polysaccharides are another type of structural polysaccharide that differs in composition from phylum to phylum. Sulphated polysaccharides are widespread in nature, occurring in a great variety of organisms. They are indeed universal in marine algae but not found in land plants, although they occur in animal tissues such as sea cucumber (Ribeiro et al. 1994) or sea urchin (Mulloy et al. 1994). Their physiological roles are incompletely understood, but may include electrolyte homeostasis, because of their anionic nature and hygroscopic properties that may prevent from dehydration upon long exposure which would explain the higher fucoidan content reported in Fucaceae (Percival & McDowell 1968). Some studies have shown a correlation between fucoidan content and the depth at which brown algae grow. It has been suggested that the closer algae are to the surface, the greater the fucoidan content (Evans 1989). Furthermore, fucoidans appear to play a role in the organization of the algal cell wall, and may also be involved in the morphogenesis of algae.

Figure 1.4 A) Chemical structure of β-D-mannuronic acid (M) and α-L-guluronic acid (G) and B) example of GGMM sequence (Vauchel 2007)
embryos, as well as being involved in the cross-linking of alginate and cellulose (Davis et al. 2003). The functions of sulphated polysaccharides actually strongly depend on the presence and spatial positioning of their sulphate groups. While glucoaminoglucans and mucins would be the most prevalent polyanionic compounds in animal tissues, fucoidan and carrageenan are representative of the algae and more specifically of seaweeds (macroalgae). The major sulphated polysaccharides of brown seaweeds are fucose-rich.

Fucoidan was first named fucoidin when it was first isolated by Kylin in 1913. Now, it is known as fucoidan according to IUPAC (International Union of Pure and Applied Chemistry) rules (‘an’ referring to the fact that it is a glycan). Fucoidan structure and composition are still not well defined due to its complexity and the different composition between the species (Li et al. 2008). The first model for the structure of fucoidan, from F. vesiculosus (source of commercial fucoidan), was described by Percival and Ross in 1950. It was based on a L-fucose polymer with mainly α-1,2 glycosidic bonds with sulphate groups at C4 of fucose units, and branches of sulphated fucose every 5 units. This model was the sole one for 40 years. In 1993, Patankar and colleagues reinvestigated the structure fucoidan from F. vesiculosus. The position of sulphate was confirmed to be at the C4 position but the glycosidic bonds were shown to be α-1,3 instead of the α-1,2 linkage described previously (Patankar et al. 1993). Moreover, this study described the branch points to occur every 2-3 fucose units (Figure 1.5) in contrast to their proposed occurrence every the 5 units, as described by Percival and Ross in 1980. According to Patankar et al (1993), this difference can be explained most likely by the different extraction protocols used. Indeed, fucoidan extracted by Percival and Ross involved an acid extraction, whereas that used by Patankar and colleagues was based on a water extraction.

More recently, structural studies of fucoidan have been conducted on different seaweed species and widely reviewed (Holtkamp et al. 2009; Ale et al. 2011; Ale & Meyer 2013; Li et al. 2008; Wang 2011). Once again, different structures have been reported for the

Figure 1.5 Patankar et al model of Fucus vesiculosus fucoidan (Patankar et al. 1993)
same species. For example, Chevolot et al. (2001) described an *A. nodosum* fucoidan with a backbone possessing a large proportion of both α-1,3 and α-1,4 glycosidic bonds. The *A. nodosum* fucoidan structure described by Marais and Joseleau was slightly different. Their fucoidan extract consisted of a highly branched backbone with α-1,3 as the main linkage and a few of α-1,4 linkages (Marais & Joseleau 2001). Fucoidan composition has been shown to change according to the species (Percival & McDowell 1968), the extraction process (Mabeau et al. 1990), as well as the season of harvest and localisation (Honya et al. 1999). In those conditions, a lot of different structures have been reported for fucoidan fractions to date, which are summarised in Table 1.2.

**Table 1.2 Chemical composition of some fucoidans (Li et al. 2008)**

<table>
<thead>
<tr>
<th>Brown seaweed</th>
<th>Chemical composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. vesiculosus</em></td>
<td>fucose, sulfate</td>
</tr>
<tr>
<td><em>F. evanescens</em></td>
<td>fucose/sulfate/acetate (1/1.23/0.36)</td>
</tr>
<tr>
<td><em>F. distichus</em></td>
<td>fucose/sulfate/acetate (1/1.21/0.68)</td>
</tr>
<tr>
<td><em>F. serrata</em></td>
<td>fucose/sulfate/acetate (1/1.01)</td>
</tr>
<tr>
<td><em>Lessonia vadosa</em></td>
<td>fucose/sulfate/acetate (1/1.12)</td>
</tr>
<tr>
<td>Macrocystis pyrifera</td>
<td>fucose/galactose (18/1), sulfate</td>
</tr>
<tr>
<td>Pelvetia cunicularia</td>
<td>fucose/galactose (10/1), sulfate</td>
</tr>
<tr>
<td>Udadia pinnatifida (Mekabu)</td>
<td>fucose/galactose (1/1.1), sulfate</td>
</tr>
<tr>
<td>Ascophyllum nodosum</td>
<td>fucose(9%), xylose(10%), GlcA(11%), sulfate</td>
</tr>
<tr>
<td>Himanthalia loeoo and Bifurcaria bifurcata</td>
<td>fucose, xylose, GlcA, sulfate</td>
</tr>
<tr>
<td>Padina pavonica</td>
<td>fucose, xylose, mannose, glucose, galactose, sulfate</td>
</tr>
<tr>
<td>Laminaria angustata</td>
<td>fucose/galactose/sulfate (9/1/9)</td>
</tr>
<tr>
<td>Ecklonia kurome</td>
<td>fucose, galactose, mannose, xylose, GlcA, sulfate</td>
</tr>
<tr>
<td>Sargassum sterophyllum</td>
<td>fucose, galactose, mannose, GlcA, glucose, xylose, sulfate</td>
</tr>
<tr>
<td>Adenocystis articulata</td>
<td>fucose, galactose, mannose, sulfate</td>
</tr>
<tr>
<td>Hizikia fusiforme</td>
<td>fucose, galactose, mannose, xylose, GlcA, sulfate</td>
</tr>
<tr>
<td>Dictyota menstrualis</td>
<td>fucose/xylose/trinic acid/galactose/sulfate (1/0.8/0.7/0.8/0.4) and (1/0.3/0.4/1.5/1.3)</td>
</tr>
<tr>
<td>Spatoglossum schroederi</td>
<td>fucose/xylose/galactose/sulfate (1/0.5/2/2)</td>
</tr>
</tbody>
</table>

### 1.3.4 Laminaran

Laminaran is a food storage polysaccharide in brown algae and was first isolated by Schmiedeberg in 1885. The laminaran content ranges from less than 2 to 34% of the algal dry weight depending on the species of brown algae. In *A. nodosum* the percentage of laminaran ranges from 1 to 7%, whereas laminaran levels in *Laminaria* sp. vary from 0.75 to 27.5% on a dry weight basis (Manners et al. 1973). Its structure is simpler than fucoidan and has been elucidated (*Figure 1.6*). The majority of laminarans contain polymeric chains of
two types: one type is built only of glucopyranose \((\text{Glc}p)\) residues (G-chains), whereas the other type is terminated with \(1-O\)-substituted D-mannitol residues (M-chains). ‘Non-reducing’ laminarans have been identified that contain terminal mannitol groups. Typically laminaran has been shown to possess between 40 and 75\% of M-chains (Percival & McDowell 1968). Differences in laminaran size, structure and composition have been identified in fractions extracted from various algal sources. The molecular distribution tends to vary with age, with more mature plants producing high molecular weight laminarans (Zvyagintseva et al. 2003). Interspecies variations may include differences in the G:M ratio (in some cases M-chains may not exist, as noted in laminaran from *Desmarestia firma* (Carlberg et al. 1978)), in the degree of polymerization (DP; from 25 up to 50 residues, with the average being ~33) and the degree of branching (Kim et al. 2000) (DB). The typical molecular mass distribution of laminaran is from 5–20 kDa (Stone, B; Clarke 1992). However, the molecular mass distribution can be as high as 40 kDa. The ratio of \(\beta\)-1,3 to \(\beta\)-1,6-linked glucose residues varies from 2:1 to 9:1, with the \(\beta\)-1,6 links being randomly distributed along the \(\beta\)-1,3 backbone (Mueller et al. 2000). Seasonal/species variations affecting the structure and composition of the marine algal polysaccharides are important considerations when harvesting the algae for industrial and scientific research purposes.

Two different types of laminaran are distinguished according to their solubility in cold water (both are soluble in hot water). Soluble and insoluble laminarans can be present in certain brown algal species. Differences have been observed between these two fractions and are well summarised by Percival (Percival & McDowell 1968). The average number of branching is higher for the soluble form (0.3 branches per molecule for the insoluble form and 1.6 for soluble one). The higher degree of branching can explain the greater solubility by the presence of more hydrogen bonds between the individual laminaran molecules and water molecules. Moreover, the soluble form contains, on average, more mannitol units than the insoluble form (2.7\% and 1.7\% respectively) (Percival & McDowell 1968).

![Schematic representation of the β-1,3-linkages between glucose units in laminaran (Kelly 2005)](image-url)
1.3.5 Phenolic compounds

 Phenolics compounds in seaweeds (or macroalgae) are referred as phlorotannins, since most of them are polymers of phloroglucinol (1,3,5-trihydrobenzene), are secondary metabolites and are not directly involved in primary metabolic processes like photosynthesis, cell reproduction and division (Ragan & Glombitza 1986). The monomeric phloroglucinol units are linked by aryl-aryl bonds to form groups of fucols (Figure 1.7).

![Figure 1.7 Chemical structures of A) phloroglucinol and B) an example of a fucol (Koivikko 2008)](image)

Other kinds of polyphenols (condensed phlorotannins are formed also from phloroglucinol but with different linkages: e.g phlorethols formed by arylether bonds) (Koivikko 2008). Phenolic content differs significantly between seaweed species and is related to the seaweed habitat (Connan et al. 2004). Indeed, species living in the intertidal zone (higher exposure to air and light) have a much higher phenolic content (more than 5% of the seaweed dry weight for F. vesiculosus and A. nodosum) than species living in the subtidal area (<1% in average in L. digitata) (Connan et al. 2004). This statement is reinforced by the seasonal variation of the proportion of phlorotannins in the seaweed. In all species, including L. digitata, the phenolic concentration is significantly higher in summer, when the days are longer. Phenolic content can reach up to 20% of seaweed dry weight in some species (Targett & Boettcher 1995). These polyphenols can have molecular weights ranging from 320 to 420,000 Da in A. nodosum and include fucols, phlorethols, fucophlorethols, eckols as well as halogenated compounds such as bromophenols (Glombitza & Grobe-Damhues 1985).
1.4 Extraction processes for recovery of seaweed biomolecules

Application of the most appropriate extraction process(es) is a very important consideration in approaches to determine the structures, the types, diversity and seasonal variation of the biochemical compounds and biomolecules in seaweed. The development and optimisation of extraction protocols is a difficult task. Indeed, the ideal extraction process should allow maximum extraction of the desired compounds without affecting (or at least having minimum effect on) the native structure of the target compounds.

1.4.1 Polysaccharide extraction

The extraction of enriched or even purified polysaccharides from seaweeds, in a form that resembles their native composition and structure, still remains a significant challenge, and is especially important yet challenging for the extraction of fucoidans. Many different extraction protocols have been tested on different seaweed species for the extraction of polysaccharides (including fucoidan), often resulting in different extraction yields and frequently yielding different final products in terms of composition and size. Marcel Tudor Ale and Anne. Meyer recently published (2013) a comprehensive summary of the history of fucoidan extraction between 1913 (first extraction by Killing) and 1952.

Table 1.3 Development of fucoidan extraction methodology from 1910 to 1952 (Ale & Meyer 2013)

<table>
<thead>
<tr>
<th>Year</th>
<th>Brown Seaweed sp.</th>
<th>Reported PCSPs Composition</th>
<th>Extraction Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1913</td>
<td>Laminaria and Fucus</td>
<td>Fucoidan contaminated with mannitol, alginate and laminaran</td>
<td>Dilute acetic acid extraction</td>
</tr>
<tr>
<td>1915</td>
<td>Laminaria digitata</td>
<td>Fucoidan contained methylpentose, l-fucose, and some pentoses</td>
<td>Dilute acetic acid extraction</td>
</tr>
<tr>
<td>1915</td>
<td>Macrocystis pyrifera</td>
<td>Dominantly alginic acid, with fucose-sulfate</td>
<td>Seaweed was soaked in 2% Na₂CO₃ for 24 h, filtered, HCl was added, the precipitate filtered, and then resolved in 2% Na₂CO₃</td>
</tr>
<tr>
<td>1931</td>
<td>Laminaria digitata</td>
<td>Substantial amounts of calcium sulfate and uronic acid</td>
<td>Soaking of seaweed in water, precipitation of crude sulfate by ethanol</td>
</tr>
<tr>
<td>1931</td>
<td>Macrocystis pyrifera</td>
<td>Methylpentose monosulphate polymer with mainly fucose and alginate contaminants</td>
<td>Repeated extraction with 2% HCl at room temperature for 48 h, precipitated with 90% EtOH, Prepared by precipitating the droplets exuded from seaweed in boiling ethanol</td>
</tr>
<tr>
<td>1937</td>
<td>Laminaria digitata</td>
<td>Sulfate residue must be substituted by fucose or another sugar residue</td>
<td>Acid pH 2.4-2.6, 70°C for 1 h, 3-times; or aqueous at 100°C for 24 h, lead acetate treatment, barium hydroxide addition</td>
</tr>
<tr>
<td>1950</td>
<td>Fucus vesiculosus, Fucus spiralis, Himanthalia lorea, Laminaria clustoni</td>
<td>Substantial amount of fucose and sulfate; and small amounts of uronic acid, galactose and xylose; ash was mainly calcium sulfate, Fucose, ash, sulfate</td>
<td>pH 2.2-2.5, 70°C for 1 h, 3-times</td>
</tr>
<tr>
<td>1952</td>
<td>Fucus vesiculosus</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
(extraction by Black, which is still the reference method because the main commercial source is extracted using this method) which is presented in Table 1.3. The first fucoidan was extracted using dilute acetic acid by Killing in 1913 and was reported to contain mannitol, laminaran and alginic acid. Further investigation was necessary to define those compounds as contaminants and not as component parts of fucoidan. Acid extraction remained the only extraction protocol that was used for some time afterwards, with the variation between the methods residing in the nature of the acid and the concentration used. Finally, in 1952, Black defined a multi-step extraction protocol with three successive extraction steps using 1 M HCl solution at pH 2-2.5 for 1 hour at 70°C. The extracted product contained fucose, sulphate and ash; the composition of the commercial source currently available consists of 44.1% fucose, 26.3% sulphates and 31.1% ash (Nishino et al. 1994). More recently, alternative protocols have been developed using aqueous extraction solutions, sometimes enriched (or not) with calcium chloride or sodium chloride, with extractions applied at different temperature and for different durations. The resulting products are very heterogeneous between the extraction methods, and even for one method applied to the same species. Indeed, it is very difficult to compare the techniques and methodologies due to the combination of variations between the species (Holtkamp et al. 2009), the seasonal timepoint (Rioux et al. 2009), the environmental localisation (O’Connell 2008) and the extraction protocol used (Ale & Meyer 2013).

Concerning the extraction of alginates, these polysaccharides have been commercialised for many years, so a reference method have been established to optimise the yields (Figure 1.8). Basically, seaweeds are washed with mineral acid (usually HCl) to remove counterions (Ca\(^{2+}\), Mg\(^{2+}\), etc.). Then, alginic acid is neutralised by alkali solution (KOH, NaOH or Na\(_2\)CO\(_3\)) to form sodium alginate. After centrifugation to remove residual
solids (residual seaweed biomass), the sodium alginates are precipitated using acid or CaCl$_2$ to form alginic acid or calcium alginates, respectively (both are insoluble forms of alginates). Finally, the pellet is resuspended using sodium carbonate (Pawar & Edgar 2012).

Finally, the extraction of laminaran probably involves the most basic methodology. Indeed, laminaran is probably the most soluble polysaccharide present in brown seaweed. A water extraction at room temperature (RT) is usually sufficient to extract the soluble form of laminaran as explained in Section 1.3.4. A hot extraction process allows the extraction of the two forms of laminaran, one of which will precipitate when the solution cools down (Elyakova et al. 1995).

1.4.2 Extraction of Phenolic Substances

Since there is no single protocol for extracting tannins from all plant materials, the procedures used for tannins are widely variable (Hagerman 1988). The solvents most commonly used to extract phlorotannins from *F. vesiculosus* have been aqueous mixtures of ethanol and acetone (Ragan & Glombitza 1986). A well-known feature of free phlorotannins is that they are prone to rapid oxidation. In many studies where individual phlorotannins are isolated, extracted phlorotannins are treated with an acetic anhydride-pyridine mixture (Glombitza & Pauli 2003), potassium disulphite (Keusgen & Glombitza 1997) or ascorbic acid (Shuyun & Jay-Allemand 1991) to protect them from oxidation. More recently, an extraction method has been developed that uses methanol/water (60:40 v/v) as solvent to extract total phenolic compounds from fresh seaweed, after grinding or pulverising the biomass in liquid nitrogen (Connan et al. 2004) (Queguineur et al. 2012). In this latter method, phenolics are extracted twice two 2 h incubations at 40°C in the dark. The extracts are then dried and kept at -20°C until analysed.

1.5 Bioactivity of seaweed extracts and purified seaweed compounds

1.5.1 Seaweed and seaweed extract uses

The use of seaweeds is quite widespread and diverse and the oldest records of an application for seaweeds dated back to 14,000 BC; seaweed remains have been excavated from a Shaman hut in Monte Verde in Chile, implying that it was even back then used as food or even a form of natural medicine (Dillehay et al. 2008).

One of the main applications developed worldwide for the seaweed resource is in horticulture for the production of plant/fruit/vegetable, where the seaweeds or seaweed
extracts are used as soils conditioners, mineral sinks and more recently plant biostimulants. Cast seaweeds have been harvested on the beaches all over the west coasts of European countries (Scotland, Ireland, France, Spain, etc.), generally to be mixed with sand to provide soil amendments for use by local farmers. Products based on cut and dried seaweeds have consequently been developed for application in other non-coastal areas. Some companies based especially in Scotland, started to produce liquid extracts (Chapman & Chapman 1980). The main seaweed species used in these types of horticultural applications are *A. nodosum*, *Laminaria sp. Ecklonia maxima*, *Macrocystis pyriphera*, and *Lessonia species* (McHugh 2003).

Food is another main application area for seaweeds. On the Asian market only, the value of the seaweed-based food sector is worth about US$6 billion per year. The main uses are for production of sushi and sea vegetables, which can be fresh or included in soups (Tseng & Fei 1987). In Western countries, seaweeds have also been used in foods but are more related to famine periods and therefore carry a pejorative image. In fact, the fungus (*Phytophthora infestans*) associated with potato blight in Ireland in this period is thought to have originally been an endosymbiont of seaweeds that were used in this period to fertilize poor soil in coastal areas, which further reinforces the negative image of seaweed in this context. One of the main commercial use of seaweeds for some time has been their use as raw materials for colloid production; colloids are structural polysaccharides like alginates, agars and carrageenans extracted from seaweeds, and used as stabilizers and/or thickening agents in many market sectors ranging from food, drinks, to paints and cosmetics (Bixler & Porse 2010).

Cosmetic and Spa products are another growing sector where seaweeds are used more and more, either in whole form or extracted in water or relevant solvents for cosmetic applications. The majority of these products, however, containing low amounts of raw material and therefore, this sector in reality remains a niche market (McHugh 2003).

The use of seaweeds in the production of natural feed and food stuffs for health of animals and humans is another new and high-value niche market. Some functional properties of seaweed extracts or specific seaweed compounds such as fucoidans and laminarans have been described, and include their recognition as strong natural bioactives, with immuno-stimulating, antiviral and in some cases, anticancer activities (Holdt & Kraan 2011). A few companies, especially in Asia, are currently developing functional foods and drinks using some of these seaweed compounds.
1.5.2 Alginates

Alginates are hydrocolloids with many applications in the food, pharmaceutical, cosmetics and biotechnology industries, where they are used as gelling agents, thickeners or stabilizing and emulsifying agents (Hernandez-Carmona et al. 2000). As mentioned before, the use of alginates as a thickener as a food ingredient remains the main commercial market for seaweed extracts. But alginates have been shown to have interesting bioactive properties and health benefits also based on their gelling properties. Indeed, sodium alginates, by action of gastric acid, are turned to insoluble alginic acid and form a barrier on the stomach surface (Hampson et al. 2005). This gelatinous layer can act as a rate-limiting barrier for drug release as it retards drug efflux and water influx, and prevents matrix disintegration. If the drug is water-soluble it will dissolve as the matrix hydrates and diffuse out of the alginate gel layer (Dettmar et al. 2011). Moreover, alginates have been shown to have an effect on glycaemia with the hypothesis that alginates act as an inhibitor of amylase. Studies have shown that they have potential also in cholesterol management; the presence of alginates in the intestine decreases the absorption of fats which consequently reduces the plasma cholesterol levels (Brownlee et al. 2005). Alginates are also finding increasing use in new hydrogels for chronic wound management, where it has been suggested they have both an antibacterial function and can stimulate wound healing. Alginates are also used in dental applications in the production of pastes for making teeth imprints, for example (Srivastava et al. 2012).

1.5.3 Laminarans

Different bioactivities have been identified for laminarans. Immunomodulatory effects have been shown on macrophages from the anterior kidney in salmon (Dalmo & Seljelid 1995). Indeed, cells treated with laminaran showed different morphological characteristic and the production of superoxide anions were enhanced which induced a higher activity of some enzymes, especially acid phosphatase. Furthermore, an *in vivo* study on cod showed that labelled-laminaran, after intravenous administration, accumulated in the heart, spleen and kidneys (Dalmo et al. 1996). In plants, laminarans have been identified to be a strong elicitor of defence responses. Different sizes of laminaran have been tested and the smallest active fraction was shown to be laminaripentaose (Klarzynski et al. 2000). In previous collaborative work done in the DNA Damage Response group (Carty laboratory; Ryan 2007)) and Tuohy laboratory (Kelly 2005; O’Sullivan 2008), NUI Galway, laminaran from *A. nodosum* was shown to modulate bone cell function (unpublished work – IP in progress).
1.5.4 Fucoidans

The list of bioactivities suggested for fucoidans have been and are still widely investigated, although much of the work to-date suffers from a lack of characterisation of the extracts tested and limited information on the extraction procedures used. Indeed, bioactivity studies have been mainly carried out on crude fucoidan preparations, making the relationship between activity and composition difficult to ascertain (Li et al. 2008).

Nevertheless, many different activities have been identified and the anticoagulant property of fucoidan is by far the most widely studied. Different researchers have compared the anticoagulant activities of fucoidans extracted from different brown seaweed species against heparin as a control (Nishino & Nagumo 1987) (Cumashi et al. 2007). Through different experiments, Nishino et al. (1987) investigated the relationship between anticoagulant activity and the degree of sulphation of fucoidans and concluded from their findings that the sulphate esters are important for this activity, but an over-sulphated fucoidan has a lower activity. Fucoidan size is also an important parameter and different results have been observed. A low molecular weight fucoidan (MW = 3,090 Da) showed in vitro anticoagulant activity (Chevolot et al. 2001). Another study on Lessonia vadosa fucoidan showed that the native fucoidan (MW = 320 kDa) gave much higher activity than a depolymerized fraction (MW = 32 kDa) (Chandia & Matsuhiro 2008).

The antiviral activity of sulphated fucans was also identified with no direct effect being observed on the virus itself. However, the fucoidans are able to inhibit viral induced syncytium formation (Mandal et al. 2007).

Many other bioactivities of fucoidans have been shown (mainly in vitro) including as antioxidant, anti-inflammatory, antiangiogenic (Cumashi et al. 2007), inhibitor of sperm binding to oviductal monolayers (Gaboriau et al. 2007), and potential anti-cancer properties (Foley et al. 2011).

1.5.5 Seaweed Phlorotannins or phenolics

Phenolic compounds have been shown to have potent activity against stress-induced oxidation and have activity in the natural control of biotic stresses such as grazing or settlement of bacteria (Stengel et al. 2011). Moreover abiotic stresses also be reduced by the presence of these phlorotannins, arising from their ability to absorb UV radiation or adsorb metals. Seaweed phenolic substances are also able to protect human liver cell lines against oxidation (Queguineur et al. 2012). In addition to their powerful antioxidant properties, seaweed phenolics also have been shown to have antiviral properties (Kwon et al. 2013), and may have potential as active ingredients to prevent tumorigenesis (Hiqashi-Okaj et al. 1999).
1.6 Potential prebiotic properties of seaweed extracts and compounds

1.6.1 Definition of a prebiotic

The first definition of a prebiotic was suggested in 1995, and was as follows: a “non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bifidobacteria in the colon, and thus improves host health” (Gibson & Roberfroid 1995). This definition was updated in 2004 to include different factors in the definition, such as resistance to gastric acidity, fermentation by intestinal microflora and selective stimulation of bacterial growth. Thus the definition was updated as follows in 2004: a prebiotic is a “selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health” (Gibson et al. 2004). Finally, the Food and Agriculture Organization (FAO) of the United Nations defined a prebiotic ingredient, in 2007, as: “A prebiotic is a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota” (Food and agriculture organization of the United Nations 2007). This same technical report also defined the criteria required to scientifically demonstrate that an ingredient is a prebiotic. These criteria include

1. Non-digestibility: testing prebiotic resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption

This criterion may be determined using in vitro or in vivo models (animals or humans), with models applicable to humans requiring:
- Recovery of non-digestible molecules following oral administration (direct assessment)
- No increase of glycemia and insulinenia following oral administration (indirect assessment).

2. Fermentation by intestinal microflora

This criterion may be assessed May be determined using in vitro or in vivo models (animals or humans), with models applicable to humans requiring:
- Breath test, to measure the concentration of gases in exhaled air following oral administration (indirect assessment)
- Measurement of the recovery of the test carbohydrate in faeces following oral administration of the carbohydrate or prebiotic

3. Selective stimulation of growth and/or activity of intestinal bacteria

This criterion may be assessed using in vitro models or in vivo models (animals or humans), with methods requiring:
- Cultivation of bacteria on selective media
- Hybridation methods: FISH (fluorescence in situ hybridisation)
- Quantitative PCR (qPCR)
- DNA and proteomics fingerprinting methods: DGGE, TGGE, DHPLC, etc.

1.6.2 Existing prebiotics

Commercial reference prebiotic ingredients are inulin and fructooligosaccharides (FOS), both of which are derived from plant sources. FOS consist of glucose linked by α,β-1,2 bonds to fructose to form GF2, GF3, etc. according to the number of fructose units. The main difference between FOS and inulin resides in the molecular size. Indeed, FOS are generally smaller molecules with a degree of polymerization (DP) between 2 and 8 whereas the degree of polymerization (i.e. number of covalently linked sugar units) or DP of inulin can reach 60 (Huebner et al. 2008).

The other commercial prebiotics in use are the galactooligosaccharides (GOS). GOS consist of glucose linked to galactose via α-1,4 bonds, as the starting building block, with further galactose units added to reach a maximum DP of 8, similar to FOS. GOS are found naturally in abundance in legumes. A comparison of the prebiotic activity of the commercial sources shows relatively similar effects for FOS, inulin and GOS on the bifidobacteria species tested, except for B. bifidum, for which only the FOS gave a positive effect (when compared to glucose). With the lactobacillus species investigated, GOS generally gave higher results than either of the other two ‘prebiotics’ (Huebner et al. 2007).

1.6.3 Prebiotic activity of seaweed extracts

The best approach to determine the prebiotic potential of an ingredient is until lately by an in vivo study. This method is generally long and costly, so not appropriate for a first screen to compare different samples in order to identify the best candidate for in vivo evaluation. Therefore, the pre-selection of one or a few samples is generally done in vitro.

A first test ideally should investigate the stability or transformation of the potential prebiotic when exposed to conditions resembling those of the stomach and gastrointestinal tract. For seaweed extracts, an in vitro study of the resistance of laminaran extracted from S. latissima to digestive enzymes showed that the polysaccharide remained intact after enzymatic and acid treatment (Devillé et al. 2004).

The second test is ideally to evaluate the selective effects on intestinal microbiota. Beneficial (lactobacilli and bifidobacteria) and pathogenic (E.coli, enterococcus) bacteria are generally grown in pure culture on the seaweed extract as the only carbon source and growth profiles are compared to those on a simple carbon source, such as glucose. More than thirty
years ago, Salyers and co-workers showed that laminarans and alginates could be degraded by human colonic bacteria (Salyers, West, et al. 1977; Salyers, Vercellotti, et al. 1977). Other studies have been performed on seaweed extracts but mainly on extracts from red seaweed species (Rhodophyta). This in vitro approach is a good way to pre-screen different extracts, but might be not representative of the behaviour of the strains in the milieu that exists in the intestinal tract.

In vivo studies to evaluate the potential of seaweed extracts as prebiotic ingredients have been conducted only on animals (in laboratory or in farms). The extracts tested were mainly dry seaweed (especially A. nodosum and Laminaria sp) with most of the time, little information about the chemical and biochemical composition of the sample, the harvesting time of the seaweed, the location from which the seaweed was harvested and the methods (if any) used to process the seaweed into an animal feed format. Tests in farms have usually been done on pigs to evaluate the gut microbiota after being fed with seaweed; post-feeding tests are usually done on by faecal analysis. Moreover, an important parameter is the weight of the pigs/animals after treatment. In general, in terms of gut microflora, an increase in the Lactobacillus population has been observed, while Escherichia coli numbers tended to decrease. Moreover, the pigs/animals were generally in good health with, according to the experiments, a gain, a marginal loss or no effect on the weight (O’Sullivan et al. 2010).

1.7 References


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Stone, B; Clarke, A., 1992. *Chemistry and biology of 1,3-β-Glucans*,


Chapter 2: Materials and Methods
Unless otherwise stated, all reagents and chemicals were purchased from Sigma-Aldrich Ireland Ltd., Dublin, Ireland. A detailed list of all of the materials and reagents used in this project is given in Appendix 1.

2.1 Seaweeds

Freshly harvested seaweed biomass was used to prepare all the extracts. Three different species were harvested: *Ascophyllum nodosum* and *Fucus vesiculosus* (both Fucales) and *Laminaria digitata* (a Laminariales sp). The seaweeds were hand-harvested from a site in An Spiddeál (*Trà Sàlin*), Co. Galway, by J. Brebion or, for the larger batches of *A. nodosum*, by professional harvesters (with J. Brebion) through the industry collaborator on this project, *Oilean Glas Teoranta* (OGT), Ballymoon estate, Kilcar, Co. Donegal, Ireland (*Figure 2.1*). The seaweeds (or algae) were subsequently processed in the laboratory in Tuohy Laboratory, NUI Galway, or in OGT’s factory/processing facility in Co. Donegal.

2.1.1 Laboratory processing of seaweeds

Seaweeds processed in the laboratory were hand harvested in Spiddeál (*Trà Sàlin*), on the west coast of Ireland (GPS: N53°14'38.606", W9°18'20.593’). After being washed with fresh water, the seaweed was cut with a scissors into small pieces (≈ 1 cm²) and frozen.

![Figure 2.1 Map of Ireland with seaweed harvesting site A) Dungloe bay and B) Tra Salin in Spiddeal](image-url)
at -20°C until required. Three different species were harvested in Spiddeál and processed in the laboratory: *A. nodosum*, *F. vesiculosus* and *L. digitata*.

2.1.2 Industrial processing of seaweeds

Seaweeds that were industrially processed were hand harvested in Dungloe bay, Co. Donegal (GPS: N54°57’0.958”, W8°21’28.411”), on the North West coast of Ireland. After being washed with fresh water, the seaweed was cut into homogenous 1 mm pieces and frozen at -20°C until use. Only biomass from *A. nodosum* was processed in the OGT factory/processing facility.

2.2 Preparation of Seaweed extracts

A total of 4 different procedures for the preparation of water-soluble extracts from the seaweed biomass will be described in the following sections. One of the methods (Protocol 1) was developed through previous work in the Tuohy Laboratory, NUI Galway (Foley et al. 2011; O’Connell 2008), while the other three methods (Protocols 2-4) were developed during the course of this research.

2.2.1 Protocol 1: Procedure for preparation of a fucoidan-rich solution

Polysaccharides were extracted from seaweed processed in the laboratory at NUI Galway using a multi-step, low-chemical extraction process (Foley et al. 2011; O’Connell 2008), for the initial studies discussed later in Chapter 3 (see Figure 2.2). Seaweeds were initially freeze-dried before extraction. A 5 g quantity of dry seaweed was mixed with 50 mL of 80% ethanol (Lennox Laboratory Supplies, Dublin, Ireland) for 12 h at room temperature. After centrifugation (10 min, 6 000 rpm, 4°C), the liquid fraction (S1) was recovered and the seaweed pellet was again mixed with 50 mL of 80% ethanol solution for a period of 8 h at 70°C (the liquid fraction S2 was recovered). The polysaccharide-enriched fractions were then successively extracted from the seaweed pellet in three steps using distilled water (80 mL per step for each of two successive steps; S3 and S4) for 7 h at room temperature, followed by a final extraction (80 mL distilled H2O) for 4 h at 70°C. The solutions (supernatants) obtained after centrifugation (same conditions as described earlier) were labelled S3, S4 and S5, respectively. S3-S5 were combined into a mixed solution (Scombine; Sc) of known volume V and a corresponding volume V of a 4M CaCl2 solution was added in order to precipitate the alginates. The precipitated calcium alginates (insoluble in water) were recovered by centrifugation (as above). The supernatant fraction was
carefully removed and labelled S6. The pellet fraction (precipitated alginates) was mixed with a 4 M NaH$_2$PO$_4$ solution to generate the water-soluble form of the alginates, the Na-alginates. The final S6 solution (fucoidan-rich fraction) was subjected to dialysis (10 kDa Molecular weight cut-off membrane) to remove salts and small molecules; the retentate solution (labelled LF) was stored at 4°C until required. A schematic of this protocol is shown in Figure 2.2, above. This protocol was used to identify the best seaweed species from which to extract fucoidan (See Chapter 3, Section 3.2.1).

The total carbohydrate content and monosaccharide composition of fucoidan in solution S6 was analysed (results in Chapter 3, Sections 3.2.1.2 and 3.2.1.3).
2.2.2 Protocol 2: Time course extraction

Time course extraction protocol was undertaken to (i) investigate the effect of time on the yield of extract (water-soluble biomolecules) in a one-step extraction process at 70°C, and (ii) enable the optimisation of conditions for the one-step extraction method. A 100 g quantity of fresh, industrially processed seaweed was mixed with 500 mL of distilled water. A 10 mL aliquot of sample (seaweed + liquid) was removed at different time points (T0, T1, T2, T3, T4, T5, T6 and T24 h). These aliquots were centrifuged for 10 min at 4700 rpm to pellet the residual seaweed biomass. The liquid supernatant fractions were stored at 4°C until analysis and residual seaweed biomass was prepared for analysis by microscopy (preparation as described in Section 2.3). An investigation of the main water-soluble compounds extracted is presented in Chapter 3, Section 3.2.5. The final solution obtained after 24 hours of extraction was used as a starting material for the comparison of two methods to precipitate alginates and the results are discussed in Chapter 3, Section 3.2.3.

2.2.3 Protocol 3: Extraction scale up

Extraction scale up was performed in collaboration with the company Oilean Glas Teo (OGT). The company provided the fresh seaweed biomass and the extraction equipment. Figure 2.3 shows the fresh seaweed (*A. nodosum*) after the industrial cutting process. The seaweed was cut into homogeneous pieces of 1 mm (as mentioned above) and stored in cold room at 4°C. Extraction was done with 100 kg of fresh seaweed. The mixing tank, shown in Figure 2.4, was filled with water (300 L) and warmed at 65°C. The tank was
equipped with a stirrer and a system for temperature regulation. When the desired
temperature was reached, the seaweed (100 kg) was loaded and the temperature was
recorded every hour. Moreover, pH, conductivity, total solids content, carbohydrate and
phenolics contents were measured for each hourly sample (for the results, see Chapter 3,
Section 3.2.6). After 7 hours of extraction, the tank was emptied into a clean container.
Residual solids were separated from liquid by filtration using fine sieves and muslin (Figure
2.5) and a brown liquid was recovered. This liquid extract was stored in a 1000 L container
and taken to the Moorepark Technology Ltd pilot plant facility at Teagasc Food Research
Cente (Moorepark Fermoy, Co. Cork, Ireland) for further clarification and
fractionation/purification. The first step involved a clarification step (Figure 2.5) to remove
very small solid particles that passed through the filters during the filtration step at OGT,
and some insoluble complexes formed between compounds (potentially the more
hydrophobic substances, including pigments).

Figure 2.6 shows the different colours of the extract before (A) and after (B)
passage through the clarifier. After clarification, the extract a 150 L of a clear, dark solution
was obtained, with solids and insolubles having been separated.

The final step conducted at the MTL facility involved successive ultrafiltration
(fractionation) steps using 1 kDa and 10 kDa MWCO membranes, in a pilot scale
ultrafiltration system, to obtain three different soluble extracts. The first extract was the
fraction below 1 kDa, the second the fraction between 1 and 10 kDa and finally, the last
above 10 kDa. Those three fractions were used to test different amberlite resin to adsorb
phenolic substances and results are presented in Chapter 3, Section 3.2.4.

Figure 2.5 Separation of residual seaweed after
extraction through muslin on a fine sieve

Figure 2.6 Clarification system to remove insoluble
compounds A) extract before clarification and B) after
2.2.4 Protocol 4: Experimental design towards developing an optimised procedure for extraction

In order to define the effect of extraction parameters on different responses, such as extraction yields, extract composition or extract bioactivity, a full factorial experimental design was developed (the description and calculation of a full factorial design is presented in Section 2.7). The parameters investigated were extraction time (1 or 6 hours), temperature (25°C or 70°C) and the ratio of water (extractant) to seaweed biomass (2 or 5), which corresponding to the volume of water (mL) used per gram of fresh seaweed. In the full factorial design, all combinations of extraction parameter levels were tested which corresponded to 8 different protocols conducted in triplicate (24 extractions). The 8 protocols are described in detail in Section 2.7.2. After extraction, residual seaweeds were removed by centrifugation (10 min, 4700rpm at 4ºC) and filtration. The volume of extract (E0) was recorded and the solids content in extract was measured. Extract E0 was dispensed into aliquots and stored at -20ºC until required for analyses.

2.3 Preparation of the biomass for analysis by microscopy

The seaweed cells were analysed by microscopy directly after extraction (see Section 3.2.5). Prior to analysis the samples had to be fixed and stained.

2.3.1 Fixation of the seaweed cells

The first step in the preparation of the seaweed cells consisted of fixing them to avoid any degradation over time.

During the time course extraction, samples were collected at different time points over a 24 h period. The samples consisted of a liquid fraction plus residual seaweed biomass. The residual seaweed was separated from the liquid by centrifugation (10 min, 6000 rpm, RT). Once isolated, seaweed cells were fixed using primary fixative (2% glutaraldehyde, 2% paraformaldehyde in 0.01M sodium cacodylate/HCl buffer) by overnight incubation at room temperature. The biomass was then kept in water until further treatment.
2.3.2 Paraffin wax infiltration

After being fixed, the seaweed cells were treated with wax to prepare thin tissue layers. First of all, the cells were dehydrated by successive treatments with ethanol and xylene as described in Table 2.1. Finally, the tissue samples were cut into 5 µm thick layers using a microtome and placed on microscope slides before staining. The wax treatment and cutting was kindly done by Mr. Mark Canney, Centre for Microscopy and Imaging, NUI Galway.

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Durations</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% Ethanol</td>
<td>Overnight</td>
</tr>
<tr>
<td>80% Ethanol</td>
<td>30 min</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>30 min</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>1 h</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>1 h</td>
</tr>
<tr>
<td>100% Ethanol/Xylene</td>
<td>30 min</td>
</tr>
<tr>
<td>Xylene</td>
<td>1 h</td>
</tr>
<tr>
<td>Xylene</td>
<td>30 min</td>
</tr>
<tr>
<td>Paraffin Wax</td>
<td>3 h</td>
</tr>
<tr>
<td>Paraffin Wax</td>
<td>Overnight</td>
</tr>
</tbody>
</table>

2.3.3 Staining of Polysaccharides

Alcian blue was used to stain acidic polysaccharides (fucoidans and alginates) in the prepared seaweed tissue sections (Section 2.3.2). Before starting the staining, the seaweed cells had to be rehydrated in a series of baths with different ethanol concentration (100%, 90%, 80%, 70%, 60%, 50% and 0%). The slides were successively immersed in decreasing concentrations of ethanol for 2 minutes and finally in a water bath (0% ethanol) for another 2 min. In parallel, a 0.5% w/v aqueous solution of alcian blue was prepared and the slides were then placed in the stain for 10 min. After that time, slides were placed in running water to remove excess alcian blue stain and the cells were again dehydrated using the successive ethanol bath for 2 minutes but starting on this occasion with the 50% ethanol solution through to 100% ethanol. Finally, the slides were immersed in a xylene bath for 10 min. The slides were kept at room temperature before light microscopy analysis. Alternatively, some
slides were also counter-stained with neutral red. After removing the excess alcian blue in running water, in this case the slides were submerged in a 0.5% w/v aqueous solution of neutral red for 2 min. Once again, a running water bath allowed removal of any excess neutral red stain. The slides were then treated similarly to dehydrate the cells.

2.4 Preparation of enriched fucoidan fractions

2.4.1 Precipitation of Alginates

An investigation of different chemical options for alginates precipitation was conducted on extract obtained at the end of the time course extraction (Protocol 2, Section 2.2.2). A 5 mL fraction of extract was mixed during 30 min with 5 mL of glacial acetic acid or 5 mL of a 4M CaCl$_2$ solution. After centrifugation (15 min, 4700 rpm, 4°C), the pellet was recovered and resuspended in 5 mL of a 0.5% NaOH solution. The volumes of both the supernatant and re-solubilised pellet solutions were recorded; the samples were then stored at 4°C before analyses. Samples were hydrolysed (see Section 2.5.7) with trifluouracetic acid prior to separation and analysis of monosaccharides and sugar alcohols by High Performance Anion Exchange Chromatography (HPAEC; see Section 2.5.8) to determine the influence of the chemical precipitants used on the residual sugars and on the composition of fucoidan in the supernatant. The results of this experiment are presented Section 3.2.3.

2.4.2 Removal of Phenolic substances using Amberlite® resin

First of all, a preliminary study was conducted to define the optimum quantity of dry resin to use to achieve removal of phenolics from a 20 mL volume of seaweed extract. To 20 mL of extract (retentate from pilot-scale 1 kDa ultrafiltration step of Protocol 3), different known weights of dry amberlite® XAD7 resin were added (from 0 to 10 g in 1 g increments), and the mixture was stirred gently at RT for 10 min. The mixtures were then centrifuged (10 000 rpm, 5 min) to separate the resin from the liquid fraction. After centrifugation, the volumes of supernatant were recorded and the phenolics content was quantified using the method of Zhang et al (2006), as describes in Section 2.5.3.2.

When the ratio amberlite/extract (g/mL) was identified that gave optimum removal of phenolics, a range of different amberlite® resins were compared with different extracts to identify the best resin for removal of phenolics. The resins tested were (all amberlite®): IRA-900, XAD-2, XAD-4, XAD-7HP, XAD-16 and IRC-50. After centrifugation, polyphenols and total sugars were quantified (see Section 2.5.3). The results are presented in Chapter 3, Section 3.2.4.
Finally, an efficiency score was calculated to determine the best resin for adsorption of phenolics with reduced or limited adsorption of sugars, in order to identify the best option to use to remove phenolics, yet recover the maximum concentration of sugars in the liquid fraction. The score was defined as the ratio of the percentage of phenolics removed to the percentage of sugar removed from the starting liquid fraction. The higher the score the more effective and selective the resin is for removal of phenolics with minimal loss of sugars from the liquid extract.

2.5 Biochemical and Chemical Analyses

2.5.1 Measurement of Dry weight and solids content

Both the dry weight of seaweed before the extraction and the solids content of the extract after the extraction were systematically determined. An empty weigh boat was weighed (mass of cup), a quantity of seaweed or liquid extract was added (sample mass) to the weight boat, and the weight boat was then placed at 50°C for 24 h. After 24 h (or until a constant weight was obtained), the boat containing the dried sample was weighed again (dry sample + cup), and the dry weight or solids content was calculated as follows (Equation 2.1):

$$\text{Dry weight (solid content)} = \frac{(\text{dry sample + cup}) - \text{mass of cup}}{\text{sample mass}}$$

For both the initial seaweed and extracts, the dry matter content was measured and expressed as a percentage by weight relative to the dry weight of the freshly harvested seaweed sample.

2.5.2 Ash content determination

An empty crucible was weighed (mass of cup) and a quantity of seaweed or liquid extract was added (sample mass); the crucible containing the seaweed or liquid extract was then placed at 550°C for 180 minutes. After cooling down in a desiccator (to avoid any rehydration of the sample), the crucible containing the ash was weighed again (dry sample + cup) and the ash content was calculated using Equation 2.1 above (see Section 2.5.1).

The ash content was measured and expressed as a percentage of ash in the initial starting sample (mg/g of liquid or solid sample), which depending on the extract/sample, could be the initial seaweed biomass or crude extract recovered from the seaweed biomass.
2.5.3 Colorimetric assays

The microplates used for all of the colorimetric assay absorbance measurements were flat-bottomed, low protein-binding, high optical quality clear polystyrene 96-well plates (Sarstedt, Sinnottstown, Co. Wexford, Ireland).

2.5.3.1 Total carbohydrate determination

The total carbohydrate content of each fraction was assayed using a microassay version of the DuBois assay (DuBois et al. 1956). Each sample was analysed in triplicate as follows. In a 1.5 mL clear microcentrifuge tube, 50 µL of extract were mixed with 50 µL of a 5% phenol solution (in a fumehood, using the appropriate protective clothing and nitrile gloves); the mixture was kept at 4°C for 10 min. Then, a 250 µL volume of concentrated sulphuric acid was added (from a dedicated dispenser, in the fumehood), the tube was capped and the mixture was boiled in a water bath for 15 min, before being cooled to RT. Finally, a 250 µL aliquot from each tube was dispensed into individual wells in a 96-well microplate plate (Sarstedt, Wexford, Ireland; see Appendix 1), and the optical density was recorded at 490 nm using a BioTek Powerwave XS2 microplate reader. Quantification was achieved using appropriate standards, according to the extract tested, e.g. fucose for a fucoidan-rich extract, glucose for a laminaran-enriched extract, etc. The response of sugars varied according to the sugar standard tested as shown in Figure 2.7 above, which provides representative examples of standard curves for relevant sugars.

![Figure 2.7 Standard curves for different sugar standards over the concentration range from 0 to 500 µg/ml](image-url)
2.5.3.2 Quantification of Total polyphenol content

Quantification of total phenolic substances was performed according to the protocol described by Zhang and colleagues (Zhang et al. 2006). A 100 µL volume of sample (in triplicate) was mixed with 50 µL of Folin-Ciocalteau reagent, 200 µL of a 20% (w/v) NaCO₃ solution and 650 µL of distilled water. The mixtures were incubated at 37°C for 40 minutes (using the incubation facility in the BioTek Powerwave XS2 microplate reader) and optical densities were finally read at 765 nm. Quantification was achieved by reference to a phloroglucinol standard curve from 0 to 100 µg/mL (Figure 2.8).

![Figure 2.8 Standard curve of phloroglucinol over the concentration range from 0 to 100 µg/mL](image)

2.5.4 Pigment quantification by HPLC

Pigment analysis was kindly done by Dr. Solene Connan in Dr. Dagmar Stengel’s laboratory using HPLC analysis with UV detection. The system used was an Agilent 1200 series HPLC system. Pigments were separated at 40°C by Reverse Phase (RP)-HPLC on a Spherisorb ODS2 (25 cm x 4.6 mm ID, 5 pm particle size) column, and eluted using a linear gradient of solvent A, methanol/ammonium acetate (80/20) and B, acetonitrile/water (90/10). The gradient started with 100% of A and finished with 100% of B within a 4 min elution period. The column was then re-equilibrated with 100% of solvent A for 5 min before injection of the next sample (Division et al. 1991). All solvents and chemicals used were of HPLC grade.
2.5.5 Sulphate quantification by ion chromatography (IC)

Sulphates were quantified by ion chromatography using an ICS-3000 ion chromatography system (Dionex, USA). Ions were separated on an IonPac AS22 column (and guard column IonPac AS22), 4 x 250 mm (Dionex, USA) and eluted with a ready to use the appropriate dilution of an AS22 eluent concentrate containing 0.45 M carbonate and 0.14 M bicarbonate (sodium salts), under isocratic conditions at 1.2 mL/min. The conductivity of the eluting anions was measured after cation suppression using an ASRS300 suppressor (Dionex, USA). The principle of suppressor action is shown in Figure 2.9.

![ASRS 300 in AutoSuppression Mode](image)

*Figure 2.9 Cations auto suppression with the ASRS30 (Dionex)*

Basically, in this approach the cations are attracted to a negative electrode (cathode) and displaced by water molecules. Quantification was achieved by reference to a known concentration of different anions in solution (anions standard: Dionex, USA).

2.5.6 Size fractionation of extracts

The extracts were filtered using a 0.2 µm syringe filters (Sarstedt, Sinnottstown, Co. Wexford, Ireland). A 2 mL fraction of each of the relevant extract filtrates was size-fractionated by ultrafiltration using centrifugal Vivaspin 2 units (Sartorius, Ireland) with different MWCOs (i.e. 10, 30, 50, 100 and 300 kDa). The individual UF units were
centrifuged (Beckman Coulter, O'Callaghans Mills, Co. Clare, Ireland) for 30 min, 4°C at 4700 rpm. Both the filtrate and retentate volumes were adjusted to 2 mL and stored at 4°C until required for analysis.

### 2.5.7 Polysaccharides hydrolysis for analysis of monosaccharide composition

Polysaccharides were hydrolysed using a solution of 4M solution of trifluoroacetic acid (TFA), formic acid and MilliQ water in the proportion 1/3/2, respectively, to break the glycosidic bonds and liberate the monosaccharides prior to separation, analysis and quantification by HPAEC with Pulsed Amperometric Detection (PAD). A 200 µL aliquot of sample was mixed with 200 µL of the acidic hydrolysis solution in a tightly capped microcentrifuge tube and incubated in a boiling water bath for 4 h. After cooling down of the mixture to RT, the residual acids were evaporated by co-evaporation with ethanol. A 200 µL volume of ethanol was added to the hydrolysed sample and the mixture was evaporated under vacuum using a miVac concentrator (Genevac Ltd, Ipswich, UK) over a 2 h period. The last step (addition of ethanol and evaporation) was repeated two more times with shorter evaporation cycles (1 hour and 30 minutes) to remove any residual acid. The dry product obtained was kept at 4°C before analysis by HPAEC-PAD.

### 2.5.8 Monosaccharides analysis by High Performance Anion Exchange Chromatography (HPAEC) with Pulsed Amperometric Detection

Two protocols were used to quantify the monosaccharides in each sample.

**Protocol 1:** Monosaccharide composition was determined by anion exchange chromatography using an Ion Chromatography System 3000 (ICS-3000) (Dionex, USA) equipped with an ED-40 pulsed amperometric detector and a gold electrode. The column used for monosaccharide separation was a CarboPac PA100, 4 x 250 mm (Dionex, USA), equipped with a guard cartridge or column. The samples were separated and eluted with HPLC grade 10 mM NaOH under isocratic condition at 1mL/min and 30°C.

**Protocol 2:** This protocol used a different column for monosaccharide separation and analysis. The monosaccharides were separated on a CarboPac SA10 (4 x 250mm) equipped with guard column and eluted with 1mM KOH under isocratic conditions at 1mL/min at 25°C.

Quantification was achieved by reference to the appropriate monosaccharide standard curves (mannitol, fucose, galactose, glucose, xylose and mannose). Standard curves were prepared for each monosaccharide and are included in Appendix 2.
2.5.8.1 Monosaccharide separation on CarboPac columns

Neutral sugars behave as weak acids, and thus are at least partially ionised at high pH (negative charge), which allows their separation by anion exchange techniques. Using HPAEC, neutral sugars can be separated in their oxyanionic form, at alkaline pH, on high performance pellicular anion exchange resin. The resins exhibit a rapid transfer, high pH stability (pH 0-14) and high mechanical stability that permits pressures of up to 4000 psi.

2.5.8.2 The HPAEC-PAD method

HPAEC is a chromatographic technique that was developed to separate carbohydrates. Coupled with pulsed amperometric detection (PAD), it permits direct quantification of non-derivatised carbohydrates. HPAEC is a selective, specific and sensitive method for sugar separation and quantification. Indeed, pulsed amperometry only detects components containing functional groups that are oxidisable at the detection voltage employed, meaning that carbohydrates can be selectively detected using the appropriate voltage.

2.5.8.3 Mechanism of separation

Although anion exchange has been used extensively in the field of glycobiology to analyse glycopeptides and acidic carbohydrates, it was not commonly used for the analysis of neutral sugars. Examination of the pKa values of neutral monosaccharides shows that carbohydrates in fact behave as weak acids (e.g. pKa of glucose: 12.28, mannose: 12.08 and xylose: 12.15). At high pH, these sugars are at least partially negatively charged and can thus be separated by anion exchange chromatography.

2.5.8.4 Pulsed amperometric detection (PAD)

Pulsed amperometric detection (Figure 2.10) offers excellent signal to noise detection of carbohydrates at very sensitive detection levels. Carbohydrates are detected by measuring the electrical current generated by their oxidation at the surface of the gold electrode. The amplitude of the electrical signal is stoichiometric with the amount of sugar present in the sample. However, the oxidation product has to be removed from the electrode surface in between measurements. Removal is accomplished by first raising the potential to a level sufficient to oxidise the gold surface. This cause desorption of the oxidised carbohydrates. The potential is then lowered to such a level that facilitates reduction of the
gold surface to its initial detection state. Pulsed amperometric detection employs a repeating cycle of three potentials E1, E2 and E3.

2.6 Data analysis and calculation of carbohydrate concentrations in seaweed extracts and enriched fractions

2.6.1 Calculation of the mannitol concentration following HPAEC-PAD

The mannitol concentration was determined by HPAEC using the protocol described in Section 2.5.6 above and a representative chromatogram is shown in Figure 2.11. Non-hydrolysed samples were used to determine the concentration of free mannitol in solution (brown algae are known to contain high levels of free mannitol as well as other sugar alcohols). Quantification was achieved by to reference to the appropriate mannitol standard (see Appendix 2 for the mannitol standard curve relevant to quantification by HPAEC). The concentration of ‘bound’ mannitol, i.e. mannitol units present as covalently-linked terminal substituents on laminaran, was determined as described in Section 2.6.3 below.
2.6.2 Calculation of the fucoidan concentration by HPAEC-PAD

Fucoidan was also quantified by HPAEC-PAD by quantification of the combined concentrations of specific monosaccharides shown previously to be present in fucoidans.
from *A. nodosum* (Foley et al. 2011; O’Connell 2008). After acid hydrolysis (see Section 2.5.7), monosaccharides were separated and quantified as explained in Section 2.5.6. Based on earlier work on fucoidans from *A. nodosum*, and work in this research with more purified fucoidans from this seaweed, in the Tuohy Laboratory, NUI Galway, fucoidan composition was based on the combined amounts of fucose, galactose, rhamnose, xylose and mannose. The concentrations of each individual monosaccharide were quantified by quantitative comparison of the respective peak areas to those of the corresponding standards (Appendix 2). A representative chromatogram for a hydrolysed seaweed (*A. nodosum*) extract from this research is shown in Figure 2.12 above. The concentrations of fucose, galactose, rhamnose, xylose and mannose were collectively combined to calculate the fucoidan concentration.

### 2.6.3 Calculation of laminaran concentration

Laminaran concentration was also quantified by HPAEC. Laminaran is composed of glucose and mannitol. To determine the quantity of mannitol linked to the laminaran, non hydrolysed (*Figure 2.13-A*) and hydrolysed (*Figure 2.13-B*) samples were used. The quantity of mannitol was determined by subtracting the quantity of mannitol before hydrolysis from the quantity after hydrolysis. The mannitol concentration was then combined with the quantity of glucose present (on hydrolysed samples) to give the overall laminaran concentration. Free glucose was not present in the unhydrolysed sample as can be seen in *Figure 2.13-A*, and as any cellulose in the seaweed cell wall would be insoluble and likely to remain in the biomass after aqueous extraction, the assumption was made that the glucose detected by HPAEC following acid hydrolysis of the extracts, was due to laminaran present.

![Figure 2.13 Representative chromatograms of a A) non hydrolysed and B) hydrolysed seaweed extract used to quantify the laminaran concentration](image-url)
2.6.4 Calculation of extraction yields and total solid content in extracts

After quantitative determination of the volumes and dry weights of the extracts obtained from each extraction protocol (including extracts from any intermediate steps), an approach was taken to calculate and determine the total extraction yields and extract composition on a dry weight basis relative to (a) the initial seaweed dry weight, and (b) to the total dry weight of the relevant extract. Figure 2.14 shows the calculations done per extract and for each individual compound or analyte quantified.

First of all, the exact quantity of fresh seaweed used for the extraction had to be recorded (Ms) and the dry weight measured (DWs). These two measurements allowed the quantity of dry seaweed used for each extraction to be calculated. After extraction, centrifugation and filtration, the recovered volume of liquid (Ve) was noted and the solids content was determined (as %Se or g solids per 100 mL of recovered extract). Using these two results, the mass of total solids in the extract could also be calculated.

Finally, analysis of the biomolecule (denoted as compound in Figure 2.14) composition in each extract gave a concentration, in mg/mL, for each compound. This concentration was multiplied by volume of liquid (Ve) recovered for each extract to give the concentration of each compound present in the extract. This concentration was then be divided by the total dry weight of seaweed used to obtain the extraction yield. In a same

![Figure 2.14 Calculations of extraction yields (% seaweed dry weight) and compound contents (% of total solids)
way, the compound concentration was divided by the solids content of liquid (%Se) to find out its proportion on a weight basis relative to the total solids content of the extract. This approach was made possible by the fact that the solids contents of the extracts reached a maximum of 3%, which was too low to affect the density of the solution. The density of the solution or extract would therefore, in effect, still be close to a value of one. Meaning that 1 mL of volume of liquid would be expected to weigh approximately 1 g, making it possible to express the concentration (mg/mL) also in mg/g of extract.

One further calculation was also performed in relation to the yields. The total extraction yield was expressed as ratio of the mass of total solids in extract to the mass of dry seaweed used.

The calculations outlined in this section have been used in the analysis of data to investigate the influence of extraction parameters on extract and compound yields and on the composition, the results of which are presented in Chapter 4.

2.6.5 Carbohydrate Polyacrylamide Gel Electrophoresis (C-PAGE)

The electrophoresis technique used to identified oligosaccharides produced by enzymatic digestion of alginites with specific alginate lyases isolated from the marine bacteria: Zobellia galactanivorans (Thomas et al. 2013) was Carbohydrate Polyacrylamide Gel Electrophoresis, abbreviated C-PAGE. A 500 µL aliquot of seaweed extract was incubated individually with 5 µL of purified enzyme (AlyA1: specific G block lyase or AlyPa: specific M-block lyase) overnight at 30°C.
A 5 µL aliquot of the digested extract was mixed with 15 µL of a 10% solution of sucrose with phenol red and samples were applied to individual lanes in a 36% polyacrylamide gel (Table 2.2 shows the composition of the gel, Buffer 20X: 1M Tris, 20 mM EDTA). In-gel oligosaccharide separation was conducted at 200 V in a 50 mM Tris, 1 mM EDTA migration buffer at pH 8.7. Electrophoresis was allowed to continue until the phenol red reached approximately 2 cm from the bottom of the gel. The power was switched off and the gel was removed from the gel apparatus and placed in a 0.5% (w/v) alcian blue solution for 10 min to stain the negatively-charged carbohydrates; the gel was rinsed several times with water. The gel was finally placed in a 0.4% (w/v) silver nitrate bath (no shaking) and rinsed 3 times, for 1 minute each time, with water. The stain was then developed in a solution of 7% (w/v) sodium carbonate and 0.08% (v/v) formaldehyde. The developing reaction was stopped by the addition of glacial acetic acid. The C-PAGE results are presented in Chapter 5, Section 5.2.4.
2.6.6 Evaluation of the alginate content in seaweed extract by the action of alginate lyases

The action of alginate lyase leads to the formation of double bonds in the oligosaccharide that are produced in the reaction from alginate; the double bonds absorb at 235 nm. Based on this property, a 1 mL aliquot of seaweed extract (or an enriched fraction) was incubated at 30°C with 50 µL of enzyme in a quartz cuvette and the absorbance was read every 2 seconds at 235 nm. The absorbance increase was compared to the increase obtained by enzymatic hydrolysis of commercial purified alginate, at different concentration under identical reaction conditions. The results of this analysis are presented in Chapter 5, Section 5.2.4.

2.7 Experimental design to investigate the effects of extraction parameters on extract yield and composition

2.7.1 Definition of the experimental design

The design of an experiment (or experimental design) is by definition a precisely organized sequence of experiments conducted in order to define with a minimum number of tests and maximum accuracy, the influence of some process parameters on a specific end-point or end-points, e.g. yield, purity, efficiency or cost, etc.

Experimental design is used widely in industrial studies, as well as in research and development with ultimate goals such as the determination of key parameters in a process line, optimisation of parameter settings or prediction of results by establishing a mathematical model for the process.

A rigorous experimental design approach was taken in this work to investigate the effects of a number of extraction parameters on the yields of seaweed extracts, yields of enriched fractions and on the biomolecule composition in one-dimensional (1D or on an individual parameter basis), two-dimensional (2D or interaction between two parameters) and three-dimensional (3D or interaction between three parameter) levels. Chapters 4 and 5 present the results of this multi-factorial approach to investigate the role and relevance of the extraction parameters applied in the study. The ultimate goal was to develop a robust mathematical relationship between extraction parameters and the yield and composition of a specific extractor enriched fraction that could be used in the future to predict these endpoints (with the specific seaweed) equally for laboratory or industrial production of the specific extracts or specific enriched polysaccharide fractions from *A. nodosum*. 
2.7.2 Parameters and responses of an experimental design

The first step of an experimental design is to define the parameters. Three different kinds of parameters can be identified as shown in Figure 2.15. Controllable parameters are fixed and defined by the operator (in this work, these parameters were: quality of water used, stirring technique and intensity). Uncontrollable parameters cannot be fixed and some variations occur, for example in this experiment, uncontrollable parameters were the quantity of water needed to wash the seaweed or time required to defrost the cut seaweed samples. Finally, in a specific area of investigation, studied parameters are the ones investigated to figure out if and how they influence the responses. In this research, the three parameters investigated were: time (1 and 6 h), temperature (25°C and 70°C) and the ratio of water/ seaweed biomass (2:1 and 5:1). The ratio was expressed as the volume of water (in mL) per gram of seaweed used for the extraction. For example, a ratio of 2 indicates that 2 mL of water and 1 gram of seaweed (or more especially in this case, 200 mL of water and 100 g of seaweed).

Responses are the physical quantities measured after each experiment. The experimental design aims to find out which parameters influence the response and its evolution of the response according to the parameters investigated in the subject/area of interest. Different responses were investigated (ranging from %yield relative to the total dry weight of the initial seaweed biomass to the specific effects on biomolecule (e.g. polysaccharide) composition; and the calculations used are explained in Section 2.7.4.

![Figure 2.15 Schematic representation of the different kind of parameters identified for an experimental design](image-url)
2.7.3 Establishment of a design of experiment

In order to investigate the influence of the specific extraction parameters, a full factorial experimental design was set up. A factorial design is a sequence of experiments containing two or more studied parameters at different values (or levels), in which it is possible to investigate all possible combinations of levels across all factors. This design enabled the investigation of the effect of parameters on specific responses, as well as the effects of interactions between parameters. The number of experiments required for a full factorial design is defined by $l^n$ with $l$ corresponding to the number of level and $n$, the number of studied parameters. In this research, three parameters were studied at two different levels, labelled “low level” (-1) and “high level” (+1). Thus, the number of experiments (extractions) required was $2^3=8$. All experiments required were summarized in a matrix called a “Hadamard matrix” (square matrix whose rows that are mutually orthogonal) which is presented in Table 2.3. In this matrix, values of parameters are expressed as -1 or +1 corresponding to low or high level of the topic/area being investigated. The values of parameter levels are shown in the last two rows. Each row explains the protocol to follow with the level of each parameter and the result obtained for the response (Y). For example, extraction 1 used the ‘low level’ for each parameter, which corresponds to an extraction time of 1 h at 25°C at an extractant/seaweed biomass ratio of 2 (200 mL of water for 100 g of seaweed). This matrix was also used to calculate the effect of each parameter as explained in Section 2.7.4.2. A full factorial design of three parameters can also be presented as a cube in which the corners represent the experiments that need to be done (Figure 2.16).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>A: Time (h)</th>
<th>B: Temperature (°C)</th>
<th>C: Ratio water (mL) / seaweed (g)</th>
<th>Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction 1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>Y1</td>
</tr>
<tr>
<td>Extraction 2</td>
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<td>-1</td>
<td>+1</td>
<td>Y2</td>
</tr>
<tr>
<td>Extraction 3</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>Y3</td>
</tr>
<tr>
<td>Extraction 4</td>
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<td>+1</td>
<td>+1</td>
<td>Y4</td>
</tr>
<tr>
<td>Extraction 5</td>
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<td>-1</td>
<td>-1</td>
<td>Y5</td>
</tr>
<tr>
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<td>-1</td>
<td>+1</td>
<td>Y6</td>
</tr>
<tr>
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<td>+1</td>
<td>-1</td>
<td>Y7</td>
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<td>+1</td>
<td>Y8</td>
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<td></td>
</tr>
<tr>
<td>Level +1</td>
<td>6</td>
<td>70</td>
<td>5</td>
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</tr>
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</table>

Table 2.3: Hadamard matrix of the factorial plan of the experimental design for the specific experimental area proposed in this research
2.7.4 Statistical analysis

Statistical analysis was used to identify the most influential parameters for the specific response studied. Qualitative analysis was performed by interpretation of the main effect plot and the interaction plot drawn by the software Minitab. The effects of each parameter and interaction were calculated to define influential effects and to support the development of a statistical model for the extraction.

2.7.4.1 Qualitative analysis of the influence of extraction parameters

For each parameter, a graph containing two curves was prepared and analysed (Figure 2.17). The first curve (horizontal) was obtained by calculating the average of the response of all experiments and the second one shows the difference between the experiments done using the low and high levels of the particular parameter. In this example, the effect of time was investigated. Responses using the same extraction time were averaged to draw the second curve. The slope of this curve indicated if the parameter was potentially influential. A low slope, giving two curves almost parallel, showed a low effect of the parameter on the response. The slope could also be negative and indicated that the effect gave greater results at the low level. The evolution between the two parameter levels was
supposed to be linear (no intermediate points were investigated so might not reflect the reality) in the area of the experiment.

\[\text{Figure 2.17 Representative graph of qualitative analysis of main parameter effect}\]

Once the qualitative analysis of the main parameter was done, a second graph, using the same software and showing the effect of the interactions between the parameters was analysed. A representative graph of the interaction plot is presented in Figure 2.18. This figure is composed of three graphs corresponding to the three possible first level interactions. The first level interactions were the interactions 2 by 2 between the main parameters (Time/Temperature, Time/ratio and Temperature/ratio. The second level interaction would be, in our case, the interaction between the three parameters (Time/Temperature/ratio). The principle of this analysis was very similar to the previous analysis as the comparison was done between two curves. For example, the interaction Time/Temperature, the black curve was drawn by averaging the results obtained after 1 h extraction and 25°C against the results obtained after 1 h extraction at 70°C. The red curve was obtained doing the same calculation except that time used was 6 hours. Thus, it was possible to analyse the interaction between the effect(s) of time on a specific target and the temperature. The interaction was considered as insignificant when the two curves were parallel like the interaction Time/Ratio in this example (Figure 2.18), contrary to the interaction between Time/Temperature and Temperature/Ratio, both of which appeared to be important.
2.7.4.2 Quantitative analysis of parameters and interaction effects

This step was essential to confidently identify influential parameters and to compare the effects of interaction between them. A value was calculated for each effect and interaction, and was compared to a confidence interval using the variance between the three

![Figure 2.18 Representative graph for qualitative analysis of interaction effect](image.png)

Table 2.4 Table used for the effect of parameters and interactions calculation

<table>
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<th>Extraction</th>
<th>A</th>
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<th>C</th>
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<td>6</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>Y6</td>
</tr>
<tr>
<td>7</td>
<td>+1</td>
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<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>Y7</td>
</tr>
<tr>
<td>8</td>
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<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
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<tr>
<td>Effects</td>
<td>$x_a$</td>
<td>$x_b$</td>
<td>$x_c$</td>
<td>$x_{ab}$</td>
<td>$x_{ac}$</td>
<td>$x_{bc}$</td>
<td>$x_{abc}$</td>
<td>$x_0$</td>
</tr>
</tbody>
</table>
replicates done per extraction. An extension of the Hadamard matrix (Table 2.4, above) was used to calculate the effects of parameters and interactions between parameters.

For each extraction, the level of each parameter was noted in the matrix (using A for time, B for temperature and C for Ratio). For the interactions, the coefficients were obtained by multiplying the coefficients for the individual parameters. For example, in extraction 1, the interaction AB was obtained by multiplying the coefficient of A by the coefficient of B (-1 x -1 = +1). Responses (calculated as explained in Section 2.6) were labelled Y followed by the extraction number and the effects were named x. Effects were calculated by adding the individual values of all the responses with the appropriate coefficient and dividing by the number of experiments (8 in this case). That gave:

For time:
\[ x_a = \frac{(-Y_1 - Y_2 - Y_3 - Y_4 + Y_5 + Y_6 + Y_7 + Y_8)}{8} \]
For temperature:
\[ x_b = \frac{(-Y_1 - Y_2 + Y_3 + Y_4 - Y_5 - Y_6 + Y_7 + Y_8)}{8} \]
For ratio:
\[ x_c = \frac{(-Y_1 + Y_2 - Y_3 + Y_4 - Y_5 + Y_6 - Y_7 + Y_8)}{8} \]

Same calculations were done for the interactions and \( x_0 \) was the average of the response.

A confidence interval (at 95%) was then calculated. The interval was the same for each parameter because the calculations were based on the standard deviation between the replicates. Table 2.5 summarises the calculations done to define the interval. For each response, a standard deviation was calculated using the three replicates done for each protocol. Standard deviations were called \( s_i \) with the “i” corresponding to the extraction number (Table 2.5). For each extraction, the variance was calculated (square of standard deviation). A sum of all the variances was then divided by 3, corresponding to the number of replicates. Finally, the interval was calculated at 95% by dividing the square root of the previous value by the square root of the number of experiment done in total (8 extractions in triplicate) and multiplied by 2.12 which was the value given by the student table for a risk of 5% and N-k degrees of freedom (where N was the number of experiments and k the number protocols tested: 24-8=16). Effects were then expressed as a range of values, for example \( x_a \pm \) confidence interval. If the interval contained the value zero, the effect was considered as insignificant.
2.7.4.3 Statistical Model Equation

For each parameter, if the interval contained the value 0, the effect was considered as non-influential and was removed from the equation. The equation was proposed on the basis of the statistical model to anticipate or predict the evolution of a response according to the parameters. The response Y was defined as the sum of all significant parameters and interactions multiplied by their effect. Parameters were expressed as a centred variable (see next Section). The value x₀ corresponded to the average of all experiments.

\[ Y = x_0 + x_a \cdot A + x_b \cdot B + x_c \cdot C + x_{ab} \cdot A \cdot B + x_{ac} \cdot A \cdot C + x_{bc} \cdot B \cdot C + x_{abc} \cdot A \cdot B \cdot C \]

2.7.4.4 Natural variables and centred variables

In the model equation, the variables used should be centred variables. The range of centred variables was between -1 and +1 corresponding to the low and high levels in the experiment (Table 2.6). If the centred variable is denoted \( t \) and the natural variable is \( T \) (real value of the parameter), then to switch between natural and centred variables, the following formula is used, where \( T_0 \) represents the middle value between the low and high level and \( \Delta T \) is the difference between \( T_0 \) and the low value:

\[ \text{Table 2.5 Calculation of the confidence interval} \]

<table>
<thead>
<tr>
<th>Extractions</th>
<th>Standard deviation ( s_i )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction 1</td>
<td>( s_1 )</td>
</tr>
<tr>
<td>Extraction 2</td>
<td>( s_2 )</td>
</tr>
<tr>
<td>Extraction 3</td>
<td>( s_3 )</td>
</tr>
<tr>
<td>Extraction 4</td>
<td>( s_4 )</td>
</tr>
<tr>
<td>Extraction 5</td>
<td>( s_5 )</td>
</tr>
<tr>
<td>Extraction 6</td>
<td>( s_6 )</td>
</tr>
<tr>
<td>Extraction 7</td>
<td>( s_7 )</td>
</tr>
<tr>
<td>Extraction 8</td>
<td>( s_8 )</td>
</tr>
<tr>
<td>Variance ( s^2 )</td>
<td>( \sum s_i^2 / 3 )</td>
</tr>
<tr>
<td>Interval</td>
<td>( 2.12 \sqrt{s^2 / 24} )</td>
</tr>
</tbody>
</table>
59

\[ t = \frac{T - T_0}{\Delta T} \]

Table 2.6 T0, \( \Delta T \), natural and centred variables for each parameter studied

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Time</th>
<th>Temperature</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural variables</td>
<td>1hr</td>
<td>25°C</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6hrs</td>
<td>70°C</td>
<td>2</td>
</tr>
<tr>
<td>Centered variables</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
</tr>
<tr>
<td>T0</td>
<td>3.5hrs</td>
<td>47.5°C</td>
<td>3.5</td>
</tr>
<tr>
<td>( \Delta T )</td>
<td>2.5hrs</td>
<td>22.5°C</td>
<td>1.5</td>
</tr>
</tbody>
</table>

2.8 evaluation of prebiotic activity

Prebiotic activity of activity of seaweed extracts was evaluated using a microplate bioassay system developed and optimised in the Tuohy Laboratory, NUI Galway to test the effects of plant-derived extracts on microbial growth, in a statistically relevant manner.

2.8.1 Preparation of polysaccharide-rich fractions from crude extracts

In order to investigate more the role of seaweed polysaccharides in bacterial growth, a number different fraction were prepared: Fraction \( S_0 \) (crude extract with no purification step), Fraction \( S_1 \) (the S0 fraction dialysed at 2 kDa MWCO using UF), \( \text{Alginate-rich} \) fraction, \( \text{fucoidan-rich} \) fraction and \( \text{laminaran-rich} \) fraction. Preparation of these fractions was as follows:

A 35 mL aliquot of the S0 extract was dialysed by using a 2 kDa MWCO UF unit to remove salts and small molecules like mannitol to obtain Fraction \( S_1 \). In parallel, another aliquot of the S0 fraction was mixed with glacial acetic acid to reach pH2. This step allowed recovery of the \( \text{alginites} \) by precipitation. After centrifugation, the supernatant was retained for preparation of the fucoidan fraction, and the pellet was resuspended in 0.5 N NaOH until all of the pellet was dissolved (and the pH returned to neutral). The solution was then dialysed using a 2 kDa MWCO filter and freeze dried.

The supernatant (after alginate precipitation) was subjected to precipitation by ice-cold ethanol (-20°C) to recover the fucoidan. Two volumes of ice-cold ethanol were added to one volume of the supernatant. After centrifugation, the precipitated \( \text{fucoidan} \) was recovered in water, dialysed (2 kDa MWCO) and freeze dried. Excess ethanol in the supernatant was evaporated under vacuum. The concentrated \( \text{laminaran-rich} \) extract was also dialysed and freeze dried until required.
2.8.2 Bacterial cultures

Bacterial cultures were received either as lyophilized cultures or as agar plate stocks. Lyophilized cultures were resuscitated according to the instructions received from the culture collection (i.e. DSMZ, Germany or NCIMB, UK). For routine cultivation and preparation of inocula for liquid broth cultures MRS-agar was used as an optimum agar support medium for the cultivation of Lactobacilli strains and LB-agar for the opportunistic pathogens (Oxoid, Hampshire, UK). Plates were inoculated aseptically (plate streaking technique) with approx. $1 \times 10^6$ cfu/mL from resuspended stocks, using a sterile loop. Cultures were incubated at 37°C for approximately 12-24 h. Where agar plate culture stocks were received, a single colony was taken and used to aseptically inoculate a fresh agar plate.

Two further cycles of fresh agar plate cultures were prepared to generate active seed cultures for the inoculation of liquid media (broth cultures).

A single colony was transferred to approx. 25 mL of the appropriate broth medium (see Table 2.7), and incubated at 37°C overnight, with shaking at 200 rpm, under aerobic conditions. Subcultures were prepared daily by inoculating a fresh 25 mL of appropriate sterile broth media with 200 µL of the overnight culture.

Three species were used for the first screening: *Escherichia coli* is the main potentially pathogenic strain used for the prebiotic activity (O’Sullivan et al. 2010), *Pseudomonas aeruginosa* is known to digest the alginates (Farrell & Tipton 2012) and *Lactobacillus plantarum* is the most beneficial strain used (Tsuda & Miyamoto 2010; Gupta et al. 2011). Details on the optimum growth conditions for those strains are summarised above in Table 2.7. To standardise the inoculum density for the susceptibility (or bioactivity) test, the culture were grown to an optical density of between 0.08 and 0.13 which corresponded to a bacterial cell concentration between 1 and $2 \times 10^8$ cfu/mL. This approach (i.e. seed culture density for bioactivity testing) has been validated and published by the clinical and laboratory standards institute (Clinical and Laboratory Standard Institute 2006).

<table>
<thead>
<tr>
<th>ATCC number</th>
<th>name</th>
<th>Optimum growth media</th>
<th>Optimum growth temperature</th>
<th>biosafety level</th>
<th>oxygen requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25922</td>
<td><em>Escherichia coli</em> (gram-)</td>
<td>LB broth</td>
<td>37°C</td>
<td>1</td>
<td>aerobic</td>
</tr>
<tr>
<td>ATCC 10145</td>
<td><em>Pseudomonas aeruginosa</em> (gram-)</td>
<td>LB broth</td>
<td>37°C</td>
<td>2</td>
<td>aerobic</td>
</tr>
<tr>
<td>ATCC 14431</td>
<td><em>Lactobacillus plantarum</em> (gram+)</td>
<td>MRS broth</td>
<td>37°C</td>
<td>1</td>
<td>aerobic</td>
</tr>
</tbody>
</table>
2.8.3 Bioactivity tests to assess the effects of the seaweed extracts and enriched fractions on bacterial growth and culture viability

All experiments were performed under a strictly sterile environment. All solutions were sterilized in an Autoclave at 105°C for 30 minutes and bacterial cultures were manipulated under a laminar flow hood (validated DanLaf hood fitted with carbon and Hep filters). The growth response of bacterial strains to a selection of seaweed extracts was evaluated over a minimum of a 24 hour bacterial growth assay.

Briefly, 20 µL of a seed bacterial culture at the right concentration (see above) was inoculated into 180 µL minimal medium M9 (description of growth media used is provided in Appendix 3) containing 0.1% w/v of the test sample or control prebiotic compounds as the only added carbon source, in replicate wells in a sterile 96-well microplate. The bacteria were then grown at 37°C for 24-48 h, in a GeNIOS system (Tecan), with periodic shaking (200 rpm). The OD at 595 nm of the cultures was evaluated every 30 min using software that is part of the GeNIOS system (Tecan). This system allowed four different measurements to be taken per well (four different locations in the well) and calculated the average of the 4 readings to have a good representation of the culture OD in a given well.

A total of 40 fractions, all isolated from *A. nodosum* (8 different extraction protocols as explained in Section 2.7.3 and 5 fractions per extraction as explained in Section 2.8.1) were evaluated, along with cultures grown on a standard prebiotic carbohydrate fraction, fructo-oligosaccharides (FOS).

In addition the growth response to glucose as the sole carbon source was also evaluated (positive control culture). Growth curves for each strain in response to each compound were generated (OD595 vs time). Each sample was evaluated in triplicate (minimum) per assay (a template of a typical plate layout is shown in Appendix 4). At the end of the experiment, plates were stored at 4°C and collected or delivered daily to an external laboratory (CLS: Complete Solution Laboratory, Ros Muc, Ireland) to have an independently validated assessment of colony counts (enumeration of live bacteria) by plate counting.

Cultures were also sent to CLS periodically to validate purity. In addition, in house checks were conducted by Polymerase chain reaction (PCR)-based approaches using universal oligonucleotide primers designed to amplify specific regions of the DNA (referred to as rDNA) encoding the 16S rRNA subunit. PCR products were purified and sent for sequence analysis to LG Genomics (Germany). Bioinformatics analysis of the sequence data was used to confirm the identity of the sequence, and consequently the bacterium.
2.8.4 Statistical analysis of bacterial growth

Bacterial growths, based on measurement of the optical density over time, were statistically analysed using Minitab software. First of all, the three growth phases, the lag phase (phase during which the bacteria needed to adapt to the media before starting to grow actively), the log phase (phase during which the bacteria are extensively growing) and the stationary phase (phase during which the growth was finished or remained very slow) were defined by comparing the optical density pairwise, in a one way ANOVA. During the lag phase, the growth was minimal giving a low OD variation between two points. During the log phase, the growth was fast and optical density statistically varied between time points and, finally, the OD was almost stable during the stationary phase. When the growth phases were defined, the optical densities obtained were compared to evaluate the extracts. Finally, trends were analysed to define the impact of the most influential extraction parameters on the bacterial growth. A statistical analysis of the different growth phases is described later in Section 6.2.3.1.

2.9 References

Clinical and Laboratory Standard Institute, 2006. Methods for broth dilution susceptibility testing of bacteria isolated from aquatic animals; Approved guideline, Dionex, Product Manual for ASRS 300 and CSRS 300,


O’Connell, E., 2008. *Purification and characterisation of an algal polysaccharide (fucoidan) and enzymes involved in modification of this polysaccharide*. National University of Ireland, Galway.


Chapter 3: Application of an existing method for extraction of seaweeds and comparison of approaches to recover enriched polysaccharide fractions


3.1 Introduction

Even though similar compounds are found in almost all of the brown seaweeds, their relative proportions are often different and vary according to the species (Connan et al. 2004), season (Adams et al. 2011), localisation and other environmental factors (reviewed by Stengel et al. 2011). Furthermore, the extraction technique is also a key factor and different protocols regularly yield different extracts with respect to composition (Ponce et al. 2003; Chee et al. 2010). Comparison of extracts from different seaweed species, using the same extraction protocol frequently reveals differences (Rioux et al. 2007). Moreover, significant inter-species differences can exist in polysaccharide compositions as note for fucoidans. Indeed, fucoidans have been reported to be mainly composed of fucose and sulphates in *Fucus vesiculosus* (Nishino et al. 1994) whereas, additional monosaccharides are found in fucoidan extracted from *Ascophyllum nodosum* (Marais & Joseleau 2001; Li et al. 2008). Furthermore, fucoidan composition had been reviewed to vary a lot and is dependent on parameters such as seaweed species, seasonality and extraction process (Ale & Meyer 2013). Furthermore, purification processes can also affect polysaccharide yield and/or composition (Paskins-Hurlburt et al. 1978).

In this study a known extraction method and different approaches for the production of enriched algal polysaccharide fractions from different brown seaweeds were investigated. The extraction of fractions rich in polysaccharides, especially fucoidans, was conducted using a protocol developed previously in the Tuohy laboratory (O’Connell 2008; Foley et al. 2011). In the first part of the study, the method was used to compare biomolecule yields and composition from some of the main brown seaweeds found on the west coast of Ireland, *Ascophyllum nodosum, Fucus vesiculosus* and *Laminaria digitata*. This protocol involves a multi-step procedure using different solvents (ethanol and water). The first investigation aimed to identify the best seaweed species to use to extract sugars. In parallel, the contaminant levels (polyphenols, pigments) in fractions recovered from each step of the extraction were determined. The final stage of the extraction protocol involves a step that recovers fucoidans by precipitation of alginates using calcium chloride. Two different insoluble forms of alginate were precipitated: alginic acid and calcium alginate. A comparison of calcium chloride and acetic acid as precipitating agents was performed to see the effect of those treatments on the yields of other sugars (fucoidans, laminaran and mannitol). Moreover, the main contaminants found in the extracted fractions were phenolic compounds. A comparison of hydrophobic resins able to adsorb these compounds was conducted and the effect on the percentage of sugar recovered was determined. Finally, a preliminary investigation on the effect of the duration of extraction on the yield of different
compounds and laboratory versus industrial scale extractions was completed. The findings presented in this chapter represent initial research that informed the focus of the research in each following chapter. Consequently, the main outcomes of the research in this chapter were reinvestigated more in detail and validated in the following chapters.
3.2 Results

3.2.1 Comparison of seaweed species for extraction of fucoidan

A multi-step extraction involving two successive ethanolic extractions (S1 and S2) and three successive aqueous extractions (S3, S4 and S5), as described in Extraction protocol 1 (see Section 2.2.1) was used to extract fucoidan independently from three seaweed species harvested in An Spiddeál in June and processed in the laboratory (as explained in detail in Section 2.1.1): *A. nodosum*, *F. vesiculosus* and *L. digitata*. The results of the extraction process on total carbohydrate (or sugar) yields, from each seaweed, are presented in the following sections. The total carbohydrate concentration in extract samples was determined using the phenol-sulphuric acid method (Section 2.5.3.1).

3.2.1.1 Analysis of sugar yields after each extraction step

*Figure 3.1* shows the total sugar yields following extraction, which are expressed as the percentage of total sugar relative to the seaweed dry weight (*Figure 3.1-A*). Dry weights were determined after each extraction step on the different seaweed species (i.e. the extract was dried and the weight of the solids recovered was obtained; see Section 2.5.1). The quantity of sugar extracted was expressed in mg per gram of dry seaweed and the values are given in the inset table (*Figure 3.1-B*). Yields obtained after ethanol extraction steps (S1 and S2) were low with the average value being slightly higher (1.14%) for the higher extraction temperature (S2) than for the corresponding room temperature extraction (0.85%).

![Figure 3.1 A) Comparison of sugar yields (as a % of dry seaweed) obtained in extraction steps used in Extraction Protocol 1 for F. vesiculosus, L. digitata and A. nodosum, and B) Quantity of sugar extracted (in mg) per gram of dry seaweed (n=3)](image-url)
Moreover, sugars extracted during S1 and S2 phases were mainly monosaccharides, especially sugar alcohols like mannitol as shown in *Figure 3.2*.

This figure shows representative High Performance Anion Exchange (HPAE) chromatograms for the S1 (A) and S2 (B) fractions (the HPAEC profile for these fractions were similar between the three species). The difference in the Y axis scale (which is proportional to the sugar concentration) between both chromatograms confirmed the higher sugar content in S2, and also revealed that there was 5 times more mannitol in S2 than S1 (area under peak was 32.7 and 149.8 nC*min respectively). Some other monosaccharides such as galactose were also found in these two fractions and even, likewise galactose at a similar concentration. In the aqueous extracts (S3, S4 and S5), sugar extraction yields were higher than the previous two steps. Despite differences in the extraction conditions, some yields appeared to be similar (*Figure 3.1-B*).

*Table 3.1* summarises the statistical analysis of the sugar concentrations and shows the *p* values obtained for the different fractions for the same species (A) and the *p* values between the species for the same extraction step (B). For *F.vesiculosus* (denoted FV), the maximum sugar yield was obtained after extraction S3 (6.08%) but significant yield differences were only observed between extracts S3 and S4. In comparison for *L.digitata*...
(LD), each extract gave a significantly different yield. A longer extraction at high temperature (S4) provided the highest sugar yield (5.93% of the total dry seaweed weight) and the lowest yield for the shorter extraction time (3% for S5). No significant differences were noticed between the stepwise yields for extracts from A. nodosum (AN). Each step allowed the extraction of approximately 6% total sugar (relative to the dry weight of seaweed). As Table 3.1-B shows, significant differences were obtained between species for the same extraction step. Between FV and LD, the only significant difference was obtained for the cold aqueous extraction step S3 although a difference (not significant) was observed between both seaweeds for yields obtained in fraction S5 (5.58% for FV and 3.01% for LD, Figure 3.1-B).

Extraction yields obtained with LD and AN were not significantly different across all of the extraction steps. However, a difference was noticed in the combined fraction, Sc (p value 0.004). As explained in Section 2.2.1, Sc is the solution mixture obtained by pooling S3, S4 and S5 (as will be seen in the following sections, Sc is used as the starting material for the recovery of enriched alginate and fucoidan polysaccharides). Finally, statistical differences were observed between FV and AN for the S4 and Sc fractions. Overall, the maximum yield of total carbohydrate was obtained using A. nodosum (Figure 3.1-B), with significant differences noted between this seaweed and the two other species (FV and LD).

3.2.1.2 Sugar analysis of the fucoidan rich fraction S6 fraction

Alginates were then precipitated from the combined Sc mixture (as mentioned previously, this mixture is a combined pool of the three aqueous extracts S3, S4 and S5) using calcium chloride (CaCl$_2$), as described in Section 2.2.1. The residual liquid fraction (called S6), was then analysed by a combination of hydrolysis with TFA (Section 2.5.7) followed by separation and quantification of the resulting monosaccharides by HPAEC (Section 2.5.8) to determine the fucoidan content (based on the quantities of relevant sugars present in the sample). The results showed that the S6 fraction was mainly rich in fucose and sugars known to be present in fucoidans (i.e. fucoidan-rich), with smaller amounts of sugars known characteristic of laminarans.

Figure 3.3 shows the yields of main sugars (i.e. mannitol, fucoidans and laminarans) still present in solution after precipitation of the alginates. The yields of the respective sugars are based on HPAEC data, and values are expressed on a weight basis relative to the dry weight of seaweed used for the initial extraction. For each remaining carbohydrate fraction, the relative quantities present were different according to the seaweed used. A maximum mannitol yield of above 1% was observed for L. digitata. With A. nodosum, the average mannitol yield was 0.84% and was not statistically different than the values obtained for the
other species ($p = 0.082$ and $p = 0.078$ for comparisons with $L. digitata$ and $F. vesiculosus$, respectively). Mannitol levels were statistically different only between FV (average = 0.6%) and LD with a $p$-value of 0.008. The laminaran yield was more uniform between the species with average values of 0.96%, 0.76% and 1.08% for $F. vesiculosus$, $L. digitata$ and $A. nodosum$, respectively. Statistical analysis revealed a significant difference only between $L. digitata$ and $A. nodosum$ ($p=0.04$). In contrast, all species gave statistical differences for the comparative fucoidan levels. The highest yield was obtained with $A. nodosum$ (2.08%) while the lowest yield was observed for $L. digitata$ (0.62%). An intermediate value of 1.65% was obtained for the $F. vesiculosus$ fucoidan yield.

### 3.2.1.3 Analysis of the monosaccharide composition of the fucoidan-rich S6 fraction

The relative proportions of the individual monosaccharides present in the S6 sample were investigated. After dialysis (10 kDa MWCO) as described in Section 2.2.1, the fucoidan-rich S6 monosaccharide composition was determined by TFA hydrolysis followed by separation and analysis of the monosaccharides present by HPAEC (see Sections 2.5.7 and 2.5.8). HPAEC analysis using a CarboPac PA100 column did not allow separation of xylose from mannose. Figure 3.4 provides representative chromatograms for the S6 fucoidan-enriched fraction of $F. vesiculosus$ (A), $L. digitata$ (B) and $A. nodosum$ (C). The presence of fucoidan was confirmed by the fucose, galactose and xylose/mannose peaks. The presence of laminaran was indicated by the glucose and mannitol peaks. Unidentified
peaks appearing before the mannitol are probably due to other sugar alcohols that did not correlate with the standards used (brown seaweeds are known to contain different types of sugar alcohols, including some unusual compounds, e.g. the 7-carbon sugar alcohol Volemitol (Pfetzing et al. 2000)). The relative proportions of each sugar in the respective HPAEC profiles were investigated in detail to determine if the relative amounts of each monosaccharide in fucoidan, and the relative concentrations of fucoidan and laminaran, were similar or different between the three brown seaweeds.

Fucoidan levels, composed of fucose, galactose and xylose/mannose, were higher than laminaran (glucose) in the *F. vesiculosus* and *A. nodosum* S-6 extracts, in contrast to the *L. digitata* S-6 extract in which the two polysaccharides seemed to be present in similar relative proportions (*Figure 3.3*). In terms of the composition of fucoids from all three seaweeds, fucose was the main monomer in each case. The proportion of xylose + mannose was similar to the concentration of galactose in both the *F. vesiculosus* and *L. digitata* extracts but was higher in the corresponding *A. nodosum* sample. *Figure 3.5* shows the fucoidan composition, as a percentage of the total quantity of the relevant monosaccharides (histogram) and the molar composition. Calculations of the polysaccharide composition from the raw data are presented in Appendix 5. Mass analysis.
showed significant differences between the three species. As mentioned, fucose was the main monomer in each case, with a maximum of 79.2% in *F. vesiculosus* and a minimum of 58.3% in *A. nodosum*. Galactose and xylose/mannose were present in similar mass proportions in *F. vesiculosus* (9.23% and 11.59%) and *L. digitata* (13.93% and 15.48%) enriched fucoidan samples, whereas, in *A. nodosum*, the proportion of galactose was lower than xylose/mannose (6.16% versus 35.6%). Molar ratio analysis, expressed relative to fucose (assigned a value of 1), confirmed the mass analysis. Indeed, the molar proportions of galactose and xylose/mannose were similar in *F. vesiculosus* and *L. digitata*. The main difference between these two species resided in the fucose concentration (see Figure 3.5). Moreover, the galactose content (as a molar composition) was almost identical in Fucus and Ascophyllum (0.1).

![Figure 3.5 Mass and molar ratios for the monosaccharide composition of fucoidan extracted from the different seaweed species (n=3)](image)

No correlation could be made concerning the mannitol due the dialysis process that removed the small compounds (*Figure 3.4*). Dialysis should have removed all of the free mannitol in the S6 samples. Therefore, the mannitol in the chromatograms should be the levels released from laminaran; however, as this could not be determined absolutely (some mannitol can remain after dialysis), assumptions were not made in terms of the origin or relevance of the mannitol concentration.
A. nodosum is the only seaweed that is processed industrially in Ireland and the results obtained in this study have showed that there is a higher amount of extractible fucoidan in this species. Taking these two aspects into consideration, further investigation on the extraction process which is presented in the following sections was done only on industrially-processed A. nodosum.

3.2.2 Analysis of the composition and yields in intermediate fractions

Extraction protocol 1 (see Section 2.2.1) was done in duplicate on the same seaweed batch of A. nodosum. While this protocol was aimed at the extraction of polysaccharides (complex sugars), other compounds (contaminants) were extracted. This section focuses on a more detailed analysis of the S1-S6 fractions generated in protocol 1 with the purpose of investigating levels of some of the main contaminants (e.g. phenolic substances) present. Each analysis was performed in triplicate on each fraction. General measurements (volume, solids content of solutions, etc.) were investigated in parallel with sugar and phenolics contents to evaluate the reproducibility of the extraction.

3.2.2.1 General properties and total extraction yields

Both extractions were done on 100 g of dry seaweed. Table 3.2 shows the general properties of the fractions in terms of volumes recovered and solids content of solution.

Solution S1 was obtained using 100 g dry seaweed and 1 L of 80% (v/v) ethanol over an extraction period of 12 h. Approximately 750-770 mL of solution was recovered after 12 h of extraction at room temperature, meaning that around 250 mL was required to hydrate 100 g of seaweed. Despite similar volumes obtained for the replicates at each extraction step, differences were noted in the solids content values, especially for fractions S4 and S5. Unlike the aqueous extracts, the fractions obtained by extraction with ethanol

<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>Solid content</th>
<th>Volume (ml)</th>
<th>Solid content</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S1</strong></td>
<td>770</td>
<td>0.34% +/- 0.02%</td>
<td>750</td>
<td>0.57% +/- 0.21%</td>
</tr>
<tr>
<td><strong>S2</strong></td>
<td>1085</td>
<td>1.45% +/- 0.16%</td>
<td>1150</td>
<td>1.36% +/- 0.11%</td>
</tr>
<tr>
<td><strong>S3</strong></td>
<td>1130</td>
<td>1.16% +/- 0.31%</td>
<td>1300</td>
<td>1.01% +/- 0.25%</td>
</tr>
<tr>
<td><strong>S4</strong></td>
<td>1046</td>
<td>1.14% +/- 0.27%</td>
<td>1073</td>
<td>0.72% +/- 0.01%</td>
</tr>
<tr>
<td><strong>S5</strong></td>
<td>1820</td>
<td>0.83% +/- 0.21%</td>
<td>1800</td>
<td>0.45% +/- 0.24%</td>
</tr>
<tr>
<td><strong>Sc</strong></td>
<td>3890</td>
<td>0.79% +/- 0.15%</td>
<td>4059</td>
<td>0.83% +/- 0.09%</td>
</tr>
</tbody>
</table>
were more concentrated after high temperature extraction and had the highest solids contents overall (1.4% solids content). For the aqueous extracts, the solids content values were quite heterogeneous between the replicates (except for fraction S3). The final combined solution S\text{combine} (Sc) contained around 0.8% solids.

Figure 3.6 shows the total extraction yields obtained after each extraction step (the total extraction yield is the ratio of the total solids content in the liquid extracts relative to the quantity of dry seaweed used for the extraction, as explained in Section 2.6.4). Good reproducibility was observed in the first three steps of extraction. For both fractions S4 and S5, the first extraction seemed to have extracted more solids, even though no statistical differences could be identified. The effect of temperature seemed to be significant in the ethanol extraction steps contrary to the aqueous extractions. The lowest extraction yield was obtained for the S1 step (less than 5%). The yields in the steps thereafter were between 10 and 15% despite different parameters in terms of time and temperature. At the end of the process, around 4 L of the final extract (S\text{combine}) containing 0.8% solids were recovered. Total extraction yields for the aqueous phase reached more than 30%. Using this multi-step protocol, around 50% of the total seaweed dry weight was extracted (S1+S2+Sc). Further analyses were required to find out which compounds were extracted in each step.
3.2.2.2 Total carbohydrate extraction

Total carbohydrate present was quantified using the Dubois method (see Section 2.5.3.1) using a mixture of monosaccharides as standards (fucose + glucose + galacturonic acid which are, or are similar to, monomers in fucoidan, laminaran and alginates, respectively).

As shown previously, Figure 3.7 shows that more sugars were extracted in the aqueous extractions than in the ethanolic steps. No statistical differences were observed between the replicates of each extract. Moreover, results obtained on these batches were very similar to the previous extraction presented in Section 3.2.1.1. More than 20% of the total carbohydrate present in the starting seaweed sample was extracted during the protocol (S1 + S2 + Sc) with more than 90% of this amount in the aqueous phases (Sc). Ethanolic extraction at high temperature extracted 5 times more carbohydrates than the room temperature extraction with ethanol. For the aqueous extracts, the extraction yields were more similar regardless of time and temperature of extraction. Figure 3.8 shows the total carbohydrate proportion in the solids recovered from each fraction. Although the yields were different in the ethanolic extracts, the total carbohydrate content of S1 and S2 was quite similar at ~18%. In contrast, the total carbohydrate contents relative to the solids content values of the aqueous extracts were different. At room temperature (S3), the total carbohydrate level reached 40% of the total solids content. At higher temperature, extraction duration seemed to have an effect on the proportion of total carbohydrate recovered. Indeed,
the S4 fraction (8 h extraction) was composed, on average, of 55% total carbohydrate. In comparison, the S5 fraction contained 42% total carbohydrate. In the Scombine fraction, the average total carbohydrate content reached 59% of the dry matter.

### 3.2.2.3 Pigment extraction

Different contaminants were also extracted during the successive extraction steps of protocol 1. The main contaminants were phenolic substances, free monosaccharides and sugar alcohols (not covalently part of polysaccharide or glycoconjugate structures) and pigments (quantified by HPLC as described in Section 2.5.4). Table 3.3 shows the pigment concentrations in µg/mL in each extract. The HPLC chromatograms showed specific peaks for chlorophyll A, chlorophyll c2 and fucoxanthin, as well as fragments of these pigments (labelled Chl a deriv and fuco deriv in the table). Following the S1 extraction, different pigments were found in the extract: chlorophyll A, fucoxanthin, chlorophyll c2 and also some other β-carotenes. Extractions were performed in a clear glass beaker so the extracts were not protected from light, which would induce damage to the pigments. Approximately 50% of the chlorophyll A extracted was damaged after the first step of extraction (correlating with the levels of chl a derivative). Fucoxanthin was more resistant to light-induced damage and 90% was still intact (see amounts of Fucoxanthin versus fuco deriv). After the S2 extraction, at high temperature, all of the chlorophyll A extracted was degraded due to the light and the temperature (chlorophyll A is thermosensitive). Fucoxanthin was not degraded more during this step (approx. 10% fuco deriv). The other pigments were extracted
more effectively during the first step than the second one. Chlorophyll c2 was 10 times less concentrated than the chlorophyll A and the fucoxanthin represented more than 50% of the total carotenoids detected. No pigments or β-carotenes were extracted during the aqueous phases, as could be expected due to their lipophilic chemistries. The values obtained for extract S3 may be due to remaining ethanol in the seaweed biomass after extraction S2. The total pigment reached respectively 1% and 0.09% of total solids in extracts S1 and S2. Yield of pigment extracted (S1 + S2) was 0.05% of the dry seaweed.

### 3.2.2.4 Phenolic substances

The other contaminant extracted was the phenolic substances. The yield of total phenolic substances extracted was between 4.5 and 5.0% (S1 + S2 + Sc). Figure 3.9 shows the repartition of phenolic substance extraction yields across the different extraction steps. Similar to observations with the extraction of carbohydrates, the ethanol extraction step at high temperature yielded more phenolic substances than the solvent step at room temperature. Moreover, solution S2 had the highest yield which represented approx. 46% of the total quantity of phenolic substances extracted (S1 + S2 + Sc) in comparison to 6% for the S1 fraction. The remaining amount was extracted during the aqueous extractions, with 23%, 14% and 11% for S3, S4 and S5, respectively. The quantities of phenolic substances extracted decreased after aqueous extraction step, with a final yield of 2-3% of the seaweed dry weight. The yield of phenolic substances (i.e. yield per dry weight of seaweed) was higher in the Sc combined solution than in the S2 extraction step, and although the replicate

<table>
<thead>
<tr>
<th>Pigments (µg/mL)</th>
<th>Extraction</th>
<th>Chlorophyll A</th>
<th>Chl a deriv</th>
<th>Fucoxanthin</th>
<th>Fuco deriv</th>
<th>Chl c2</th>
<th>β-carotenes</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>1</td>
<td>12.013</td>
<td>10.414</td>
<td>3.279</td>
<td>0.591</td>
<td>1.196</td>
<td>5.207</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>13.871</td>
<td>12.070</td>
<td>7.393</td>
<td>0.705</td>
<td>1.336</td>
<td>6.790</td>
</tr>
<tr>
<td>S2</td>
<td>1</td>
<td>n.d</td>
<td>8.115</td>
<td>3.451</td>
<td>0.274</td>
<td>0.527</td>
<td>1.005</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>n.d</td>
<td>8.781</td>
<td>4.264</td>
<td>0.330</td>
<td>0.613</td>
<td>0.851</td>
</tr>
<tr>
<td>S3</td>
<td>1</td>
<td>n.d</td>
<td>0.414</td>
<td>0.177</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>n.d</td>
<td>0.432</td>
<td>0.155</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>Sc</td>
<td>1</td>
<td>n.d</td>
<td>0.160</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>n.d</td>
<td>0.153</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
</tr>
</tbody>
</table>
extractions yielded similar results for S3-S5, differences were observed in the yield for the Sc fractions for both replicate extractions (see Figure 3.9).

In terms of phenolic substance content relative to the solids content of each of the extracts, the results showed that the ethanol-extracted fractions were more enriched in phenolic substances than the aqueous-extracted fractions, as shown in Figure 3.10. The solids content of the first extract contained up to 10% by weight of phenolic compounds.

![Figure 3.9](image1.png)  
**Figure 3.9** A) Phenolic substance ds in intermediate extraction solutions of A. nodosum and B) quantity of polyphenol extracted (mg) per gram of dry seaweed (n=3)

![Figure 3.10](image2.png)  
**Figure 3.10** Proportion of phenolic substances relative to the total solids content for all of the intermediate extraction steps for A. nodosum (n=3)
while the corresponding values for the second extract (S2) reached more than 12%. The effect of temperature seemed to vary according to the extraction solvent used. Indeed, the higher temperature ethanol extraction gave higher yields and higher levels relative to solids content whereas, in water, an opposite phenomenon was observed. The phenolic content of the first aqueous extract was 6-8% on a dry solids basis, decreasing to between 4 and 6% for S4 to a final phenolic content of 2-4% for the last extraction step (S5). Finally, on a dry solids basis, the phenolic substances represented between 7 and 9% of the total solid content in the Sc solution.

### 3.2.3 Comparison of methods for alginate precipitation

Two insoluble forms of alginates were be generated from the Sc fraction, one acidic form, alginic acid, while the second form was calcium alginate. Therefore, two types of alginates were present that could be precipitated in acid or calcium-rich media. Adding chemicals to seaweed extracts could affect the solubility and recovery of other compounds, especially polysaccharides. For this reason, investigation of alginate precipitation (see Section 2.4.1) on other polysaccharides was performed to anticipate the potential loss of other sugars (e.g. fucoidans) in the S6 fraction. The extract used for the precipitation was prepared as described in a time course extraction protocol (see Section 2.2.2), which involved aqueous extraction for 24 h at 70°C. Figure 3.11 shows the resulting extract after addition of acetic acid (A) and CaCl₂ (B) before shaking to mix the contents. The mix of extract and acetic acid is quite homogenous and the precipitate is throughout the solution. In contrast, in the solution with CaCl₂, the precipitate was localised only in the upper layer or phase, probably due to an increase in solution density.

Figure 3.12 shows the fucoidan, mannitol and glucose content (to provide an estimation of laminaran content) of the resulting precipitates and supernatants from both precipitation methods. Each precipitation was repeated in triplicate and the analysis done on both fractions (supernatant and pellet) for each replicate. However, the analysis of the starting extract had been conducted only once so no standard deviation could be calculated for this fraction. A 5mL quantity of starting extract contained 14.51 mg of fucoidan, 6.02 mg
of mannitol and 1.67 mg of glucose was mixed with 5 mL acid or calcium rich medium. After precipitation, in both cases (acetic acid and CaCl₂), the supernatants contained around

![Bar chart showing carbohydrate composition](image)

Figure 3.12 Carbohydrate composition of the seaweed extracts before and after two different treatments for alginate precipitation (n=3, except for the fraction extract)

9.3 mg of fucoidan, which corresponded to 64% of the total fucoidan, with higher standard deviations for the acid precipitation. Concerning the glucose levels, acetic acid and CaCl₂ treatments precipitated 23% and 30% of the ‘laminaran’, respectively. The main difference was observed for mannitol. Indeed, CaCl₂ treatment did not precipitate mannitol. With acid, 20% of the total mannitol was precipitated. To summarise, 75% of the total mannitol + fucoidan + glucose content was recovered in the supernatant after calcium chloride precipitation in comparison with 69.7% when using acetic acid. Use of either solvent did not seem to affect total fucoidan content but did affect the composition of the fucoidan recovered in supernatant (see Figure 3.13).

For that reason, the monosaccharide composition of fucoidan in each fraction was investigated as shown in Figure 3.13. Monosaccharides were analysed by HPAEC using the protocol 2 described in Section 2.5.8. Monosaccharides were separated on a CarboPac SA10 column and allow the separation of xylose and mannose contrary to the results presented in Section 3.2.1.3. Fucoidan in the starting extract contained 53.5% fucose, 8.4% galactose, 27.9% xylose and 10.2% mannose. After acid treatment, the proportion of fucose was slightly higher (57%) in the pellet and slightly lower (51.7%) in the supernatant. Proportionally, the xylose and mannose content were higher in supernatant (28.6% and 11.2%) and lower in pellet (26.5% and 8.4%). The galactose was similar between the fractions (8.5% in supernatant and 8.1% in the pellet). Using CaCl₂, greater differences were
noticed. Indeed, the main compound type of fucoidan precipitated was richer in xylose (44%) than fucose (38.3%). This fucoidan was also richer in galactose (10.9%) and poorer in mannose (6.8%) comparing to the fraction before precipitation. The proportions of fucose and mannose in the supernatant fucoidan were the highest between all fractions (Figure 3.13), with values of 62.1% and 12.1%, respectively. Equivalently, galactose and xylose were the lowest with accounting for 7% and 18.8% of the total monosaccharides quantified. Overall acid treatment to precipitate alginate did not appear to affect the average composition of fucoidan, contrary to calcium treatment, following which the composition of recovered fucoidan was different.

3.2.4 Adsorption of phenolic substances using amberlite resins

Previous studies have shown that amberlite resins can be used to adsorb phenolic compounds from plants (D’Alvise et al. 2000; Gray 1978). In this study, different types of amberlite resins were investigated for their potential to adsorb phenolic compounds present in the seaweed extracts with the aim to see if this approach could be used to remove residual phenolic substances from the carbohydrate-rich extracts. As phenolic substances can have reported bioactivities (Queguineur et al. 2012), their removal would be essential prior to tests to determine the potential bioactivity of the seaweed carbohydrates (e.g. fucoidan, alginate or laminaran).
3.2.4.1 Optimisation of the best quantity of dry resin / volume of extract ratio

The extract used to investigate the potential of adsorption chromatography for removal of phenolic substances was prepared according to Protocol 3 (scaled-up extraction, described in Section 2.2.3) and processed by ultrafiltration using a 1 kDa (MWCO PES membrane) to remove salts and small molecules. To investigate adsorption of the phenolic substances to the resin, the adsorption experiment was conducted in a batch-wise format.

Table 3.4 Volume of extract recovered according to the quantity of dry resin added to 20 mL of starting seaweed extract

<table>
<thead>
<tr>
<th>Ratio (% : amberlite quantity / volume extract)</th>
<th>Volume extract (ml)</th>
<th>Amberlite quantities (g)</th>
<th>Volume recovered (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>20</td>
<td>1</td>
<td>19.5</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>2</td>
<td>19.5</td>
</tr>
<tr>
<td>15</td>
<td>20</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>4</td>
<td>17.5</td>
</tr>
<tr>
<td>25</td>
<td>20</td>
<td>5</td>
<td>16.5</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>35</td>
<td>20</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>40</td>
<td>20</td>
<td>8</td>
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</tr>
<tr>
<td>45</td>
<td>20</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>50</td>
<td>20</td>
<td>10</td>
<td>13.5</td>
</tr>
</tbody>
</table>

Table 3.4 shows the quantities of dry amberlite resin XAD7 added to 20 mL of extract (as outlined in Section 2.4.2) and the volumes recorded after centrifugation.

A decrease in the liquid volume recovered was observed when the quantity of amberlite used was increased. Only a 0.5 mL loss in volume was observed for the 5% and 10% amberlite/extract ratios and 6.5 mL volume loss for the 50% ratio, which represents 32.5% of the starting 20 mL volume. Figure 3.14-B represents the volume recovered according to the quantity of amberlite added, and the slope value of -0.76 means that 0.76 mL of extract was required to hydrate 1g of dry resin. The quantities of phenolic substances were then quantified in each fraction and the percentage of compounds removed from the starting extract is shown in Figure 3.14-A, relative to the amberlite/extract ratio used. The quantities of phenolic substances removed increased significantly when the quantity of amberlite increased, especially up to a ratio of 30%. Indeed, with a ratio of 5%, 40% of the initial phenolic substances content was removed and 83% with a ratio of 30%. At higher amberlite/extract ratios, the percentage of phenolics removed continued to increase, but
more slowly and a maximum was reached at a ratio of 50% with 89% of the phenolic substances in the extract being adsorbed to the resin. As noted previously, a higher quantity of resin reduces the volume of extract recovered, so the best compromise appeared to be a ratio of 30% that gave a loss of 4 mL of extract with removal of 83% of the phenolic substances in the extract.

3.2.4.2 Evaluation of different amberlite resins for removal of phenolics from different extracts

Six amberlite resins were evaluated: IRA-900, XAD-4, XAD7HP, IRC-50, XAD-2 and XAD-16 (please refer to Section 2.4.2 for information on the resins). Tests were done on three different extracts obtained using Protocol 3 (Section 2.2.3). The crude extract, obtained after clarification was filtered using a 1 kDa (MWCO) PES membrane (solution 1). The filtrate was kept at 4°C and the retentate was subjected to another filtration at 10 kDa; both fractions from this step (retentate and permeate; solutions 2 and 3) were used. A ratio (resin/extract) of 30% was applied and phenolic substances and total carbohydrate contents (Sections 2.5.3.1 and 2.5.3.2) were quantified in each solution before and after adsorption to the resin. An efficiency score was calculated to identify the best resin to use (Section 2.4.2). This score was determined by dividing the quantity of polyphenols by the quantity of carbohydrate removed. A score >1 meant that the resin removed more polyphenols than sugar and the opposite for a score <1. Figure 3.15 shows the percentage of phenolic substances and carbohydrates removed by the respective amberlite resins for each fraction. All resins removed phenolic substances and sugars from the low molecular weight fraction (solution 1). The maximum levels of phenolic substances removed were reached using the amberlite XAD7HP resin, with 82% removal from the starting extract, while minimum
removal (6%) was obtained using the IRC-50 resin. With regard to carbohydrate levels, all resins removed at least 30% of the total amount in the extract, with the maximum (62%) observed for the XAD-16 resin. Resins XAD-4, IRC-50 and XAD-2 removed more sugars than phenolic compounds, while resins IRA-900 and XAD7HP seemed the best for removing phenolics without removing significant carbohydrates from the extract. For the fractions containing substances between 1 and 10 kDa in size (permeate; solution 2), fewer compounds were removed, in general; 11 to 37% for the sugars and from 11 to 74% for the phenolic substances. For this fraction as well, resin XAD7HP allowed most efficient removal of phenolic substances. Minimum sugar adsorption was obtained with the XAD-16 resin. For the permeate, the IRA-900 and the XAD-4 removed more sugars than phenolics. The best resin for this fraction seemed to be the XAD7HP and the IRC-500 (which adsorbed the smallest amount of carbohydrates. Finally for the higher molecular fraction (retentate from the 10 kDa UF step; solution 3), almost no phenolics were removed using the XAD-4, IRC-500 and XAD-2 resins. The XAD-4 resin did not remove any carbohydrates either. Maximum phenolics removal was obtained with the XAD7HP resin, with 40% removal of phenolics in the retentate. 

Table
3.5 shows the efficiency scores (percentage of polyphenol removed divided by the percentage of carbohydrates removed) calculated for each resin against the different fractions. Only two resins were identified to have a positive effect (removing more phenolics than sugars) for each fraction: XAD7HP and XAD-16. The XAD7HP resin had the best score for the fractions above 1 kDa molecular weight, while for the fraction with lowest molecular weight compounds present (<1 kDa), the IRA-900 resin was most efficient. No adsorption of phenolics from the high molecular weight fraction was obtained with the XAD-4 and IRC-50 resins.

Table 3.5 Efficiency score of amberlite resins for the different size fractions from the seaweed extract

<table>
<thead>
<tr>
<th>Extracts</th>
<th>XAD-2</th>
<th>XAD-4</th>
<th>XAD-7</th>
<th>XAD-16</th>
<th>IRC-50</th>
<th>IRA-900</th>
</tr>
</thead>
<tbody>
<tr>
<td>X&lt;1kDa</td>
<td>0.74</td>
<td>0.32</td>
<td>1.74</td>
<td>1.13</td>
<td>0.20</td>
<td>1.96</td>
</tr>
<tr>
<td>1kDa&lt;X&lt;10kDa</td>
<td>0.29</td>
<td>2.59</td>
<td>3.06</td>
<td>2.87</td>
<td>2.11</td>
<td>0.41</td>
</tr>
<tr>
<td>10kDa&lt;X</td>
<td>0.17</td>
<td>0.00</td>
<td>3.53</td>
<td>1.29</td>
<td>0.00</td>
<td>1.27</td>
</tr>
</tbody>
</table>

3.2.5 Microscopic analysis of the effects of time course extraction

A first investigation of effect of extraction duration (at 70°C) was performed on industrially processed seaweed (*A. nodosum*) harvested in June. At each time point (T0, T1, T2, T4, T6 and T24h), residual seaweed was separated from liquid by centrifugation and seaweed cells were fixed using primary fixative as explained in Section 2.3.1. Different analyses were done on the recovered liquid fraction to evaluate the comparative extraction of some soluble compounds.

3.2.5.1 Environmental Scanning electron microscopy (ESEM) of seaweed before and after extraction

Environmental scanning electron microscopy allowed direct investigation of the seaweed cell shape after extraction (wet samples, no sample pre-treatment or coating). Figure 3.16 shows representative segments of seaweed before (A and B) and after (C and D) 24 hours extraction at 70°C. Before extraction, meristoderm cells were well organised and could even be individually identified (*Figure 3.16-A*). Nevertheless, the industrial cutting process induced some damage as can be seen in *Figure 3.16-B*. Indeed, on the left part of this image, the meristoderm layer has been destroyed and cortex cells are visible. After extraction at high temperature (*Figures 3.16-C and D*), cells organisation has totally disappeared. These structural features have not been reported previously for industrially-cut seaweed segments before extraction.
3.2.5.2 Light microscopy of seaweed cells during the extraction time course

During the extraction time course, residual seaweed cells were fixed (as explained in Section 2.3.1) and subjected to wax cutting (Section 2.3.2) to obtain thin layer cells. Alcian blue was used to stain the acidic polysaccharides, such as fucoidans and alginates. Figure

![Figures 3.16 A) and B) ESEM images of seaweed surface before extraction. C) and D) ESEM images of seaweed surface after 24 h extraction at 70°C.](image)

![Figure 3.17 Light microscopy pictures of seaweed cells stained with alcian blue after A) 2 hours and B) 6 hours of extraction.](image)
3.17 shows the shape of the cells stained with alcian blue (see Section 2.3.2 and 2.3.3) after 2 (A) and 6 h (B) of extraction at 70°C. In the left image (2 h of extraction), the colour remained intense in the cells suggesting that a low amount of acidic polysaccharides had been extracted. After 6 h, the coloration was significantly less, suggesting that time is an important parameter for the extraction of acidic polysaccharides. Moreover, the coloration covered all the surface of the cells at the beginning of the extraction (2 h), while after 6 h, the coloration was mainly located at the middle lamella regions. The first polysaccharides extracted are probably located in the cell walls, while those in more intracellular and intercellular locations may require more time or a chemical treatment to be fully extracted.

By adding a counter stain (neutral red), all compounds that were not stained by the alcian blue will stain red. Once again, after 2 h of extraction, the coloration was still intense as shown in the left image in Figure 3.18. After 6 hours, the coloration was located mainly between the cells suggesting that the cell walls and intracellular compounds were extracted but the matrix polysaccharides (mainly composed by cellulose) were not.

3.2.5.3 Evolution of the release of compounds over time

A preliminary study of effect of extraction time was done for the release of selected compounds, i.e. mannitol, fucoidan and phenolic substances. The glucose concentration (in TFA-hydrolysed samples) was also followed over time to have an approximation of the effects on laminaran release. Figure 3.19 shows the concentration of the specified compounds over a 24 h extraction time period at 70°C. For the compounds investigated, time appeared to have an effect only on fucoidan concentration. Indeed, mannitol and glucose had a similar release profile, with a maximum concentrations reached after 1 h of extraction (1.35 and 0.39 mg/mL, respectively). A slight decrease was observed after 6 h but plateaued thereafter (1.20 mg/mL for mannitol and 0.34 mg/mL for glucose). Concerning the
release of phenolic substances, most of these compounds were extracted during the first hour (92% of the total concentration), but the concentration continued to increase slightly to finish at 2.58 mg/mL. Finally, in terms of the effects on fucoidans, the result showed that these polysaccharides were extracted throughout the duration of the extraction process. The first hour of extraction was also the most efficient and the concentration reached 0.81 mg/mL. Then, the extraction was linear up to the final 24 h time point and a final concentration of 2.90 mg/mL. The linear part of the fucoidan extraction profile had a slope of 0.098 which represented an extraction rate of almost 0.1 mg/mL/h.

3.2.5.4 Evolution of the monosaccharide composition of fucoidans during time-course extraction

As seen previously, fucoidans appeared to be the compounds most influenced by extraction time. Therefore, an investigation of monosaccharide composition of the total fucoidans extracted at time points during extraction was performed. Figure 3.20 shows the results obtained. As expected, fucose was the main monomer followed by xylose, then galactose and mannose in similar proportions (as noted previously in Section 3.2.3). The relative proportion of xylose was constant over time, starting at 28.4% after 1 h and finishing at 27.85% after 24 h. More differences were observed in the time-course release of the other three sugars. Indeed, the fucose content clearly increased over time. Fucoidan extracted after 1 h contained 43.5% fucose. Fucoidan extracted after 24 h was more than 10% richer in
fucose (final fucose concentration was 53.54%). Proportionally, mannose and galactose contents became lower with a decrease of 4.77% and 4.73% for mannose and galactose respectively which corresponded to the fucose increase (xylose variation was only ±0.59%).

The results obtained on effect of extraction obtained by this experiment were in correlation with the statistical analysis done in Chapter 5. Indeed, longer extractions tended to increase the fucose content, to decrease the galactose and mannose and had no effect on xylose. Detailed analysis (see Section 5.2.2), revealed that the extraction time was not significantly influential for most of the monosaccharide proportion in fucoidan.

### 3.2.6 Extraction scale up

As the ultimate goal of this research was to obtain fractions suitable for evaluation of prebiotic function in both *in vitro* and *in vivo* tests, the reproducibility of extraction at industrial scale and the influence of process parameters on the recovery of the target polysaccharides needed to be investigated. Extraction scale-up was performed in the OGT industrial processing plant, as described in extraction protocol 3 (Section 2.2.3). General parameters were followed including temperature, pH, conductivity and solids content of the recovered liquid fraction. Moreover, total sugar and total phenolics were quantified in liquid extracts recovered at each time point. The results are summarised in Table 3.6. Although the extraction was to be conducted at approx. 65°C (maximum feasible at scale), at T0, just after seaweed loading, a decrease in temperature to 47°C was observed. Temperature levels were back to c.65°C after 2 h and stayed constant until the end of extraction. The total solids
content at T0 was 2.17%, which may reflect extraction during the time taken to load all of the seaweed; 100 kg of fresh seaweed were loaded and it took between 15 and 20 min to achieve this operation. The solids content increased slowly thereafter and the difference between time points became less noticeable. After the first hour of extraction the solids content increased by 0.6% but only by 0.02% by the final timepoint, to finish at a final solids content in the extract of 3.30%. Extraction of seaweed compounds resulted in a slow decrease of pH from 6.2 to 5.7 after 7 h. Accumulation of charged compounds (salts and polysaccharides) in the extract clearly increased the conductivity very fast at the beginning of extraction, with a measurement at T0 of 9.05 mS/cm. After 7 h, conductivity was 10.02 mS/cm suggesting that most of the charged compounds, or compounds with high electronegativity, were extracted in the initial period. In terms of extract composition (expressed as percentage of total solid content in solution), as shown in the previous section, the content of phenolic substances remained relatively consistent between 9 and 10% in contrast to sugar concentration, which increased from 35% to 68% over time.

<table>
<thead>
<tr>
<th>Time</th>
<th>Temperature (°C)</th>
<th>Solid content</th>
<th>pH</th>
<th>Conductivity (mS/cm)</th>
<th>Sugar content</th>
<th>Polyphenol content</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>47</td>
<td>2.17%</td>
<td>6.2</td>
<td>9.05</td>
<td>35.66%</td>
<td>9.64%</td>
</tr>
<tr>
<td>60</td>
<td>49.9</td>
<td>2.77%</td>
<td>6.1</td>
<td>9.92</td>
<td>53.85%</td>
<td>8.95%</td>
</tr>
<tr>
<td>120</td>
<td>62</td>
<td>3.07%</td>
<td>6.0</td>
<td>10.04</td>
<td>58.73%</td>
<td>8.86%</td>
</tr>
<tr>
<td>180</td>
<td>64.6</td>
<td>3.15%</td>
<td>5.8</td>
<td>10.10</td>
<td>56.60%</td>
<td>10.11%</td>
</tr>
<tr>
<td>240</td>
<td>65</td>
<td>3.18%</td>
<td>5.9</td>
<td>10.20</td>
<td>56.53%</td>
<td>9.63%</td>
</tr>
<tr>
<td>330</td>
<td>65.5</td>
<td>3.28%</td>
<td>5.7</td>
<td>10.30</td>
<td>62.18%</td>
<td>9.94%</td>
</tr>
<tr>
<td>420</td>
<td>65</td>
<td>3.30%</td>
<td>5.7</td>
<td>10.05</td>
<td>67.66%</td>
<td>9.92%</td>
</tr>
</tbody>
</table>
3.3 Discussion

The first investigation was done to define the best seaweed species to extract polysaccharides. Different works showed differences between the species in terms of extract composition using the same protocol (Rioux et al. 2007). Differences were noticed between the different orders (Fucales, Laminariales…) but also inside the same orders (Demirel et al. 2012; Shekhar et al. 2011). In this study, Ascophyllum nodosum and Fucus vesiculosus (fucales) gave higher sugar extraction yields than Laminaria digitata (Laminariale) in aqueous extracts. A study performed in Canada showed similar results using multi-step extraction (but the solvent changed after each step) on the same two fucale species but using the laminarial Saccharina longicuris (Rioux et al. 2007). Moreover, despite a high extraction yield noticed in the ethanol extracts, those extracts contained small amount of sugars, and was mainly monosaccharides. Ethanol was used as solvent to extract pigments or polyphenols (Henriques et al. 2007) but also to purify polysaccharides by precipitation. Polysaccharides are generally not soluble in ethanol which explains the low sugar content, mainly monosaccharides such as sugar alcohol, in those fractions. Polysaccharides were so extracted during the three water steps. Mannitol is present in all brown seaweed and can reach up to 25% in laminaria species, that could explain the highest extraction yields obtained for Laminaria digitata compared to the other two fucales species. Laminaran extraction yields were higher F. vesiculosus and A. nodosum than L. digitata one. Laminaran content in seaweed is subjected to high variations according to the season. Indeed, this storage polysaccharide starts to be accumulated in the seaweed cells in June for a maximum peak in July and August (Adams et al. 2011). Moreover, fucales are less subjected to seasonal variations, probably due to a slower growth rate (Percival & McDowell 1968). The laminaran content in the cell walls was so more consistent over the year and did not decrease as much as in the Laminariaceae during winter time. Fucoidan content had been shown to be higher in seaweed living on the upper surface of their seawater habitat so in seaweed living in the intertidal zone like the fucales F. vesiculosus and A. nodosum (Evans 1989). Under the same extraction conditions, fucoidan yields were twice and one and a half higher for respectively A. nodosum and F. vesiculosus comparing to L. digitata. Fucoidan extracted from F. vesiculosus was the most studied and is still the main commercial source available. The monosaccharide composition of this fucoidan had been reported to contain only fucose (Li et al. 2008) contrary to the analysis on our fucoidan which contained other monosaccharides like galactose, xylose and mannose, even though the fucose was predominant (around 80%). Laminaria species were not as used as fucales for fucoidan
extraction and small amount of data were found on fucoidan extracted from *Laminaria
digitata*. Nevertheless, other *Laminaria* species were studied and composition showed the
presence of other monosaccharides like galactose or xylose (Kitamura et al. 1991) in minor
proportion comparing to fucose. Fucoidan from *A. nodosum* was probably the one in which
more monosaccharides had been identified. Likewise the fucoidan extracted in this study
that contained mainly fucose (58%) but also a high proportion of xylose and mannose (35%)
in correlation to some characterized in the literature (Marais & Joseleau 2001).

Two protocols were compared to purify the fucoidan by precipitation of the
alginites based on their solubility properties. Unlike, the precipitation using CaCl₂, an acid
treatment precipitated some mannitol as well as alginites. Under acidic condition, mannitol
solubility had been shown to decrease (Peña et al. 2000). Moreover, the hydroxyl groups of
mannitol can be esterified in acidic solution to form polyesters which are less soluble (Dhein
et al. 1983). The amount of fucoidan and laminaran precipitated was similar between the two
treatments but more interestingly, the compositions of the precipitated fucoidan were
different. Indeed, if an acid treatment resulted in an average composition of fucoidan in the
supematant similar to the one before the purification step, the use of calcium chloride gave a
fucoidan richer in fucose. Differences of fucoidan composition according to the extraction
solvent had been extensively reported. By opposition to the fucoidan composition after
alginate precipitation, which was richer in fucose by CaCl₂ precipitation, fucoidan extracted
using calcium chloride were reported to be poorer in fucose than acidic extraction in *A.
nodosum* (Cumashi et al. 2007; Nakayasu et al. 2009) but also in some other brown
seaweeds like *Adenocystis utricularis* (Ponce et al. 2003). By precipitating the alginites,
different fucoidans were so precipitated as well according to the solution used. The fucose
polymer precipitated using CaCl₂ contained actually more mannose, which can be a
fucomannan more than a fucoidan. Further investigations need to be done to find out if those
compounds were linked to the alginites and to explain the differences between the two
treatments. A NRM analysis of all solutions should give us a better idea of the potential
interactions between the molecules. To extract fucoidan, the main solvent used are
hydrochloric acid or calcium chloride so alginites are not extracted, the fucoidan
purification is so done by ethanol precipitation.

Investigation of polysaccharide fraction by removing the polyphenols showed that
amberlite resin could be used but a compromise had to be done due to the fixation of sugars
on the resin as well as phenolic compounds. Study on the capacity of resin to absorb
polyphenols was investigated on land plant extract with the monitoring of the protein
recovery. Comparison of different resin showed a higher efficiency of the amberlite IRA900 (66%) comparing to the amberlite XAD16 (38%) for the polyphenol removal. The protein recovery was also higher using the IRA900 (83% against 75% with XAD16) (D’Alvise et al. 2000). Those results are similar to one obtained on the lowest size fraction of seaweed extract with a higher fixation of sugar on the amberlite XAD16. The same study also investigated the best ratio between the quantity of resin and the volume of extract. Contrary to the results obtained on seaweed extract, where the optimum ratio was found to be 30%, on plant extract, the most efficient percentage was 10%. The evolution of polyphenol removed according to the quantity of resin is however similar between the two experiments. Study done on apple extracts showed that the percentage of polyphenol removed varied according to the concentration of amino acids and saccharides (Kammerer et al. 2010). Addition of saccharides (glucose, fructose, sucrose) reduced the quantity of phenolic compounds by 5-10% according to the compounds. The quantity of sugars in the lowest size fraction of the seaweed extract was lower than the highest ones (seaweed extracts are mainly composed of polysaccharides) which explain a higher efficiency of the resin on this fraction. The different amberlite resins are efficient on different size fractions (Aldrich 1991). The resin XAD2, 4 and 16 are very similar in terms of composition and dedicated applications, and results obtained on those resin were pretty similar except for the absorption of phenolics of the high molecular weight fraction on XAD4. The resin XAD7 is designed to absorb higher molecular weight compounds and was so twice more efficient than other resin on the fraction above 10 kDa.

The two extractions realized at the lab scale and at the pilot scale showed good correlation in terms of compounds extracted overtime. In both case, phenolic compounds were extracted at the beginning and reach the maximum concentration after 1 hour while the sugar content kept increasing. Water has been shown not to be the best solvent to extract phlorotannins (Koivikko 2008) so only a small fraction of the total phenolic content of the seaweed had been extracted. This fraction was either soluble compounds or phenolics linked to polysaccharides. Indeed, brown seaweed phlorotannins are linked to alginates and sulphated fucans (Salgado et al. 2007). Nevertheless, as the fucoidan content kept increasing overtime, the hypothesis of soluble phenolic fraction seems more coherent. The fucoidan composition changed overtime so the time appears to have an effect on the type of the fucoidan extracted, more detailed are presented in the chapter 6.
3.4 References


Aldrich, S., 1991. Amberlite XAD Polymeric resins,


O’Connell, E., 2008. Purification and characterisation of an algal polysaccharide (fucoidan) and enzymes involved in modification of this polysaccharide. National University of Ireland, Galway.


Chapter 4: Influence of extraction parameters on main compound extraction yields and crude extract composition from the brown algae *Ascophyllum nodosum*
4.1 Introduction

Optimisation of an extraction process and especially extraction yields is fastidious work but can have important consequences. From an industrial point of view, optimisation of extraction yields is very relevant in determining the production costs and, consequently, can have an influence on the sale price, in a competitive market. Additionally, a factory processing natural products has to face the impact of seasonal variation on their products, so knowledge of how the extraction parameters influence the yield, at different time points of the year, is very important for optimisation of harvesting and processing times as well as for process sustainability. Furthermore, pre-treatment of biomass (alteration of particle size, drying of samples, etc.) could also have an effect on the quantities of compounds extracted (Maisuthisakul & Pongsawatmanit 2004; Luthria 2008). If the optimisation of the extraction yields is essentials to determine the production cost of seaweed extracts, the composition of the extracts and the understanding of the variation between batches are also very important. Indeed, in processing natural biomass, a good understanding of the interaction between induced and uncontrolled extrinsic factors such as seasonality and controllable variables (extraction parameters) is fundamental to anticipate or predict, as far as possible, the final extract composition.

Moreover, polysaccharides from brown seaweed have been mainly extracted by multi-step extractions using chemicals (acidic or alkaline media, calcium chloride) to-date. The diversity of methods for the extraction of fucoidans was recently reviewed by Ale et al (2011) (Ale et al. 2011b). Getting a decent yield is also important at the research scale to minimize the number of batches required, to reduce potential inter-batch variability and compositional differences between the tested extracts, and to obtain enough material to run detailed characterization experiments and downstream bioactivity tests for example. In the literature, the use of chemicals usually increases the extraction yields, but it can also damage the structure of native molecules in the plant (Ale & Meyer 2013).

To date, different studies on the influence of individual extraction parameters have been done, with these targeted on specific compounds. Ale and co-workers investigated the influence on the extraction time, temperature and acid concentration on fucose-containing sulphated polysaccharides yields from Sargassum sp. With this species, temperature was the most influential parameter and gave an increase in the response (extraction yield) when the extraction temperature was increased. The same effect was also noticed for the influence of extraction time. The acid concentration effect was negative meaning that an increase in the acid concentration decreased the fucose-containing sulphated polysaccharide yield.
Moreover, interactions between these parameters also influenced the extraction yield, with the exception of the combined effects of time and temperature (Ale et al. 2011a).

In other reports, the effects of the same extraction parameters as those investigated in this study have been evaluated on polysaccharide and polyphenol extraction yields but the interaction of both parameters was not analysed. Nevertheless, similar conclusions were made between the different studies. For example, in each cases, an increase of the duration of extraction increased polysaccharide yields from pumpkins (Sun et al. 2011; Xianzhe et al. 2011), from the mushrooms Grifola frondosa (Yang et al. 2013) and Fomes officinalis (Mingdong et al. 2013), and from the land plants Peonia sinjiangensis (Tian et al. 2011) and Limonium sinense (Tang et al. 2011). These same studies showed that the yields are generally higher with an increase in the extraction temperature, as well as the ratio between volume of solvent used for extraction and the quantity of biomass. Two investigations done on the extraction of polysaccharides from pumpkins gave the exact same results, i.e. that the yields increased when the ratio of extractant to biomass was increased but only up to a ratio of 20 (Sun et al. 2011; Xianzhe et al. 2011). After that, the extraction yields decreased. A similar observation was made following polysaccharide extraction from the mushroom F. officinalis, except that the optimum ratio, beyond which yields decreased, was determined to be 10.

Time has been shown to increase the quantity of phenolic substances extracted from pomegranate but the extraction time range tested was very narrow (from 0 to 10 minutes) (Wang et al. 2011). The same study revealed that the effects of the extractant to biomass ratio, as well as the extraction temperature, were positive on the yield (i.e. a higher yield at larger extractant volumes). A similar effect of temperature on phenolics yields was also seen with litchi (Ruenroengklin et al. 2008) but an opposite effect was observed with parsley with a decrease in phenolics extraction yields at higher temperature (Luthria 2008).

The effects of particle size have been investigated previously on different types of biomass during the extraction of phenolic substances and polysaccharides. Indeed, extraction yields of phenolic substances from parsley (Luthria 2008) were higher when the particle size was smaller. Moreover, the yield of phenolic compounds extracted from pomegranate was higher when the extraction was done on small size particles, but only if the extraction time was less than 3 hours (Wang et al. 2011). For longer extraction times, particle size was no longer influential.
In Chapter 3 (Section 3.2.5.3), the duration of extraction differentially influenced the extraction yields of polysaccharides and other compounds (including phenolic substances) from seaweed. For this reason, a detailed investigation was initiated, using a single step water extraction method to establish how some extraction parameters may influence the extraction yields of the main compounds of interest from brown seaweed. A detailed, statistical approach was taken in the experimental design (Section 2.7). Three extraction parameters were investigated: time, temperature and liquid/biomass ratio (i.e. extractant volume) as well as the interactions between all three parameters. To underpin the experimental outputs, the extraction experiments were done with three different *A. nodosum* batches. The first two batches used seaweed harvested in February but the seaweeds were cut differently (Laboratory process for the first batch as described in section 2.1.1 and industrially processed for the second one, see section 2.1.2) which would allow investigation of the influence of particle size. The third batch was processed identically that the second one, with the difference being that the seaweed was harvested in June to explore the effect of possible seasonal variations. Finally, a mathematical model was proposed for the influence of the extraction parameters on each compound from each seaweed batch. This model is of relevance for process control and scale-up, as well as for reproducibility in laboratory and commercial environments.

Extraction of the main water-soluble compounds present in *A. nodosum* was investigated in this study, with the exception of the alginates. As commercial standards of the alginate components (mannuronic and guluronic acid) are not available, very accurate determination is difficult. Although other hexuronic acids can be used, most methods will give differential responses to individual hexuronic acids, which prevent precise quantification. Moreover, despite several trials on different methods to quantify uronic acids in plant extracts, interference by other compounds in these assays has been identified. Nevertheless, to address the quantification and characterization of a preliminary investigation to apply and optimise a semi-enzymatic quantification and characterisation of alginates was investigated, the results of which are presented later in Chapter 6. To approximate the potential influence of extraction parameters on alginate yields, an investigation was done on the total sugar content determined by the phenol-sulphuric acid. By comparison of the results with those obtained for fucoidans and laminarans, an approximation of the alginate content could be made (the three polysaccharides are the main polysaccharides in brown seaweeds). As crude extract composition also varied according to the extraction parameters, a correlation of the influence of extraction parameters on extraction yields and extract composition was also investigated.
4.2 Results

The aim of the research in this chapter was to compare the effects of specific extraction (and seasonal) parameters, and the interactions between these parameters, on the yields of selected compounds extracted from the brown seaweed *A. nodosum*. The first comparison was done to compare the effects of different cutting processes on two batches of seaweed (batch 1 and 2) harvested in February. The first batch was processed in the laboratory as described in Section 2.1.1, while the second batch was industrially processed as described in Section 2.1.2. In the first batch, the seaweed was blended (using a hand blender) in the volume of water used for the extraction, so this mechanical action was taken into consideration.

The second comparison investigated the potential effects of seasonal variation between batch 2 (see above) and 3. The two batches were identically processed (i.e. industrially processed) but the seaweed in the third batch was harvested in June. Eight extraction protocols were done in triplicate as explained in Sections 2.2.4 and 2.7.3, on each of the three seaweed batches. The protocols tested corresponded to the number of experiments required for a full factorial experimental design (involving the parameters of time, temperature and ratio) and the statistical analysis was done as described in Section 2.7.4. The specific parameter details used in each protocol are summarized again in Table 4.1.

<table>
<thead>
<tr>
<th>Extraction N°</th>
<th>Time (hours)</th>
<th>Temperature (°C)</th>
<th>Ratio (ml/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>70</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>70</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>70</td>
<td>5</td>
</tr>
</tbody>
</table>

End-points investigated were the total yield of extract on a dry weight basis, the ash yield, as well as the extraction yields of the main compounds of interest, i.e. total carbohydrate, individual polysaccharides (laminaran and fucoidans), mannitol and phenolics (see Section 2.6 for relevant analytical methods). Extraction yields of individual compounds
were defined as the ratio of the mass of pure compounds extracted to the dry weight of seaweed biomass used for extraction. Total extraction yields were expressed as a percentage of the seaweed dry weight. The calculation of extraction yields is explained in Section 2.6.4.

4.2.1 Comparison of the extraction yields of the main compounds in crude extracts from the three batches

The values for the extraction yields of the target compounds from the eight individual extraction protocols (conducted in triplicate), for each seaweed batch (i.e. batches 1-3), complete with standard deviation and confidence interval values, are provided in Appendices 6A-C. Table 4.2 provides a summary of the findings for each batch. In this table, the yield values for each respective compound category from the 8 protocols have been averaged; the minimum and maximum results obtained are displayed between brackets, below the average value. Batches were compared by one way-ANOVA and values that do not share the same letter in a column are significantly different. Comparison of the first two batches revealed an effect of the seaweed cutting process (scissor cut and blending for the first batch versus use of an industrial cutter for the second batch). Seasonal variation was investigated by comparison of batch 2 (seaweed harvested in February) and 3 (seaweed harvested in June).

4.2.1.1 Comparison of the main compound extraction yields between the batches

The average total sugar extraction yields were the only endpoint that did not reveal significant differences between the batches; total sugar yield averages were 4.93%, 4.90% and 5.26% respectively for batches 1, 2 and 3. The effect of blending the seaweed in the water used for extraction generally increased the yields of all compounds, except the polysaccharides. Indeed, batch 1 gave significantly higher values for average total extraction

Table 4.2 Batch comparison of the extraction yields expressed as a percentage of the dry seaweed mass (see 2.6.4), values are the averages of the 8 protocols with minimum and maximum values between brackets (min – max)

<table>
<thead>
<tr>
<th>Batches</th>
<th>Total</th>
<th>Ash</th>
<th>Polyphenols</th>
<th>Total sugars</th>
<th>Mannitol</th>
<th>Fucoidans</th>
<th>Laminarans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>20.43a</td>
<td>5.12a</td>
<td>2.99a</td>
<td>4.93a</td>
<td>3.63a</td>
<td>0.63b</td>
<td>1.90b</td>
</tr>
<tr>
<td></td>
<td>(13.46 – 30.68)</td>
<td>(2.99 – 7.03)</td>
<td>(1.71 – 4.85)</td>
<td>(3.38 – 7.42)</td>
<td>(1.94 – 5.27)</td>
<td>(0.27 – 1.67)</td>
<td>(1.24 – 2.71)</td>
</tr>
<tr>
<td>Batch 2</td>
<td>15.82b</td>
<td>2.63c</td>
<td>1.85b</td>
<td>4.90a</td>
<td>1.52b</td>
<td>0.75ab</td>
<td>2.28a</td>
</tr>
<tr>
<td></td>
<td>(12.48 – 20.70)</td>
<td>(2.63 – 3.60)</td>
<td>(1.46 – 2.31)</td>
<td>(3.40 – 7.06)</td>
<td>(0.75 – 2.67)</td>
<td>(0.35 – 1.40)</td>
<td>(1.59 – 2.82)</td>
</tr>
<tr>
<td>Batch 3</td>
<td>21.22a</td>
<td>3.41b</td>
<td>3.10a</td>
<td>5.26a</td>
<td>1.96b</td>
<td>0.99a</td>
<td>2.37a</td>
</tr>
<tr>
<td></td>
<td>(17.36 – 25.57)</td>
<td>(2.69 – 4.19)</td>
<td>(2.53 – 3.66)</td>
<td>(3.42 – 7.22)</td>
<td>(1.32 – 3.96)</td>
<td>(0.45 – 1.81)</td>
<td>(1.43 – 3.62)</td>
</tr>
</tbody>
</table>
yields (20.43% versus 15.82% for batch 2), ash (5.12% versus 2.63%), phenolics (2.99% versus 1.85%) and mannitol (3.63% versus 1.52%) but lower values for average fucoidan (0.63% versus 0.75%) and laminaran (1.90% versus 2.28%) yields. The average fucoidan extraction yields were the only values that were not significantly different between batches 1 and 2.

Regarding a potential seasonal effect, extractions done on seaweed harvested in June gave significantly higher average yield values than the extractions done on seaweed harvested in February, with the exception of the carbohydrate yields. The average sugar extraction yields between the two batches were 1.52% and 1.96% for mannitol, 0.75% and 0.99% for fucoidans, 2.28% and 2.37% for laminarans, respectively, for batches 2 and 3. Greater differences were noticed for the average total extraction yield, ash and phenolic substances yields. Closer examination of the data in Appendices 6A-C showed that the lowest yields of phenolics, ash, total sugars, fucoidans and lowest total extraction yields, were obtained using the first protocol, which involved a short extraction (1 h), at low temperature (25°C) and a low liquid to biomass ratio (i.e. 2). The contrasting protocol (6 hours, 70°C and a ratio of 5) gave the highest yield values for the endpoint compounds listed above. Regarding mannitol and laminaran, different extremes were identified for the batches. Indeed, the minimum amount of mannitol extracted was obtained using the first (1 hour, 25°C and extractant/biomass ratio of 2), the fifth (6 hours, 25°C and ratio 2) and the sixth (6 hours, 25°C and ratio 5) protocols for batches 1, 2 and 3, respectively. Interestingly, in the two batches in which seaweed harvested in February were processed (batches 1 and 2), a smaller extractant/biomass ratio gave a lower yield response overall, contrary to the summer batch, in which a smaller yield response was obtained using a higher extractant/biomass ratio. Similarly, the lowest yield response was obtained after a short extraction period for the laboratory processed batch and after a longer extraction for the two industrially processed batches.

The maximum extraction yields for mannitol were obtained with contrasting protocols for each batch, i.e. the eighth (6 h, 70°C and an extractant/biomass ratio of 5) and the fourth (1 hour, 70°C and an extractant/biomass ratio of 5) protocols, respectively. The third batch gave the only observable difference to the other two batches, in that the lowest and highest mannitol yield responses were identified with the protocols using the highest extractant/biomass ratio.

For the laminaran yields, both February batches gave the poorer yield response at lowest temperatures (protocols 5 and 1, respectively, for batches 1 and 2), in contrast to the summer batch for which the lower results were obtained at higher temperature (protocol 7).
For all batches, the minimum laminaran yields were obtained using protocols with the lowest extractant/biomass ratio and maximum yields with the highest extractant/biomass ratio.

4.2.1.2 Comparison of main compound proportions in crude extracts from the three batches

In the previous section, the effects of extraction and inter-batch effects were compared on yields of individual compounds relative to the initial dry weight of seaweed used in each extraction. In this section, the yield of each compound relative to the total dry weight of extract recovered was calculated for all eight protocols (in triplicate) used in the extraction of each batch of seaweed. This approach provides an overview of the relative proportions of each compound in the total extract recovered. The individual values, standard deviation and confidence interval values are provided in Appendices 6D-F, while Table 4.3 below summarizes the average yield values of compounds relative to the total solids content of the extracts. Values in the same column in this table that do not share a letter are significantly different.

The average ash content values were similar for the two industrially processed batches (16.6% and 16.1%, respectively for batches 2 and 3). The additional mechanical step (blending) significantly increased the average proportion of ash (24.9%) in the extract from the first batch. Average phenolics content values were statistically higher in the summer (third) batch (14.6% versus 11.8%) that the comparable February (second) batch. Interestingly, the observed difference was counterbalanced by the additional blending action in batch 1 that significantly increased the phenolics content.

Regarding the effects of parameters on the proportions of the sugars investigated, the average mannitol yield was much higher in batch 1 accounting for 17.7% of the total solids content.
extract dry weight, yet almost identical in batches 2 and 3 (9.6% and 9.2%, respectively). Similarly, the average fucoidan content was similar in extracts from seaweed batches 2 and 3 at 4.5%, but in contrast to mannitol, was lower in batch 1 (2.8%). Finally, the average proportion of laminaran was highest in batch 2. A seasonal effect was highlighted by the significantly lower average laminaran content in the extract produced from the third batch. Moreover, the results suggest that the effect of the additional mechanical step considerably reduced the proportion of laminaran with an average value of 9.4% noted for the first batch.

As in the previous chapter, the effects of extraction parameters were investigated to determine the compound content in extract. Minimum and maximum values had been previously shown to be generally obtained after cold and hot processes, respectively. In terms of overall composition, extracts from the first batch appeared to be different than those for the other batches with, for most of the compounds, the lower extremes obtained in a cold process extract (appendix 6D). Regarding the two industrially processed batches, the phenolics, mannitol and laminaran contents in the crude extracts were lower when the extraction was performed at high temperature (appendices 6E & 6F). This observation contrasts with the pattern noted for the proportion of fucoidan, which was higher at the higher extraction temperature. Concerning the other parameters and compounds, the trend was not as obvious, and a more detailed analysis was required.

As mentioned, the results summarized in Tables 4.2 and 4.3 for each batch are derived from eight extraction protocols with different time, temperature and ratio parameters being employed, the complete datasets are given in appendices 6A-G and proposition of equation model are given in appendices 7A-C.

### 4.2.2 Total extraction yield

As seen previously, the total extraction yield was influenced by the cutting process (average value for batch 1 was higher than batch 2) but also by the seasonality (batch 3 values higher than those for batch 2). A more detailed analysis of the influence of the extraction parameters of time, temperature and extractant/biomass ratio (referred to as ratio from here), and their interactions is presented in the following section as explained in Section 2.7.4.
4.2.2.1 Influence of the main extraction parameters and inter-parameter interactions actions

Figure 4.1 shows a semi-qualitative analysis of the total extract yields for each parameter (explained in Section 2.7.4.1). As seen in Table 4.2, batches 1 and 3 gave similar results and the global average for all experiments, represented by the horizontal line in each image plate in Figure 4.1, was similar (20.43% DW and 21.22% DW for batch 1 and 3, respectively). The second batch gave a lower result with an average of 15.81% DW. The influence of the parameters seemed higher for the first batch (higher slopes) than the two other which looked quite similar, which suggests that the cutting process was more important than the seasonal variation. The three parameters appeared to influence the extraction yields for the first batch, especially temperature with a higher slope obtained than time and ratio both of which seemed similar to each other. In industrially processed batches (2 and 3), the effect of each parameter appeared similar between the two series with a higher effect noted for temperature. Finally, all effects were positive, meaning that an increase in a parameter value would increase the response.

Figure 4.2 shows the semi-qualitative analysis of the interaction effect between the main parameters (Section 2.7.4.1). Parallel lines, representing insignificant effects, were displayed for the interactions between time/temperature and time/ratio in the three series. In case of non parallel lines, the effect of interactions could be significantly influential (even if the main parameters were not) and the values of extraction parameters should be taken in consideration to propose the mathematical model (See Section 4.2.2.4). Indeed, the difference between 1 hour (black line on the two upper plates) and 6 hours (red line) on extraction yields was similar at low and high temperatures, as well as at low and high ratios.
A slight difference was nevertheless observed for the second batch. At 70°C, the differences appeared to be slightly more important between the two extraction times than extraction at 25°C (B, or the upper left plate). Concerning the interaction between temperature and ratio, higher differences were noticed (for the three batches). A higher temperature of extraction gave higher response (total extraction yield), especially when the ratio increased, suggesting that this interaction might be influential.

4.2.2.2 Quantification of the influence of main parameters and interaction effects on total extraction yield

Effects of each parameter and interaction were calculated as explained in Section 2.7.4.2. For each batch, the average value for each extraction was calculated and the results are presented in the second column of the Table 4.4. The next set of columns represents the effect of each parameter and interaction, with A denoting time, B temperature and C, ratio. The last three columns are the actual responses (total yields, %DW seaweed) obtained with each extraction protocol. Effects were finally calculated by averaging the responses for the

**Table 4.4 Calculation of the effects of parameters and their interaction on the total extraction yield response**

<table>
<thead>
<tr>
<th>Extraction N°</th>
<th>mean</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>AB</th>
<th>AC</th>
<th>BC</th>
<th>ABC</th>
<th>Yields (%/dry seaweed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Batch 1</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>13.46</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>16.19</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>19.40</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>24.35</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>18.46</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>-</td>
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<td>19.43</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>21.44</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>30.68</td>
</tr>
<tr>
<td>batch 1</td>
<td>20.43</td>
<td>2.08</td>
<td>3.54</td>
<td>2.24</td>
<td>0.02</td>
<td>0.32</td>
<td>1.31</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>batch 2</td>
<td>15.81</td>
<td>1.00</td>
<td>2.29</td>
<td>0.80</td>
<td>0.29</td>
<td>-0.02</td>
<td>0.50</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>batch 3</td>
<td>21.22</td>
<td>0.97</td>
<td>1.56</td>
<td>1.57</td>
<td>-0.03</td>
<td>-0.08</td>
<td>0.37</td>
<td>-0.01</td>
<td></td>
</tr>
</tbody>
</table>
eight protocols, corrected with the corresponding coefficient (+ or -; see appendix 6) according to the effect studied. The values for the effect values for each batch are displayed in the bottom three rows. As seen before, the maximum extraction yield was obtained, on average, with the third and first batches (21.22% and 20.43% DW, respectively). The influence of parameters was more pronounced for the first batch than both other batches, except for the interaction time/temperature, which was more important in the second batch. The maximum effect calculated was for temperature in the three batches; temperature could affect the response (total average ± effect value) up to 3.54%, 2.29% and 0.56%, respectively. The effect of time on total extraction yield was shown to be similar between the industrially processed batches (1%) but 2-fold lower than the first batch. Effect of ratio was different between batches, with a higher value noted for the first batch (2.24%) than the second (0.80%) batch. In the third batch, the effect of ratio was similar to the effect of temperature (1.57% and 1.56%, respectively). Most of the interactions gave low effects except that between temperature and ratio, especially for the batch 1 (1.31%). The interaction between the three parameters, which cannot be seen in Figure 4.2, might be significantly important for the first batch with a maximum effect on the response of 0.76%.

4.2.2.3 Confidence interval calculation and definition of the most influential parameters

Based on the standard deviation of the triplicates for each extraction, the confidence interval at 95% was calculated for each seaweed batch (see Section 2.7.4.2 for the calculation details). Standard deviations ($s_i$), variance values (sum of variances $s_i^2$) and the confidence intervals are presented in Table 4.5. Reproducibility for the third batch was the lowest with a variance 4.5 times higher than the first batch (0.058% and 0.013% respectively) and more than 7 times higher than the second one (variance of 0.007%). The consequence was a higher confidence interval, above 1% rather than 0.5% and 0.37% for the batched 1 and 2, respectively. To determine if the parameters were influential for the response, values for the confidence interval were added/subtracted from the effect values, which gave for example, for the effect of time in the first batch (2.08%±0.0497%), an effect
value between 2.03% and 2.13%. This range did not contain the ‘zero’ value so was considered as influential. In contrast, the effect of the interaction between time and temperature, with an effect calculated at 0.02%, gave a range between -0.477 and +0.517, thus containing ‘zero’ and indicating an insignificant parameter. The value for the effect plus or minus the confidence intervals for each parameter are summarised in Table 4.6. Values in a green colour indicate significant effects. Temperature and ratio were influential parameters for the three batches, in contrast to time, which was insignificant in the third batch, despite having a similar effect to the second batch; however, a lower reproducibility gave a higher confidence interval. Interactions did also not influence the response for this batch (batch 2). Interaction temperature/ratio was significantly important for the winter batches (1 and 2), and especially in the first one (2.5 times higher than the second). Moreover, the interaction between the three main parameters appeared to have an effect on the response (total extract yield), but only for the first seaweed batch.

4.2.2.4 Proposed model equations

Based on the results obtained for the effects of influential parameters on total yield, an extraction model equation was proposed for each batch (see Section 2.7.4.3). The equation gave the response Y which relates to the average value for all experiment plus or minus the effect of each parameter (all positive for this response). This equation could be applied only on the studied area (from 1 to 6 hours for time, from 25°C to 70°C for the temperature and from 2 to 5 for the ratio). Outside these ranges, the equation was not guaranteed to reflect the reality. To increase the extraction yields, the parameters should be set up at their maximum.

Batch 1:  \[ Y = 20.43\% + 2.08\% A + 3.54\% B + 2.24\% C + 1.31\% BC + 0.76\% ABC \]

Batch 2:  \[ Y = 15.81\% + 1.00\% A + 2.29\% B + 0.08\% C + 0.50\% BC \]

Batch 3:  \[ Y = 21.22\% + 1.56\% B + 1.57\% C \]
### 4.2.3 Effect of extraction parameters and their interaction on ash extraction yields and their proportion in the crude extracts

#### 4.2.3.1 Effect of extraction parameters on ash extraction yields

Concerning the ash extraction yields (results summarised in Table 4.7), the lowest response was obtained for the second batch (2.63%). Extraction of seaweed harvested in summer yielded extracts with significantly increased ash content, up to 3.41% for the third batch. This seasonal effect could be counterbalanced by the addition of a mechanical action during the cutting process (as in batch 1). Indeed, yields obtained on the first batch were, in average, twice higher than the second one (5.12%). Due to a technical problem when the first batch was analysed no replicates could be conducted making it impossible to calculate confidence intervals. Nevertheless, similar intervals were calculated for batches 2 and 3 (0.19% and 0.16%, respectively). Standard deviations obtained for each protocol were pretty similar in the third batch (around 0.2%) in contrast to those for the second batch, where increased variation was noticed (from 0.006% to 0.604%). Effects for batch 1 were more important than batches 2 and 3 and likewise, for the ash extraction yields, all coefficients were positives. In the third batch, all main parameters gave a similar effect (around 0.23%). In February batches, temperature was the most influential parameter. Moreover, time and ratio were similar (around 0.65%) and 1.5 times lower than temperature (0.89%) in the first batch. In second batch, all parameters gave different effects with temperature (0.41%) twice more important than time (0.25%) and four times more than the ratio (0.14%). The main parameters were significantly influencing the response in the third batch, as well as the second batch, with the exception of ratio. In terms of the first batch, significant effects could not be determined, but the main parameters were considered influential due to the higher effect calculated in comparison to batches 2 and 3, in which significant effect had been shown. All of the interactions having a low effect were not calculated as influential. Interestingly, except for the interaction between the three parameters, coefficients of interaction effect for the laboratory processed batch (negative for the interactions involving time and positive for temperature and ratio) were different from the two industrially processed batches. The response for ash yield could be maximised by setting all parameters

<table>
<thead>
<tr>
<th>Batch</th>
<th>Time</th>
<th>Temperature</th>
<th>Ratio</th>
<th>Time/ Temperature</th>
<th>Time/Ratio</th>
<th>Temperature / Ratio</th>
<th>Time/ Temperature/Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>0.64</td>
<td>0.89</td>
<td>0.67</td>
<td>-0.17</td>
<td>-0.10</td>
<td>0.16</td>
<td>-0.18</td>
</tr>
<tr>
<td>Batch 2</td>
<td>0.25±0.19</td>
<td>0.41±0.19</td>
<td>0.14±0.19</td>
<td>0.16±0.19</td>
<td>0.10±0.19</td>
<td>-0.07±0.19</td>
<td>-0.02±0.19</td>
</tr>
<tr>
<td>Batch 3</td>
<td>0.22±0.16</td>
<td>0.23±0.16</td>
<td>0.25±0.16</td>
<td>0.07±0.16</td>
<td>0.00±0.16</td>
<td>-0.05±0.16</td>
<td>0.05±0.16</td>
</tr>
</tbody>
</table>
at the high level, as for the previous responses studied. Despite a lower average value in the second batch relative to the third one, an increase of the temperature would affect the response more and could compensate for the difference.

4.2.3.2 Effect of extraction parameters on ash content in the crude extract

As shown previously (Table 4.3), the general average ash yield was much higher for the batch 1 (Table 4.8). Confidence intervals were pretty wide due to low reproducibility of some protocols. Interestingly, the average of the standard deviations between low and high levels of time was identical between the two industrially processed batches. Indeed, the difference of standard deviation average between the 1 hour and 6 hours extraction time points were 0.72% in both cases (higher standard deviations for the 1 hour protocols). Moreover, differences in the averages between the two other parameters (temperature and ratio) were also similar for the same batch with lower reproducibility for the low temperature and high ratio extracts. The wide intervals in batches 2 and 3 did not allow identification of the most influential parameters for the ash content response. In general, the laboratory processed batch gave higher interval than industrially processed batches 2 and 3, so an interval >1%, suggests that no parameters were influential for this batch. Effects

Table 4.8 Influential parameters and interactions for the ash content (% of total solids)

<table>
<thead>
<tr>
<th></th>
<th>Time</th>
<th>Temperature</th>
<th>Ratio</th>
<th>Time/Temperature</th>
<th>Time/Ratio</th>
<th>Temperature/Ratio</th>
<th>Time/Temperature/Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>0.83</td>
<td>-0.08</td>
<td>1.00</td>
<td>-0.83</td>
<td>-0.58</td>
<td>-0.90</td>
<td>-1.61</td>
</tr>
<tr>
<td>Batch 2</td>
<td>0.44±1.07</td>
<td>0.19±1.07</td>
<td>0.13±1.07</td>
<td>0.61±1.07</td>
<td>0.64±1.07</td>
<td>-1.01±1.07</td>
<td>-0.20±1.07</td>
</tr>
<tr>
<td>Batch 3</td>
<td>0.26±0.64</td>
<td>-0.09±0.64</td>
<td>0.04±0.64</td>
<td>0.32±0.64</td>
<td>0.02±0.64</td>
<td>-0.49±0.64</td>
<td>0.21±0.64</td>
</tr>
</tbody>
</table>

values were generally higher for the first batch, except for temperature and the interaction of temperature/ratio. However, effects values were low and insignificant in all batches. In the first batch, time and ratio gave a higher effect on the response (ash yield), with both parameters giving a positive slope but the differences between low and high levels (1 h versus 6 h for time) for these parameters were small (1.6% and 1.98% for time and ratio, respectively). In all batches, the maximum effect was calculated for an interaction. In the first batch, the interaction of the three parameters (-1.61%) represented 6.5% of the global average. For the other two batches, the maximum effect was obtained for the interaction temperature/ratio, which influenced the global ash yield by 1.01% and 0.49% for batches 2 and 3. All main parameters had a low effect on the response (ash yield) for batches 2 and 3, and slightly higher for the first batch, especially for time and ratio. No parameters were
defined as influential for this particular response. The results indicate that an extraction would contain around 25% ash using laboratory processed seaweed and between 16% and 17% for an industrially processed batch, depending on the season.

4.2.4 Effect of extraction parameters on the phenolics extraction yields and their proportion in the crude extracts

4.2.4.1 Effect of extraction parameters on phenolics extraction yields

The extraction yields of phenolic substances were highly influenced by the cutting method used in processing the seaweed as shown in Table 4.1 with, on average, an extraction yield of 2.99% for batch 1, which was significantly higher than with the yield for batch 2 (1.85%). Moreover, seasonality was also important. Indeed, the summer batch gave statistically higher yields than the February batch (3.10% on average). Values for the effects of each parameter and their interaction are displayed in Table 4.9. The confidence intervals, for each batch, are also presented and influential effects (intervals in which the ‘zero’ value was not included) are highlighted with the green colour. Reproducibility of the extractions was good as been evidenced by the low standard deviation obtained, resulting in low intervals. Standard deviations for the first batch indicated a lower reproducibility for the extraction done with a ratio of 5 than the ratio 2 (0.48% and 0.16% respectively on average; see Appendix 6). No trends were observed for the two industrially processed seaweed batches. As observed for the previous response (ash yield and content), the best reproducibility was obtained with the second batch with an interval (±0.05%) that was 2-fold less than the value obtained for the third batch (±0.12%) and almost six times lower that the first batch (±0.28%). Effects of parameters were shown to be higher for batch 1 than batches 2 and 3. The main effect was observed for time and temperature, and was similar for both, and higher than the effect of extractant/biomass ratio for the batch 1. In contrast, for the industrially processed batches (2 and 3), the effect of time and temperature appeared almost identical (0.12% and 0.15% for time, 0.16% and 0.15% for temperature), but the effect of
the ratio was higher in the third batch (0.25% versus 0.13% in the second batch). All main parameters were influential with a similar effect observed between time and temperature in all series, but contrary to the first 2 batches, ratio was the most important in the last batch. Values for the interactions were low in all batches, especially in batch 1. Nevertheless, in the second batch, the interaction of temperature with extractant/biomass ratio was statistically influential. Indeed, for this batch, the phenolics content in the crude extract was similar for the two ratios when the temperature was low. At a higher extraction temperature, the response was higher at a higher volume of solvent/extractant (see Appendix 6). Low intervals excluded all the other interactions as being significant.

4.2.4.2 Effect of extraction parameters on phenolics content of the crude extract

Analysis of the effects of the extraction parameters on phenolics content relative to the overall crude extract composition was conducted using the same approach as for the extraction yields. The most influential parameter could possibly be different, and influence the relative proportion of each compound differently; some effects could be considered as non-significant even if the extraction yield increased. The reproducibility of the effects seen for the second batch was almost twice and three times higher than third and first batches, respectively, as shown by the confidence intervals (Appendix 6 and Table 4.10). Intervals calculated were narrow enough for the two industrially processed batches (0.33% and 0.56% for batches 2 and 3, respectively) and wider in the laboratory processed batch (1.08%). For the first batch, standard deviations were higher for the extraction using an extractant/biomass ratio of 5 than the lower ratio of 2, with an average value of 1.87% and 0.71%, respectively (Appendix 6). No conclusions could be made for the most reproducible extracts in batches 2 and 3. Extraction time had the highest effect for the first batch (1%) followed by temperature (0.38%) and the interaction temperature/ratio gave a negative coefficient (-0.29%). The low reproducibility of this analysis on the first batch yielded, overall, an insignificant effect for all parameters and interactions for this batch. A similar profile of effect values for the main parameters was observed for batches 2 and 3, with the highest effect for the extraction
temperature with a negative coefficient (-0.67% and -0.36%, respectively), which means that hot process extracts contained less phenolic compounds than the cold ones (even though temperature was significant only for the second batch). Extraction duration and ratio were not important, most especially time, which had with a very low effect value (0.08% in both cases). Likewise interactions values between batches 2 and 3 showed a similar profile, which was different from the first batch. The interaction of time/temperature gave the highest effect value and was even influential for the phenolics content of the second batch (-0.52%). At a low temperature, extracts contained more polyphenols after a long extraction and the opposite was noticed at high temperature. Phenolics content variations were relatively low between the protocols tested with high standard deviations between the replicates on occasions, which may explain why influential parameters were not identified. An extract from seaweed prepared in the laboratory (batch 1) would contain 14.44% of phenolics plus or minus 1.08%. The effect of identified important parameters in the second batch could change the response up to 1.19% (0.67 + 0.52), which would correspond to 10% of the average result (1.19/11.8, average of the phenolic content between the eight protocols).

4.2.5 Effect of extraction parameters on the total sugar yields and their proportion in the crude extracts

4.2.5.1 Effect of extraction parameters on total sugar yields

The total sugar content (excluding sugar alcohol) of the eight extracts (in triplicate) from seaweed batches 1-3 was determined by the phenol-sulphuric method (Section 2.5.3.1). As alginates could not be precisely quantified in our samples, as explained in the introduction to this chapter (see Section 4.1), by determining the effects on total sugar, in combination with similar information for fucoidans (Section 4.2.7.1) and laminarans (Section 4.2.8.1), it may provide some indirect insight into the influence of parameters on alginates, or the contribution of the alginates (one of the predominant polysaccharide fractions in brown seaweeds) to the overall findings for the total sugar.

Although no obvious effects of seasonality or seaweed processing had been identified for the total sugar extraction yields (Table 4.2), Nevertheless, the effects of the different extraction parameters on total sugar extraction yields from each batch were investigated (Table 4.11 and Tables 6A-C in Appendix 6). The best reproducibility was obtained with the two industrially processed batches with confidence intervals of 0.30% and 0.43%, respectively, for batches 2 and 3. Standard deviations for the first batch were higher, which resulted in a confidence interval more than twice that of batch 2 (i.e. 0.66%). From the standard deviation values for each extraction protocol applied to batch 1, protocols 4 and
8 gave much higher confidence values (1.91% and 1.46% respectively) whereas all of the values for the other protocols were similar and much lower (around 0.4%). From this finding, it would suggest that protocols using high temperature and high extractant/biomass ratio appeared to be less reproducible than the other methods. For batch 2, no real trend was identified in the standard deviation values, whereas for batch 3, the extractant/biomass ratio seemed to be very important to obtain reproducible results. In fact, the average standard deviation of the total sugar yields for the protocols using the lower extractant/biomass ratio was more than 5 times lower than the average for protocols using a higher extractant/biomass ratio (0.15% and 0.82%). By combining the results of the effect of the parameters and the confidence intervals, statistically influential parameters were identified and summarized in Table 4.11. Despite no differences identified between the batches concerning the global average of the total sugar extraction yields, extraction parameters showed to have an effect, and even different according to the batch.

The higher values across the parameter effects for the first batch compared to the second batch (same harvesting period) suggest that the extraction parameters were more influential on the laboratory-processed batch than the batch processed in an industrial environment. However, due to a lower reproducibility between replicates for all protocols, as mentioned previously, only the temperature appeared to influence the response (total sugar yield) for the first batch (1.21%), In contrast, with batch 2 the ratio (0.50%), temperature (0.83%) and the interaction between time and temperature (0.36%) were significant. Regarding the time/temperature interaction (batch 2), the 1 hour extractions gave higher sugar yields at low temperature than those allowed to proceed for 6 hours. At 70°C, the yields increased for both extraction durations, but this increase was more important for the 6 hour extractions, which gave higher yields than the 1 hour extractions at this temperature. Regarding the third batch, the main parameters were all influential but not the interactions between parameters. Furthermore for the February batches, temperature was the most important influential parameter (1.22%). Time and ratio had similar effects with yield values of 0.66% and 0.56%, respectively. With batch 3, the interaction of time with temperature
had an identical effect value to that of the second batch, but the lower reproducibility excluded this interaction from being influential parameter overall. No significant difference in the average total sugar extraction yields was observed between the batches. Consequently, by setting up all the parameters to zero centered value (which correspond to natural variables of 3.5 hours, 47.5°C and ratio 3.5, see Section 2.7.4.4), non-statistical differences should be found between the batches.

4.2.5.2 Effect of extraction parameters on total sugar content in the crude extract

The total sugar content as a proportion of the crude extract dry weight was higher in the industrially processed seaweed batch harvested in February (batch 2), than laboratory processed one (batch 1), as shown in tables 6D-F in Appendix 6. Moreover, the February batch (batch 2) crude extract surprisingly contained more sugars than the summer batch (batch 3). Similar to the observations for the sugar extraction yields (%DW seaweed), the reproducibility of total sugar content values across the 8 protocols were low resulting in wide confidence intervals of 2.68%, 1.92% and 2.11% respectively, for batches 1, 2 and 3; Appendix 6. However, some trends could be identified for the most reproducible protocols and especially with the two industrially-processed batches. Indeed, for the second batch, the cold process extractions gave, on average, higher standard deviations (3.47%) than the extractions conducted at higher temperature (1.51%). Similarly, for the third batch, the two extractant/biomass ratios gave different standard deviations (1.57% on average for ratio 2:1 and 3.71% for ratio 5:1). No trends were observed for the different parameters for the first batch. Due to the wide confidence intervals, no influential parameters were identified for the first two batches. With the third batch, the temperature effect was the most important and statistically influential for the sugar content in crude extract (3.82%).

Each batch showed a different profile in terms of parameter effect value profiles (Table 4.12). Even though defined as insignificant, the extraction time and temperature had the most effect for the first batch (-1.62% and 1.76% respectively). The negative coefficient

<table>
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<tr>
<th></th>
<th>Time</th>
<th>Temperature</th>
<th>Ratio</th>
<th>Time/Temperature</th>
<th>Time/Ratio</th>
<th>Temperature/Ratio</th>
<th>Time/Temperature/Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>-1.63 ± 2.68</td>
<td>1.76 ± 2.68</td>
<td>-0.15 ± 2.68</td>
<td>-0.76 ± 2.68</td>
<td>0.63 ± 2.68</td>
<td>0.70 ± 2.68</td>
<td>-0.37 ± 2.68</td>
</tr>
<tr>
<td>Batch 2</td>
<td>-1.77 ± 1.92</td>
<td>0.73 ± 1.92</td>
<td>1.80 ± 1.92</td>
<td>1.81 ± 1.92</td>
<td>0.53 ± 1.92</td>
<td>-1.85 ± 1.92</td>
<td>1.88 ± 1.92</td>
</tr>
<tr>
<td>Batch 3</td>
<td>1.85 ± 2.11</td>
<td>3.82 ± 2.11</td>
<td>0.72 ± 2.11</td>
<td>1.34 ± 2.11</td>
<td>0.97 ± 2.11</td>
<td>0.12 ± 2.11</td>
<td>0.82 ± 2.11</td>
</tr>
</tbody>
</table>
for the effect of time was also found for the second batch (a value of -1.77%), whereas a positive value was observed for the third batch (1.85%). Moreover, all parameters and interactions yielded positive coefficients for batch 3, while time and the interaction of temperature/ratio were negative for the second batch and time, ratio, time/temperature and time/temperature/ratio were negative for the first batch.

4.2.6 Effect of extraction parameters on the mannitol extraction yields and their proportion in the crude extracts

4.2.6.1 Effect of extraction parameters on mannitol extraction yields

Mannitol extraction yield (%DW seaweed) was highest for the first batch (3.63%) suggesting that there may be an effect of the cutting process, while the two other batches gave similar average values (1.52% and 1.96% respectively for batches 2 and 3) which did not suggest a likely seasonality effect. The best reproducibility was obtained with the third batch which gave the narrower interval (±0.09) as shown in Table 4.13. For the February batches, an industrial cutting process seemed better to reproduce similar extractions, resulting in an interval for the second batch (0.18%) 1.5 times less than that for the first batch (0.26%). No trends could be noticed for the most reproducible protocols, and standard deviations were pretty homogeneous with 1 or 2 exceptions per batch. Influential parameters identified were the same for the two February batches with the importance of the main parameters (time, temperature and ratio) evident, but no obvious influential effects from the interactions between parameters on total mannitol yield (%DW seaweed). For the third batch, time was not one of the important factors, but the interaction between the temperature/ratio was defined as influential and the effect was even more important than the influence of extractant/biomass ratio only on total yield values (0.24% and 0.17%, respectively).

The effect calculations (Table 4.13) yielded a negative effect of time on batches 2 and 3, and positive influence on the first batch. A negative effect, in theory, is impossible to obtain unless some compounds were evaporated/lost or linked to others biomolecules during longer extraction. Nevertheless, this effect on the third batch was low (-0.08%) and most probably non influential. For the February batches, this effect was more important (0.34% and -0.23% for batches 1 and 2, respectively). The effect of temperature was 2-fold that of extraction time for batch 1 (0.63%) and more than 6 times for the third batch (0.55%). With the second batch, the effect value for temperature was similar to the time despite different coefficients (negative for time and positive for temperature). Ratio was the most influential parameter for the first two batches, being slightly more important than temperature for the
first batch, but having 2-fold the effect in the second batch. Concerning the interactions between parameters, most of them had a negative coefficient, with a low effect values (Table 4.13). For the first batch, the most important interactions were time/temperature (negative coefficient) and temperature/ratio (positive coefficient) with values of -0.16% and 0.20%, respectively. For the second batch, the main influential interaction was time/temperature (-0.19%), the other ones had an effect below -0.05%. In the same way, the only important interaction in the third batch was temperature/ratio. The difference between low and high levels of temperature was significant between extractant/biomass ratios 2:1 and 5:1 (Appendix 6). Ratio was the most important parameter on the extraction of mannitol in the February batches in contrast to the summer batch, in which temperature had a higher effect.

Table 4.13 Influential parameters and interactions for the mannitol extraction yields (% seaweed dry weight)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>0.34±0.26</td>
<td>0.26±0.19</td>
<td>-0.08±0.09</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.63±0.26</td>
<td>0.26±0.19</td>
<td>0.55±0.09</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.71±0.26</td>
<td>0.45±0.19</td>
<td>0.17±0.09</td>
</tr>
<tr>
<td>Time/Ratio</td>
<td>-0.16±0.26</td>
<td>-0.19±0.19</td>
<td>-0.02±0.19</td>
</tr>
<tr>
<td>Temperature/Ratio</td>
<td>-0.08±0.26</td>
<td>-0.04±0.19</td>
<td>0.05±0.19</td>
</tr>
<tr>
<td>Temperature/Time</td>
<td>0.20±0.26</td>
<td>0.24±0.09</td>
<td>0.01±0.09</td>
</tr>
</tbody>
</table>

In general, a high temperature and high ratio increased the mannitol extraction yield. Extraction time had a negative coefficient for batch 2, suggesting that less mannitol was extracted after 6 hours than 1 hour. This could probably due to some evaporation during the longer process extractions.

4.2.6.2 Effect of extraction parameters on mannitol content in crude extract

Mannitol content as a proportion of the dry weight of the recovered extract was significantly higher in the first batch (17.7%) than the two industrially processed batches, with contents of 9.6% and 9.2%, respectively, for batches 2 and 3. In terms of reproducibility of the extraction protocols, the summer batch gave better results across all protocols resulting in the narrowest confidence interval (0.67%; Appendix 6). With the February batches, better reproducibility was obtained on the industrially processed batch with a confidence interval of 1.09% versus 1.33% for the first batch. No trends could be identified regarding the most reproducible protocols in all batches (Appendix 6). Table 4.14 shows the average effect values for each extraction parameter and interactions between parameters for the mannitol content in crude extracts (values in green indicate the statistically most influential parameters). Except for time, where the profile of values was similar between batches 2 and 3 (negative and potentially influential), the batches using
seaweed harvested in February gave comparable profiles and contrasted with the third (summer) batch. Indeed, temperature was insignificant for the first two batches and positively influential for the third batch (1.88%). The converse influence to temperature on the batches was observed for the influence of ratio (1.69% and 2.51% respectively, for batches 1 and 2). For the February batches (1 and 2), the most important calculated effect was for ratio and this was the only really significant influential parameter for batch 1. In addition, time was also influential on the second batch (longer extraction decreased the

\[ \text{Table 4.14 Influential parameters and interactions for the mannitol content (% of total solid)} \]

<table>
<thead>
<tr>
<th>Batch 1</th>
<th>Time</th>
<th>Temperature</th>
<th>Ratio</th>
<th>Time/ Temperature</th>
<th>Time/Ratio</th>
<th>Temperature/Ratio</th>
<th>Time/ Temperature/Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.08±1.33</td>
<td>0.06±1.33</td>
<td>1.09±1.33</td>
<td>-0.77±1.33</td>
<td>-0.69±1.33</td>
<td>-0.39±1.33</td>
<td>-0.49±1.33</td>
</tr>
<tr>
<td>Batch 2</td>
<td>-1.89±1.09</td>
<td>0.32±1.09</td>
<td>2.51±1.09</td>
<td>-1.12±1.09</td>
<td>-0.26±1.09</td>
<td>-0.95±1.09</td>
<td>0.05±1.09</td>
</tr>
<tr>
<td>Batch 3</td>
<td>-0.81±0.67</td>
<td>1.88±0.67</td>
<td>0.03±0.67</td>
<td>-0.12±0.67</td>
<td>0.34±0.67</td>
<td>0.85±0.67</td>
<td>0.03±0.67</td>
</tr>
</tbody>
</table>

mannitol content). Temperature was the most important parameter for the summer (third) batch with an increase of mannitol content at high temperature. Despite a pretty low effect of temperature for batch 2, interactions involving this parameter and other parameters were the highest and even influential for the interaction time/temperature. With the second batch, at low temperature, almost no difference was noticed between short and long extraction period, but an increase in temperature was beneficial for the shorter extraction time (negative coefficient). For batch 3, temperature/ratio was influential, where a smaller ratio gave lower difference in the mannitol contents between the temperatures (25°C and 70°C). Indeed, at the higher extractant/biomass ratio, a hot extraction appeared to give a final extract with higher mannitol content. This interaction was the only significant one for this batch. Laboratory processed seaweed (batch 1) gave a response 2-fold higher than industrially processed batches (on average of the eight protocols). For the February batches, the mannitol content could be optimised by selecting an appropriate extractant/biomass ratio, whereas for the summer batch, the main influential effect on optimisation would be temperature. Moreover, using industrially processed seaweed, an increase of time decreased the mannitol content in the final extract.
4.2.7 Effect of extraction parameters on the extraction yields of fucoidans and their proportion in crude extracts

4.2.7.1 Effect of extraction parameters on fucoidan extraction yields

Fucoidan extraction yields, as a % of the total dry weight of the starting seaweed biomass, were higher for the two industrially processed batches (batches 2 and 3), even though no statistical difference was obtained between batch 1 and 2 (0.63% and 0.75% DW, respectively). In the same way, the summer batch (batch 3) gave a higher fucoidan yield (0.99%) but this was also not significantly different to the second batch (Table 4.2). Standard deviation values were relatively low for all protocols with each batch, which in turn gave small confidence intervals in general (see Appendix 6). Some of the confidence value ranges, across all of the extraction protocols for a given batch, were marginally wider for batch 3 (0.09%) and the narrower for batch 2 (0.07%). Nevertheless, some lower reproducibility was observed in the 6 hour extractions, with an average %yield of 0.11%, 0.10% and 0.16% for batches 1, 2 and 3 respectively, compared with 0.04%, 0.05% and 0.08% for the batch averages for the 1 hour extractions. All parameters and interactions (even the composite interaction between time/temperature/ratio) were identified as influential for the extraction yield (%DW seaweed) of fucoidans, using blended seaweed as the starting material in batch 1. The two industrially processed batches (2 and 3) were influenced by the same parameters (only time and temperature) with similar effect values (see Appendix 6 and Table 4.15). The overall profiles of interactions were also similar between the batches, especially between the two industrially processed ones. The interaction between time/temperature was influential in all series. Indeed, at 25°C, the fucoidan extraction yields were similar between 1 hour and 6 hour extractions. By increasing the temperature to 70°C, the differences between the two time durations became significant (higher response for the 6 hour extraction). In contrast to batch 1, interactions involving ratio were insignificant for batches 2 and 3. Interaction between ratio and time or temperature had the same profile for all industrially processed batches. A higher level of ratio increased the effect of time and temperature. Indeed, the differences between shorter and longer extraction times (or lower and higher temperatures) were more important at the higher

![Table 4.15 Influential parameters and interactions for fucoidan extraction yields (% seaweed dry weight)](image-url)
extractant/biomass ratio. When seaweeds were processed in the laboratory (batch 1), fucoidan extraction was influenced by all parameters and interactions. All coefficients of the equation were positive, so several of the parameters increased the response in terms of yield. Regarding the other two batches, a good correlation was observed between both and the seasonal effect was identified only on the total average (0.75% for batch 2 and 0.99% for batch 3), across all protocols. The effects of parameters (time and temperature) had a similar effect on the two batches.

4.2.7.2 Effect of extraction parameters on fucoidan content in crude extract

The average fucoidan content across all of the protocols showed that the industrially cut seaweed (batches 2 and 3) gave similar response (4.51% and 4.50%, respectively) and that the proportional content in the crude extract was higher than for the laboratory processed batch (2.85%; see Appendix 6). The reproducibility of the response was good resulting in narrow confidence intervals for the three batches. In all batches, similar trends were noticed for the influence of the extraction parameters on the reproducibility. In fact, in all cases, the extractions done during one hour, at 25°C and ratio 2 were more reproducible than the corresponding extractions conducted over a six hour extraction period ones, at 70°C and ratio 5. In the three batches, temperature was the most influential parameter with 0.92%, 1.14% and 1.46% content values for batches 1, 2 and 3, respectively (Table 4.16). Extraction time was also identified as influential with a higher effect on the industrially processed batches (like temperature) with 0.82% and 0.66% for batches 2 and 3, against 0.44% for batch 1 and ratio was insignificant for the fucoidan content in crude extract (effect in batch 3 was zero). The interaction between the two influential parameters was also important across protocols tested for the first and third batches. A small difference was noticed between short and long extractions at lower temperature. At a higher temperature, long extractions seemed to be more efficient in increasing the fucoidan content of the final extract. The effects of the other interactions were at least 2-fold lower than the interaction between time/temperature and were not influential. The best protocol to apply to increase fucoidan content in seaweed

<table>
<thead>
<tr>
<th>Table 4.16 Influential parameters and interactions for fucoidan content (% of total solid)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Time/Temperature</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>Batch 1</td>
</tr>
<tr>
<td>Batch 2</td>
</tr>
<tr>
<td>Batch 3</td>
</tr>
</tbody>
</table>
extract was similar for the three batches. A higher value of extraction time and temperature optimise the response (yield relative to the extract dry weight) optimally.

### 4.2.8 Effect of extraction parameters on the laminaran extraction yields and their proportion in the crude extracts

#### 4.2.8.1 Effect of extraction parameters on laminaran extraction yields

Like fucoidan, laminaran extraction yields as a % of the DW of seaweed biomass were, on average, higher for the industrially-processed batches. However, for laminaran there was a significant difference between the laboratory-processed batch and industrially-processed batches. Surprisingly, season did not seem to be important with regard to the levels of this storage polysaccharide extracted from *A. nodosum* with similar yields obtained for batches 2 and 3 (2.28% and 2.37%, respectively). Laminaran content was determined using a calculation that combines results from two analyses (see Section 2.6.3) and this may explain the lower reproducibility and generally wider confidence intervals between protocols for a given batch. With the first batch, yields from protocols using the higher extractant/biomass ratio tended to be less reproducible than those obtained with the low extractant/biomass ratio, but only for the 1 hour extractions, and the opposite observation was noticed for the 6 hour extractions. For the second batch, the main difference was mainly due to the influence of time; the low duration of extraction gave higher standard deviations than the longer extractions. And finally, standard deviations were more homogeneous across the protocols applied to the third batch, which resulted in a narrower overall confidence interval (±0.16%). The widest interval was obtained for batch 2 at ±0.24%. Batches 1 and 2 gave very similar values for the effects of each main parameter, despite a different effect values for time (which was statistically insignificant in both series), as seen in Table 4.17. Effect of temperature and ratio were significantly influential with similar values for both parameters for the same batch, e.g. 0.28% and 0.31%, respectively for the first batch, 0.26% and 0.30% for the second and 0.40% and 0.52% for the third batch. Overall, the effects of those two parameters on %yield DW seaweed were more important for the third batch. Moreover, the temperature showed a negative coefficient in the third batch, in contrast to both February batches (batches 1 and 2). Laminaran extraction yields appeared to be optimal at high temperature for seaweed harvested in February and at low extraction temperature for seaweed harvested in June. The difference between low and high levels of temperature was much bigger at a ratio of 5 than a ratio of 2. Between the two ratios, a slight increase was observed at 70°C, whereas this increase was more obvious at 25°C. Despite a lower overall average yield for the first batch 1, the yields for the February batches were very similar.
Concerning the summer batch, temperature had a negative coefficient meaning that a low level of this parameter was better to increase the response. In all cases, ratio was the most influential parameter, and should be employed at the maximum (ratio of 5 in this experiment) to optimise the laminaran extraction yield relative to the DW of the starting seaweed biomass.

4.2.8.2 Effect of extraction parameters on laminaran content in crude extract

Every batch gave, on average, a significantly different result concerning the laminaran content in crude extract (Table 4.3). The highest content was obtained for the second batch at a yield of average 14.5% of the dry weight of the crude extract, across all of the protocols (extractions 1-8; see Appendix 6). For the first and second seaweed batches, the yield average between all protocols was 9.4% and 11.3%, respectively. Due to the high standard deviation values for the first two batches, the average confidence intervals, across all protocols, were quite variable (1.09% and 0.74% for batches 1 and 2). For the first batch and protocols using the shorter extraction process (Extractions 1-4), standard deviations were higher at a higher extractant/biomass ratio (Extractions 2 and 4) than at the lower extractant/biomass ratio (Extractions 1 and 3). The opposite effect was noticed for protocols using the longer extraction time (Extractions 5-8). For batch 2, standard deviations were higher for protocols using the short extractions (1.10% on average versus 0.40% for the longer extractions). No trend was identified for the last batch (Batch 3). Regarding the temperature and ratio effect values (Table 4.18), the values for specific parameter effects were the same for the three batches (e.g. negative for temperature and positive for ratio), but those parameters were not influential with the laboratory processed batch, only for the industrially-processed batches. Moreover, no parameters or interactions could be identified that influenced the laminaran content in the crude extracts derived by the various protocols from the first seaweed batch. In the case of batches 2 and 3, irrespective of the extraction time, the same parameters were statistically influential. Time and temperature had a negative
effect in all series meaning that longer extraction and high temperature processes resulted in extracts that were poorer in laminaran content. The effect of time was higher for the February batches than the summer seaweed batch but was significant only for batch 2.

Table 4.18 Influential parameters and interactions for laminaran content (% of total solid)

<table>
<thead>
<tr>
<th>Batch 1</th>
<th>Time</th>
<th>Temperature</th>
<th>Ratio</th>
<th>Time/Temperature</th>
<th>Time/Ratio</th>
<th>Temperature</th>
<th>Time/Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-1.04±1.09</td>
<td>-0.36±1.09</td>
<td>0.47±1.09</td>
<td>0.46±1.09</td>
<td>-0.15±1.09</td>
<td>0.10±1.09</td>
<td>-0.74±1.09</td>
</tr>
<tr>
<td>Batch 2</td>
<td>-1.10±0.74</td>
<td>-1.10±0.74</td>
<td>1.15±0.74</td>
<td>0.01±0.74</td>
<td>0.54±0.74</td>
<td>-0.82±0.74</td>
<td>-0.21±0.74</td>
</tr>
<tr>
<td>Batch 3</td>
<td>-0.58±0.61</td>
<td>-2.68±0.61</td>
<td>1.74±0.61</td>
<td>-0.39±0.61</td>
<td>0.11±0.61</td>
<td>-0.92±0.61</td>
<td>-0.10±0.61</td>
</tr>
</tbody>
</table>

Temperature and ratio had similar effects, despite apparently opposite coefficients, on the second batch (-1.10% and 1.15% respectively). The interaction between those two parameters was also close to these values at -0.82%. In the third batch, temperature was the most important parameter with an effect value of -2.68% and ratio influenced the response by up to 1.74%. The interaction between temperature/ratio showed a similar profile (similar coefficients) between batches 2 and 3. At the lower extraction temperature (25°C), a significant increase was noticed between the two levels of ratio (by around 6% in batch 2 and around 5% in batch 3 with a higher response at higher ratio). At the higher extraction temperature (70°C), an increase was also observed but in lower proportion (less than 1% for both batches). For the laboratory processed seaweed (batch 1), no significant differences were observed between the different extraction protocols, and the extracts contained 9.44%±1.09% laminaran. Comparing batches 2 and 3, the February batch appeared to contain more laminaran than the summer batch but a long extraction time decreases the laminaran yield in the extracts derived from seaweed harvested in February, and has no effect on the summer batch. Finally, the effects of temperature and ratio were more important (especially temperature) for batch 3 (June seaweed batch).
4.3 Discussion

Extraction yields are an important parameter in terms of the industry point of view and a comparison between different points of the year provide invaluable information on the potential variability in the end-product and the likely implications for the cost of production. The crude extract is a mixture of the water-soluble compounds extractible from the seaweed and is the base product for many high-value applications. To-date, this type of extract has been used for the agriculture market. However, the relationship between the methods of extraction, in combination with harvest times from two different annual timepoints of biological relevance, and the yield and biomolecule composition is also of scientific interest. Much of the data published to-date on biomolecule composition in seaweeds (especially carbohydrates) has been derived using chemical-based extraction techniques, which can result in modifications to the structures and composition of the biomolecules. In addition, as many of the more bioactive compounds are water-soluble, developing an extraction method based on understanding the influence of the extraction parameters on the recovery and yield of the desired biomolecules is important to underpin sustainable extraction at scale and reproducible data in bioactivity tests (Ale & Meyer 2013; Stengel et al. 2011).

The biomass harvesting and pre-treatment is the first parameter to take into consideration. Most of the seaweed extracts are prepared from dried seaweed and some authors have shown that the effect of the drying process showed no significant differences on the crude extract yields between air and oven drying, but a freeze drying process increased the quantity of compounds extracted (Maisuthisakul & Pongsawatmanit 2004). Before extraction, the seaweed biomass is cut to increase surface extraction area, and the size of the particles have been shown to be important parameter for optimisation of the extraction yield. The effect of particle size has been investigated for different biomass types in the extraction of phenolic substances or polysaccharides. Indeed, the extraction yields of phenolics from parsley (Luthria 2008) were higher when the particle sizes were smaller. Similarly, the yield of phenolic compounds from pomegranate was higher when the extraction was done on small size particles, but only of the extraction conducted for < 3 hours (Wang et al. 2011). At longer extraction times, particle size no longer had an influence.

In this study, seaweed in batch 1 was blended using a hand blender, which resulted in smaller pieces than the industrially-processed batches (2 and 3). Total extraction and phenolic substance extraction yields were higher for the first batch than for the second one, which correlates with the results obtained for the other biomass types mentioned above. A different effect was observed for the polysaccharide extraction yields (fucoidans and
laminarans). Indeed, by comparing the first two batches, slightly higher (potentially insignificantly different) yields of both polysaccharides were obtained for the second batch suggesting that smaller pieces of biomass were not better in this case for optimum extraction. Similar results were obtained for the extraction of polysaccharides from a mushroom (*Fomes officinalis*). Polysaccharide extraction yields in that study did not show significant differences across different biomass sizes (Mingdong et al. 2013).

The seasonal comparison between seaweed harvested in February (batch 2; end of winter) and June (batch 3; summer) showed differences except for the sugar extraction yields (mannitol, fucoidans and laminarans), which was surprising. Indeed, despite some variation in the seaweed composition between both annual timepoints, the quantity of sugar extracted according to the seaweed dry weight was similar between the two batches. Seasonal variations of composition of *A. nodosum*, likewise other fucales species, tend to be less important due to their slow growth rate (Percival & McDowell 1968), in contrast to the faster-growing laminariales, which is reflected in the similar results obtained between the two batches in this research. The content of phenolic substances in seaweed is higher during the summer period (Connan et al. 2004), as seen in higher extraction yield for the summer batch in comparison with the February batches. In the same way, ash extraction yields were higher in the summer batch, which supports earlier reports on a higher ash content in seaweed during the summer period (Black 1950; Ryan et al. 2012).

The effects of extraction time on extraction yield was expected to be only positive or to have a ‘null’ effect as a negative effect would mean that less compounds were extracted after 6 hours than 1 hour. *Table 4.19* below provides an overview of the insight gained through this work on the effect of extraction time on the yields of the different compounds of interest, with a “+” for a positive effect (higher compound yield after 6 hours extraction), “-” for a negative effect (higher compound yield after 1 hour extraction) and “0” if the parameter did not influence the response. The only negative effect was obtained was for the mannitol extraction yield in the 2nd batch. Extracts prepared using longer extraction times contained on average less mannitol due to some evaporation. Thus this effect can then be considered as ’null’. Nevertheless, similarities were noticed between the two industrially processed batches and some differences between these and the laboratory processed batch. Mannitol extraction in water from olive leaves had been reported to reach a maximum after 2 hours (Ghoreishi et al. 2008). No experiments have been reported to-date where more mannitol is extracted during longer extraction times. Moreover, the time course extraction conducted in Chapter 3 showed a constant mannitol (and glucose) concentration in the extract over 24 hours (see Section 3.2.5.3). Laminaran extraction yields were not statistically influenced by the duration of the extraction. Indeed, in the three batches, extraction yields
were similar after 1 and 6 hours. Wang et al. (2011) reported on a laminaran extraction protocol that was optimised by orthogonal experiment and used for extraction of laminaran from Laminaria japonica. The optimum extraction time was defined to be 2 hours, and a longer extraction timeframe did not increase the yields (Wang 2011). Extraction of fucoidan is more complex. Indeed, a longer extraction has been shown to give a final extract with different fucoidan composition, in this study but also on other seaweed species (Ale et al. 2011a). Different hypotheses have been proposed and it has been suggested that long extractions on chordariaeaceae sp yielded a degraded fucoidan (Hakano et al. 2007). However, in this work, the fucoidan concentration in the extract from A. nodosum was shown to increase with increasing time (see Section 3.2.5.3).

Variation in fucoidan yields due to the solvent and extraction temperature has been more thoroughly investigated than the effects of time. Nevertheless, fucoidan extraction yields were significantly higher using protocols involving longer extractions. In general, polysaccharide extraction yields increased with the extraction duration (Mingdong et al. 2013). The increase reported by Mingdong (2013), even though statistically significant, was only 0.5% within 2 hours. In the work reported in this chapter, the total sugar content increased by 0.6% within 5 hours for batch 3 (summer harvest) and was not shown to be influential for the other two batches (February harvest timepoint).

Finally, results on the extraction yields of phenolic substances showed differences between the findings in this work, and previous reports in the literature. Indeed, extraction parameters appeared to be influential in the three batches (Table 4.19), which was different to the result obtained using the step-wise extraction protocol in Chapter 3, in which the phenolics were extracted in the first hour and levels stayed constant overtime (see Section 3.2.5.3). A study on the effect on extraction on phenolic compounds from pomegranate showed that phenolics are extracted at the beginning of the extraction process (in the first 2 minutes) only (Wang et al. 2011).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Total</th>
<th>Polyphenols</th>
<th>Ash</th>
<th>Total sugars</th>
<th>Mannitol</th>
<th>Fucoidans</th>
<th>Laminarans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td></td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Batch 2</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Batch 3</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.19 Summary of the effect of extraction time on the main compound extraction yields in the three batches
In the study reported in this chapter, temperature was influential for all yield responses across all batches (*Table 4.20*). Higher temperature increased the extraction yields in all cases, except for the laminaran extraction yield in the third batch, in which case the yield of laminaran extracted was higher at low temperature (*Table 4.20*). Previous work has shown that a hot extraction process allowed the extraction of two forms of laminarans (soluble and insoluble forms; (Percival & McDowell 1968), which may explain the higher laminaran yields observed at the high extraction temperature in this work. Like many of the biomolecules in brown seaweeds, the laminaran content can vary over the year, and it is thus likely that the relative proportion of soluble/insoluble forms vary as well. Even though no study has been done, to the author’s best knowledge, on the seasonal variation of each form of laminaran, a negative effect of the extraction temperature in the summer batch could signify a lower proportion of the insoluble form during this period of the year, or potential temperature lability of the soluble form. Many studies have investigated the effect of extraction temperature on polysaccharide extraction yields from different plant sources, like pumpkin (Sun et al. 2011) or the mushroom *Grifola frondosa* (Yang et al. 2013). For these biomass types, like seaweed results reported in this chapter (the total sugar yield increased by up to 1.2% from 25°C to 70°C), higher temperatures enable the extraction of more polysaccharide with an increase of 2-3% between the lowest and the highest temperature tested. With the mushroom biomass, the temperature range investigated was much higher than in this study (i.e. from 100°C to 250°C), nonetheless, the polysaccharide extraction yields increased by a few percent from 100°C to 150°C and by around 25% from 150°C to 250°C. More specifically, Ale et al. defined the effect value of the extraction temperature on the fucose containing sulphated polysaccharide (fucoids) yield extracted from *Sargassum sp.* at 0.23% (Ale et al. 2011a). In the same study, the influence of temperature was calculated to vary between 0.29% and 0.38% according to the batches. In terms of the yields of phenolic compounds, more differences were noted between different biomass types. Indeed, effect of high temperature induced higher extraction yields for seaweed (increase around 0.2% between 25°C and 70°C in this study) and for litchi fruits by 2% between 30°C

![Table 4.20 Summary of the effect of extraction temperature on the main compound extraction yields in the three batches](image-url)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Total</th>
<th>Polyphenols</th>
<th>Ash</th>
<th>Total sugars</th>
<th>Mannitol</th>
<th>Fucoids</th>
<th>Laminarans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Batch 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Batch 3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
and 80°C (Ruenroengklin et al. 2008) but had no effect on the extraction yield of phenolics from parsley (Luthria 2008).

The last parameter investigated was the effect of the volume of water used for extraction and the quantity of seaweed used in the extractions (referred to as the ratio in the text of this chapter, See Table 4.21). Polyphenol extraction yields were higher at the higher extractant/biomass ratio in all cases. This phenomenon was also observed for the extraction of phenolic compounds from pomegranate with a constant increase in the extraction yields with higher volume of solvent in a study done with ratios from 5 to 50 which resulted in an increase in yield of 5% (Wang et al. 2011) in comparison to only 0.5% at a ratio of 5 in this study. However, the increase described by Wang et al. was reported to be very low from the ratio 5 to 15 (yield increased by <1%), and more important afterward. Concerning the polysaccharides, effect of ratio was similar between the two industrially processed batches in the work reported in this chapter, with no effect concerning the fucoidan extraction yield and a positive effect regarding the extraction of laminarans. In the literature, different conclusions have been made on the effect of this parameter with for example, an insignificant influence being reported for the extraction yields of polysaccharide from *Grifola frondosa* (Yang et al. 2013). More interestingly, two separate studies were done using the same range of ratio (between 4 and 15) with *Paeonia sinjiangensis* (Tian et al 2011) and *Limonium sinense* (Tang et al. 2011), and an increase of 30% in the polysaccharide extraction yields was reported *Paeonia sinjiangensis* studies (Tian et al. 2011) and 7% for *Limonium sinense* (Tang et al. 2011). Using a higher ratio, polysaccharides yields tended to decrease after a ratio 20, as shown in the studies conducted with pumpkins (Sun et al. 2011; Xianzhe et al. 2011). By using a ratio <20, yields of polysaccharide extracted from pumpkin increased by 2-3%. In those same studies, total sugar extraction yields were also influenced by the ratio used and an increase of only 0.5% in yield was observed. Unfortunately, pumpkin polysaccharides, in those reports, were only described by their monosaccharide composition (glucose, galactose, xylose, arabinose and glucuronic acid) without reference to the relative proportion between different types of polysaccharides.

**Table 4.21 Summary of the effect of extraction ratio on the main compound extraction yields in the three batches**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Total</th>
<th>Polyphenols</th>
<th>Ash</th>
<th>Total sugars</th>
<th>Mannitol</th>
<th>Fucoidans</th>
<th>Laminarans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Batch 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Batch 3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>


polysaccharides. However, the effect of ratio on the polysaccharide extraction yields from pumpkins was similar to the effect on the laminaran extraction yields.

4.4 References


Ruenroengklin, N. et al., 2008. Effects of various temperatures and pH values on the extraction yield of phenolics from litchi fruit pericarp tissue and the antioxidant activity of the extracted anthocyanins. *International Journal of Molecular Sciences*, 9(7), pp.1333–41.


Chapter 5: Influence of extraction parameters on polysaccharide composition and size
5.1 Introduction

From reports in the literature to-date on fucoidan extraction, it is very difficult to correlate a trend in the composition of fucoidan. It is also very difficult to analyse the number of parameters to take in consideration in examining the effect of extraction on composition. Nonetheless, the first important factor to consider is the protocol used to dry the seaweed which can influence the yield and composition of polysaccharide extracted (Maisuthisakul & Pongsawatmanit 2004). The extraction solvent is also a key factor and a compromise has to be found between extraction yields and degradation of native polysaccharides. For example, although acid treatment tends to increase the yields, it also damages the carbohydrates (Ale & Meyer 2013). For both of these reasons, this study used fresh A. nodosum and polysaccharides were extracted with water, under experimental conditions that varies three different parameters: time, temperature and solvent/biomass ratio.

The accurate structure of fucoidan is still not well defined. Indeed, before analysis, fucoidans have to be extracted and the extraction protocol can have a significant influence on the fucoidan composition (Ale et al. 2011b). For this reason, different fucoidan compositions have been reported in the literature, even for the same seaweed species. Fucoidan extracted from A. nodosum, by a two-step extraction (room temperature and 70°C using aqueous CaCl\textsubscript{2} for solvent), was reported to contain (molar percentage of total fucoidan) only fucose (66%), xylose (3%) and sulphates (31%) (Marais & Joseleau 2001). Another study, on the same seaweed, using the same solvent (aqueous CaCl\textsubscript{2}) in a five step protocol (same extraction repeated 5 times for 5 hours) at a higher temperature (85°C) gave a fucoidan composed of (mass percentage of total fucoidan) 26.6% fucose, 4.7% galactose, 4.4% xylose, 2.6% mannose and 24.4% sulphates, which contrasts markedly with the fucoidan described by Marais & Joseleau above (Cumashi et al. 2007). The latter composition was supported by the composition of another fucoidan extract, prepared from A. nodosum using a two-step extraction (20 h at 20°C followed by 2 h at 100°C) in water, which contained 28.4% fucose, 5.3% galactose, 4.3% xylose, 0.8% mannose and 19.4% sulphates (Nakayasu et al. 2009).

As the fucoidan content in brown seaweeds varies over the year, its composition may also affect this variation. Indeed, two studies that investigated the seasonal variation of fucoidan composition extracted from Costaria costata (Laminariales) (Imbs et al. 2010) and Padina pavonica (Dictyotales) (Ermakova et al. 2012) showed similar trends. In both cases, the proportion of fucose and galactose increased from winter to summer, contrary to the
xylose and mannose, both of which decreased during the summer period. The only contradiction was found with respect to the rhamnose content, which was higher during winter in the *C. costata* fucoidan yet higher during summer for the fucoidan from *P. pavonica*. The effect of seasonality on fucoidan composition was investigated also for *Undaria pinnatifida* (Skriptsova et al. 2009). In the latter study, fucose content stayed constant over the year contrary to the mannose content which decreased significantly; the galactose increased from winter to summer.

Two forms of laminarans have been found in brown seaweed and both differ according to their solubility (soluble and insoluble forms). This difference of solubility has been shown to be due to compositional variation (Percival & McDowell 1968). Indeed, the soluble form has been shown to contain more mannitol units than its insoluble counterpart (Peat et al. 1958). Laminarans extracted from *Saccharina longicruris* displayed different compositions over the year. During the winter period, proportion of glucose in the laminarans was lower than in the summer period (Rioux et al. 2009).

In addition to the variation in composition, the size of fucoidans is also dependent of the extraction protocol with many different reported sizes in the literature. Size fractionation of fucoidan extracted from *Hizikia fusiforme* (*Fucale*) revealed a size range from 25 to 950 kDa but unfortunately, the relative proportion of each size variant was not described (Li et al. 2006). Different sizes have been reported for fucoidans from *A. nodosum*. Rioux and co-workers obtained a 417 kDa fucoidan after high temperature extraction (Rioux et al. 2007). After a three step extraction at different temperatures, two size fractions of 47 and 420 kDa were identified from this seaweed (Foley et al. 2011). Two independent reports isolated fucoidans with a similar size from *A. nodosum*, i.e. 13 kDa (Daniel et al. 2001) and 16 kDa (Senni et al. 2006). To date, to the author’s best knowledge, no studies have directly investigated the effect of the extraction parameters on fucoidan size. In general, sizes are generally determined on the resulting extract from a protocol with no deeper understanding of the effects of temperature or extraction time on size and composition.

The previous chapter (Chapter 4) showed that extraction parameters influenced the extraction yields of the main water soluble compounds from *A. nodosum* as well as the composition of the crude extracts (Chapter 4). Moreover, in Chapter 3, which conducted a preliminary evaluation of extraction methods for recovery of fucoidan-rich fractions, the monosaccharide composition of fucoidans changed during time-course extraction (see Section 3.2.5.4). This chapter focuses on the influence of extraction parameters investigated in Chapter 4 on the polysaccharide composition and size between the two industrially
processed batches (batches 2 and 3, Chapter 4), which has also allowed the seasonality aspect to be investigated. Based on the results, a mathematical model was proposed to define the fucoidan composition according to the extraction parameters (fucoidan was assumed to be composed of fucose, galactose, rhamnose, xylose and mannose). Sulphate content of the fucoidan fractions was also evaluated. Nevertheless, the effect of extraction parameters could be investigated but it was not possible to apply the same quantitative mathematical approach. Regarding the alginate composition, a preliminary investigation was conducted to determine the mannnuronic/guluronic acid ratio by combining different experiments using specific alginate lyases produced by the marine bacterium *Zobellia galactanivorans* (Thomas et al. 2013).
5.2 Results

The previous chapter showed, in general, somewhat better reproducibility of extracts from the industrially processed batches. Therefore, the effects of extraction parameters on composition of potential fucoids in extracts were investigated only for seaweed batches 2 and 3. The relative abundance of sugars normally associated with fucoids/enriched fucoidan fractions from *A. nodosum* was evaluated by HPAEC. Comparison of both of these batches also afforded an additional comparison to be made, which was a comparison of seasonality, i.e. February for batch 2 and summer for batch 3. The potential fucoidan composition was determined by HPAEC following TFA hydrolysis, as described in Sections 2.5.7 and 2.5.8. In this chapter, the composition of potential ‘fucoids’ in the crude extract obtained after the eight different protocols (prepared in triplicate) is based on individual and cumulative effects on monosaccharides known to be structural components of seaweed fucoids (fucose, galactose, rhamnose, xylose and mannose). The results were averaged and statistically analysed by a one way ANOVA to determine the influence of seasonality (results of individual protocols are shown in appendix 8). The results are summarized in Table 5.1 with significant differences indicated by values that do not share the same letter per column. The ranges of values obtained across the 8 protocols are indicated by the minimum and maximum values between brackets (min – max). Each monosaccharide was present in statistically different proportions in the two batches (*p* values for the comparison between the two batches for all extractions were <0.05). In both batches, fucose was the main monomer but the mixture of fucoidan-associated monosaccharides extracted from the summer seaweed batch was richer in fucose (43.3% versus 38.4% for the February batch). Concerning the other monosaccharides, seaweed extracts from the February batch contained more galactose, rhamnose and mannose (17.8%, 4.6% and 19.4%, respectively). Proportionally the pool of fucoidan-associated sugars from *A. nodosum* harvested in summer was richer in xylose (24.7% relative to 19.9% for the February batch).

<table>
<thead>
<tr>
<th>Batches</th>
<th>Fucose</th>
<th>Galactose</th>
<th>Rhamnose</th>
<th>Xylose</th>
<th>Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>February</td>
<td>38.4</td>
<td>17.8</td>
<td>4.6</td>
<td>19.9</td>
<td>19.4</td>
</tr>
<tr>
<td></td>
<td>[33.38 – 44.09]</td>
<td>[13.28 – 22.68]</td>
<td>[1.09 – 5.96]</td>
<td>[13.57 – 23.94]</td>
<td>[13.08 – 24.38]</td>
</tr>
<tr>
<td>June</td>
<td>43.3</td>
<td>13.5</td>
<td>2.5</td>
<td>24.7</td>
<td>16.0</td>
</tr>
</tbody>
</table>
5.2.1 Effect of extraction parameters on the proportion of fucose in the extracts

5.2.1.1 Influence of the main extraction parameters and interactions

The effects of the extraction parameters (time, temperature and extractant/biomass ratio) on the fucose content of the resulting extracts from the two industrially-processed seaweed batches showed similar profiles in each of the response curves (Figure 5.1). Time seemed to have a positive effect on the response (proportion of fucose was higher after longer extraction) and appeared to be more influential for the June seaweed batch than the February one. The most influential parameter appeared to be temperature, with a hot extraction giving polysaccharides richer in fucose, and with similar effects noted for the two batches (similar slopes). With regard to the ratio, no effect was observed for the summer seaweed batch, indeed, the average fucose content in potential fucoidans extracted at low and high ratio was identical, whereas a slight negative effect was noticed for the February batch, i.e. by increasing the ratio, the fucoidan extracted contained less fucose.

Information from an interaction plot graph (Figure 5.2) was similar for the two seaweed batches. Indeed, the interactions involving ratio were negligible in both cases, as indicated by the parallel lines in the top and bottom right plates in Figure 5.2). In the case of time versus temperature (upper left plate), in both cases, the extraction time appeared to have no influence on the fucose proportion at low temperature (25°C). However, by increasing the temperature, the fucose content increased and most especially in the extract obtained after 6 hours extractions.

![Figure 5.1 Effect of A) time (1 hour and 6 hours), B) temperature (25°C and 70°C) and C) ratio (2 and 5) on the proportion of fucose in fucoidan present in the crude extracts](image-url)
5.2.1.2 Calculation of main parameters and interactions effects

Table 5.2 shows the effect values (calculations are explained in Section 2.7.4.2) for each extraction parameter and the interactions between the parameters. For both batches, temperature was the most influential parameter on fucose levels with values of 3.60% and 2.64% (variation of fucose content between 25°C and 70°C extraction protocols), respectively for the February and June batches. Moreover, for the winter batch, time and ratio had a similar effect value (positive for time and negative for ratio) and the interaction between time/temperature had the second most influential effect. In terms of the summer batch, the effect of ratio was much lower (0.27%) than the other two parameters while time appeared to be important with a value 1.70% (variation of fucose content in potential fucoidan between 1 h and 6 h extraction protocols). Likewise for the February batch, the

| Table 5.2 Calculation of parameters and interactions effects for the fucose proportion in potential fucoidan (% w/w of the sum of each monosaccharide: fucose, galactose, rhamnose, xylose and mannose) |
|---|---|---|---|---|---|---|---|---|---|
| Extraction N° | mean | A | B | C | AB | AC | BC | ABC | February | June |
| 1 | + | - | - | - | + | + | + | - | 36.38 | 39.10 |
| 2 | + | - | - | + | + | - | - | + | 34.22 | 39.93 |
| 3 | + | - | + | - | - | + | - | + | 40.70 | 42.90 |
| 4 | + | - | + | + | - | - | + | - | 39.55 | 43.68 |
| 5 | + | + | - | - | - | - | + | + | 35.13 | 41.74 |
| 6 | + | + | - | + | - | + | - | - | 33.28 | 41.08 |
| 7 | + | + | + | - | + | - | - | - | 44.09 | 47.57 |
| 8 | + | + | + | + | + | + | + | + | 43.47 | 48.80 |

February: 38.35 0.64 3.60 -0.72 1.19 0.11 0.28 0.03%

June: 43.10 1.70 2.64 0.27 0.75 -0.13 0.23 0.24
only potentially influential interaction for the fucose content of fucoidan in the extracts from the summer seaweed batch was time/temperature (0.75%).

5.2.1.3 Confidence interval calculation and definition of influential parameters

Table 5.3 shows the standard deviation values for the fucose content obtained after each extraction protocol. Standard deviations were higher for the February batch than the June one, giving a 2-fold wider confidence interval. Even if extraction time had a low effect in the February batch, the reproducibility of shorter extraction time was lower than for the longer extraction (average standard deviation for shorter extractions was 3.30% versus 1.54% for longer extractions). Similarly, concerning the effect of temperature for the summer batch, hot extractions were more reproducible than cold extractions. Indeed, the average standard deviation for the hot extractions was three times lower than average standard deviation for cold extractions (0.6% and 1.74%, respectively).

Due to wide intervals, just temperature was defined as influential on the fucose content of the extracted fucoidans in extracts derived from the February batch (Table 5.4). Temperature was also the most important parameter for the summer batch but time was identified as influential as well.

5.2.1.4 Proposed model equation

The fucose content of fucoidan extracted from *A. nodosum* varied between the seasons, with a lower proportion obtained in early spring than summer. Nevertheless, a
consistent proportion could be obtained by adjusting extraction parameters. Indeed, a higher temperature of extraction would induce a fucoidan richer in fucose, and the effect of temperature was more than 2-fold higher for the early spring batch than the summer one.

Early spring: \[ Y = 38.35\% + 3.60\% B \]

Summer: \[ Y = 43.10\% + 1.70\%A + 2.64\%B \]

5.2.2 Effect of extraction parameters on the proportion of other monosaccharides in the seaweed extracts fucoidans

5.2.2.1 Compositional analysis of the extract from the February/early spring batch

The effects of the same extraction parameters on other monosaccharides regarded as components of fucoidan were investigated on the same way as for fucose (results displayed in Figure 5.3).

---

**Figure 5.3** Effect of A) time (1 hour and 6 hours), B) temperature (25°C and 70°C and C) ratio (2 and 5) on the proportions of galactose, rhamnose, xylose and mannose in extracts from seaweed harvested in February
are percentage of each monosaccharides in fucoidans as explain in appendix 5. An increase of the proportion of one monosaccharide was compensated by a decrease of one or other monosaccharides). First of all, graphs representing the effects of each of the main parameter effects were plotted (Figure 5.3 above). For each monosaccharide, temperature was the most influential parameter with either a positive or negative coefficient value. In comparison to the ‘cold’ extractions (extracts prepared at 25°C), extracts obtained at 70°C contained less galactose, rhamnose and mannose but more xylose. Regarding the galactose concentration in the extract, the effects of both extraction time and ratio had positive effects and the ratio appeared to be more influential than the duration or time taken for extraction, but was less influential overall than the temperature used for extraction. For the rhamnose concentration profile, both time and ratio showed very low negative effects, while for xylose, time had a slight positive effect on concentration and ratio a slight negative effect with the exact opposite relationship noticed for the concentration of mannose.

In terms of the galactose and xylose contents, the interactions profiles were similar but with differences in the coefficients, as shown in Figure 5.4. Indeed, no differences were noticed between the two extraction durations (1 and 6 hours) at low and higher levels of temperature and ratio. However, on closer examination of the proportion of galactose, the response was higher (i.e. higher relative concentration) using a low temperature and high

![Figure 5.4](image_url)

*Figure 5.4 Effect of interactions between time, temperature and ratio on galactose, rhamnose, xylose and mannose proportions in extracts from seaweed harvested in early spring/February*
ratio protocol. The exact opposite was observed for the xylose. Nevertheless, for these two monomers, all lines on the plots representing the effects of each parameter were parallel suggesting that no interactions were predominantly influential. Concerning the other two monosaccharides (mannose and rhamnose), the interaction between temperature/ratio (right bottom plates) appeared to have insignificant effects on the concentrations of both sugars. The interaction between time/temperature (left upper plate) showed a similar trend. Indeed, no differences were noticed between the effects of the two extraction times at low temperature but this was not the case for the higher temperature extraction protocols in which a longer extraction gave a lower response (in terms of concentration) than the shorter extraction time. Finally, for the interaction between time/ratio (right upper plates), a different analysis had to be done for rhamnose and mannose. The crude mixtures obtained after long extractions, at a low ratio of extractant to biomass were richer in rhamnose than those obtained after a short extraction period; the opposite effect was noticed at the higher extractant to biomass ratio. For mannose, no differences were observed at the higher extractant to biomass ratio, but a short extraction time and low extractant to biomass ratio gave an extract richer in mannose.

The effects of parameters and interactions were calculated for each monosaccharide and the results are displayed in Table 5.5. The effects on fucose content (determined in Section 5.2.1.2) were also added for a global view of how the extraction parameters might affect the concentrations and relative abundance of monosaccharides representing the potential fucoidan content. On average across the eight protocols, the extracts had a similar proportion of galactose, xylose and mannose (between 17% and 20%), which was around  

<table>
<thead>
<tr>
<th>Monosaccharide proportion in potential fucoidan (% w/w of the sum of each monosaccharide)</th>
<th>Fucose</th>
<th>Galactose</th>
<th>Rhamnose</th>
<th>Xylose</th>
<th>Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>average</td>
<td>38.35</td>
<td>17.85</td>
<td>4.56</td>
<td>19.87</td>
<td>19.37</td>
</tr>
<tr>
<td>Time</td>
<td>0.64</td>
<td>0.12</td>
<td>-0.18</td>
<td>0.26</td>
<td>-0.84</td>
</tr>
<tr>
<td>Temperature</td>
<td>3.60</td>
<td>-3.47</td>
<td>-1.94</td>
<td>5.30</td>
<td>-3.49</td>
</tr>
<tr>
<td>Ratio</td>
<td>-0.72</td>
<td>1.03</td>
<td>-0.17</td>
<td>-0.81</td>
<td>0.66</td>
</tr>
<tr>
<td>Time/temperature</td>
<td>1.19</td>
<td>-0.11</td>
<td>-0.28</td>
<td>0.27</td>
<td>-1.06</td>
</tr>
<tr>
<td>time/ratio</td>
<td>0.11</td>
<td>0.08</td>
<td>-0.68</td>
<td>0.06</td>
<td>0.43</td>
</tr>
<tr>
<td>temperature/ratio</td>
<td>0.28</td>
<td>0.05</td>
<td>-0.41</td>
<td>0.14</td>
<td>-0.05</td>
</tr>
<tr>
<td>time/temperature/ratio</td>
<td>0.03</td>
<td>-0.06</td>
<td>0.20</td>
<td>-0.02</td>
<td>-0.15</td>
</tr>
</tbody>
</table>
twice lower than the fucose proportion. Time had a low effect for every sugar except for mannose.

Temperature was the most influential parameter in all cases, with a negative coefficient for galactose, rhamnose and mannose. The effect value of temperature was similar between fucose, galactose and mannose (despite different coefficients). The highest value for the effect of temperature was identified in relation to the xylose content.

Ratio had a similar effect on the yields of all monosaccharides (despite different coefficients for the individual monosaccharides) but this influence was more than three times lower than the effects of temperature. The effects of all interactions were low and probably insignificant except for time/temperature in the case of mannose (-1.06%).

The calculation of confidence intervals was done based on the standard deviation for the triplicates from each extraction protocol, and the data are presented in Table 5.6. As noted for fucose, a higher average standard deviation, was observed for all monosaccharides in extracts obtained with protocols using the shorter extraction time, in comparison to the values obtained for measurements for extracts derived with protocols using the longer extraction time (2.52% versus 0.86% for galactose, 1.83% versus 1.11% for rhamnose, 2.00% versus 1.08% for xylose and 2.16% versus 0.80% for mannose). Overall, confidence intervals were similar between the other monomers and lower than that for fucose. The widest interval was determined for galactose (1.41%) and the narrowest for rhamnose (1.14%).

Calculations of representative coefficients (across the 8 protocols and parameters) showed that only temperature was significantly influential in determining the monosaccharide content of fucoidan the extract (Table 5.7). Temperature had the most profound effect on rhamnose. Indeed, the effect value was -1.94% which corresponded to

<table>
<thead>
<tr>
<th>Standard deviations $s_i$ (%)</th>
<th>Variance $s^2$ (%)</th>
<th>Confidence intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fucose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EXT 1</td>
<td>3.86</td>
<td>0.002</td>
</tr>
<tr>
<td>EXT 2</td>
<td>3.11</td>
<td>0.002</td>
</tr>
<tr>
<td>EXT 3</td>
<td>2.11</td>
<td>0.002</td>
</tr>
<tr>
<td>EXT 4</td>
<td>4.10</td>
<td>0.002</td>
</tr>
<tr>
<td>EXT 5</td>
<td>2.91</td>
<td>0.002</td>
</tr>
<tr>
<td>EXT 6</td>
<td>0.59</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 7</td>
<td>1.15</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 8</td>
<td>1.51</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Galactose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EXT 1</td>
<td>2.84</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 2</td>
<td>3.53</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 3</td>
<td>1.51</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 4</td>
<td>2.26</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 5</td>
<td>1.00</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 6</td>
<td>1.66</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 7</td>
<td>0.44</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 8</td>
<td>0.34</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Rhamnose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EXT 1</td>
<td>1.81</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 2</td>
<td>1.95</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 3</td>
<td>0.92</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 4</td>
<td>2.63</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 5</td>
<td>1.20</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 6</td>
<td>1.85</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 7</td>
<td>0.44</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 8</td>
<td>0.95</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Xylose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EXT 1</td>
<td>2.08</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 2</td>
<td>1.83</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 3</td>
<td>1.28</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 4</td>
<td>0.55</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 5</td>
<td>2.64</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 6</td>
<td>0.53</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 7</td>
<td>0.60</td>
<td>0.001</td>
</tr>
<tr>
<td>EXT 8</td>
<td>0.80</td>
<td>0.001</td>
</tr>
</tbody>
</table>
43% of the average of rhamnose content across the eight protocols (4.56%). Concerning the other monomers, the effect of temperature could affect the average across the eight protocols of galactose, xylose and mannose by 19%, 27% and 18%, of their initial value respectively.

Table 5.7 Effects of the influential parameters and interactions for fucose, galactose, rhamnose, xylose and mannose proportion in fucoidan extracted from seaweed harvested in February

<table>
<thead>
<tr>
<th></th>
<th>Time</th>
<th>Temperature</th>
<th>Ratio</th>
<th>Time/Temperature</th>
<th>Time/Ratio</th>
<th>Temperature / Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucose</td>
<td>0.64±1.91</td>
<td>3.60±1.91</td>
<td>-0.72±1.91</td>
<td>1.19±1.91</td>
<td>0.11±1.91</td>
<td>0.28±1.91</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.12±1.41</td>
<td>-3.47±1.41</td>
<td>1.03±1.41</td>
<td>-0.11±1.41</td>
<td>0.08±1.41</td>
<td>0.05±1.41</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>-0.18±1.14</td>
<td>-1.94±1.14</td>
<td>-0.17±1.14</td>
<td>-0.28±1.14</td>
<td>-0.68±1.14</td>
<td>-0.41±1.14</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.26±1.25</td>
<td>5.30±1.25</td>
<td>-0.81±1.25</td>
<td>0.27±1.25</td>
<td>0.06±1.25</td>
<td>0.14±1.25</td>
</tr>
<tr>
<td>Mannose</td>
<td>-0.84±1.18</td>
<td>-3.49±1.18</td>
<td>0.66±1.18</td>
<td>-1.06±1.18</td>
<td>0.43±1.18</td>
<td>-0.05±1.18</td>
</tr>
</tbody>
</table>

Figure 5.5 depicts the general trend for the effect of temperature on the sugars known to be present in fucoidans. Temperature had the same effect on galactose and mannose. Application of the mathematical model to take into consideration the effect of
parameter B (temperature) on the yield/concentration of monosaccharides associated with fucoidan structure or composition in extracts from *A. nodosum* harvested in early spring (i.e. February) would result in the following total yields of each of the monosaccharides.

\[
\%Fucose = 38.85\% + 3.60\%B \\
\%Galactose = 17.85\% - 3.47\%B \\
\%Rhamnose = 4.56\% - 1.94\%B \\
\%Xylose = 19.87\% + 5.30\%B \\
\%Mannose = 19.37\% - 3.49\%B
\]

Note: in this case, % refers to the relative proportion of each monosaccharide in a mixture of the five main sugars in *A. nodosum* fucoidan.

5.2.2.2 Compositional analysis of the extract from the June/summer batch

The same analysis was done on a batch of seaweed harvested in summer (i.e. June). The profiles representing the effects of the main extraction parameters on the yield of monosaccharides associated with fucoids from *A. nodosum* (*Figure 5.6*) were similar to those obtained for the early spring/February batch (*Figure 5.3*). Temperature was also the most influential parameter, with a negative coefficient obtained for galactose, rhamnose and mannose. In contrast to the early spring/February batch, time appeared to be more important, especially for rhamnose and mannose. Indeed, a longer extraction seemed to decrease the rhamnose and mannose content of the extract from seaweed harvested in summer. The ratio of extractant to biomass appeared to be insignificant for all monosaccharides.
Concerning the 2D and 3D interactions between temperature, time and the ratio of extractant to biomass (referred to as ratio throughout the text), time/temperature appeared to be significant but only for the galactose (Figure 5.7). At low temperature, a longer (6 h) extraction gave an extract richer in galactose than that obtained after a short (1 h) extraction. The opposite was observed for extractions conducted at the higher temperature (70°C). The interaction between time and ratio appeared to have an effect on rhamnose and mannose content. Regarding rhamnose, at the low ratio (2:1), no differences were observed between ratio and the duration of the extraction. At a higher ratio (5:1), a short extraction gave an extract richer in rhamnose than those obtained after the longer (6 h) extraction. For the mannose, the difference between extraction times was noticed only at low ratio, with an extract richer in mannose being obtained for the short extractions. The last interaction, temperature/ratio, seemed insignificant for all monomers.
On average, monosaccharides associated with fucoidans in the extract from the summer seaweed batch were present in the following relative proportions: 43.10% fucose, 13.46% galactose, 2.52% rhamnose, 24.72% xylose and 15.97% mannose (Table 5.8). These proportions were influenced by the extraction parameters and as seen previously, especially the extraction temperature. Table 5.8 displays the numerical value calculated for each effect and interaction between effects that could influence the proportion of monosaccharide associated with fucoidans in extracts from *A. nodosum* harvested in summer. As found for

Table 5.8 *Calculation of the effects of parameters and parameter interactions on the proportions of fucose, galactose, rhamnose, xylose and mannose in extracts from seaweed harvested in summer*

<table>
<thead>
<tr>
<th>Monosaccharide proportion in potential fucoidan (% w/w of the sum of each monosaccharide)</th>
<th>Fucose</th>
<th>Galactose</th>
<th>Rhamnose</th>
<th>Xylose</th>
<th>Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>average</td>
<td>43.10</td>
<td>13.46</td>
<td>2.52</td>
<td>24.72</td>
<td>15.97</td>
</tr>
<tr>
<td>Time</td>
<td>1.70</td>
<td>-0.06</td>
<td>-0.32</td>
<td>-0.10</td>
<td>-1.44</td>
</tr>
<tr>
<td>Temperature</td>
<td>2.64</td>
<td>-2.04</td>
<td>-0.94</td>
<td>3.32</td>
<td>-3.20</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.27</td>
<td>-0.26</td>
<td>0.13</td>
<td>-0.18</td>
<td>-0.18</td>
</tr>
<tr>
<td>Time/temperature</td>
<td>0.75</td>
<td>-0.57</td>
<td>0.11</td>
<td>-0.29</td>
<td>-0.23</td>
</tr>
<tr>
<td>time/ratio</td>
<td>-0.13</td>
<td>-0.14</td>
<td>-0.09</td>
<td>-0.42</td>
<td>0.55</td>
</tr>
<tr>
<td>temperature/ratio</td>
<td>0.23</td>
<td>0.06</td>
<td>-0.11</td>
<td>-0.09</td>
<td>-0.32</td>
</tr>
<tr>
<td>time/temperature/ratio</td>
<td>0.24</td>
<td>0.20</td>
<td>0.12</td>
<td>-0.41</td>
<td>-0.38</td>
</tr>
</tbody>
</table>
the early spring/February batch, time had a low effect on monosaccharide proportions, with the exception of fucose and mannose contents, with coefficients of effect of 1.70% and -1.44% obtained, respectively. Temperature had the main effect with a negative coefficient for galactose, rhamnose and mannose (like the early spring batch) and the maximum effect value for xylose was 3.32% which represented 13% of the average across the eight protocols of xylose content. Once again, in terms of the effect of temperature, similarities were observed between the two batches, but this effect appeared to be bigger for rhamnose. The effect of ratio on relative monosaccharide yield values was low for all monomers.

Confidence intervals were in general narrower for this batch than for the early spring/February batch as shown in Table 5.9. Moreover, in contrast to the early spring/February batch, more disparity was noticed between intervals for monosaccharides in the summer batch extracts. The widest one was obtained for mannose (1.47%) and the narrowest for rhamnose (0.42%). No trends were identified for higher average standard deviation values for this batch.

Similar to the overview analysis of the previous batch, only temperature was identified as the most influential parameter in determining the monosaccharide content of extracts relevant to fucoidan (Table 5.10), with the exception that time influenced the proportion of fucose present.
By removing the effect of time on the proportion of fucose extracted from seaweed harvested in summer, a graph to predict the relative proportions of monosaccharides associated with fucoidans in *A. nodosum* extracts composition according could be plotted and the profile is presented in Figure 5.8.

![Figure 5.8 Effect of extraction temperature on the evolution of monosaccharide composition in extracts from A. nodosum harvested in summer](image)

Application of the mathematical model to take into consideration the effect of parameters A (time) and/or B (temperature) on the yield/concentration of monosaccharides...
-associated with fucoidan structure or composition in extracts from \textit{A. nodosum} harvested in summer (June) would result in the following total yields of each of the monosaccharides.

\[
\begin{align*}
\%Fucose &= 43.10\% + 1.70\%A + 2.64\%B \\
\%Galactose &= 13.46\% - 2.04\%B \\
\%Rhamnose &= 2.52\% - 0.94\%B \\
\%Xylose &= 24.72\% + 3.32\%B \\
\%Mannose &= 15.97\% - 3.20\%B 
\end{align*}
\]

5.2.3 Effect of extraction parameters on the sulphate content in potential fucoidans present in the seaweed extracts

Anions were separated using the appropriate HPLC column and were detected by conductivity after cation suppression as outlined Chapter 2, Section 2.5.5. This analysis could be done only on the summer batch. The investigation of the main extraction parameter effects on sulphate content (Figure 5.9) showed that temperature had a major impact on the sulphate content by decreasing the proportion of sulphate (in potential fucoidans) extracted. The same effect (higher parameter inducing a lower response) was noticed for the ratio but the slope of the curve representing the effect was lower the one for temperature. Finally time had the only positive effect, suggesting that longer extractions extracted polysaccharide (e.g. fucoidans) richer in sulphates, but as the slope of the curve was low, the effect was probably insignificant.
The 2D and 3D extraction parameter interactions between the three parameters appeared very low in all cases (Figure 5.11). Indeed, the response from the two extraction durations (two upper plates) decreased in a similar manner for the 2D interaction between both the extraction temperatures and ratio.

Mathematical representation of the effects of parameters (Table 5.11) confirmed the results of the previous analysis, with the highest effect (negative value) obtained for the temperature (-4.63%: variation of sulphate content in fucoidans varied by 4.63% between

![Interaction Plot for Sulphates in Fucoidans](image)

**Figure 5.10** Effect of interactions between time, temperature and ratio on sulphate proportion in extracts (most likely associated with fucoidan) from seaweed harvested in summer

<table>
<thead>
<tr>
<th>Extraction No.</th>
<th>mean</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>AB</th>
<th>AC</th>
<th>BC</th>
<th>ABC</th>
<th>%SO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>46.9</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>39.3</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>35.0</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>36.1</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>44.9</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>46.8</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>40.1</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>29.6</td>
</tr>
<tr>
<td>Batch 1</td>
<td>39.85</td>
<td>0.49</td>
<td>-4.63</td>
<td>-1.86</td>
<td>-0.86</td>
<td>-0.25</td>
<td>-0.47</td>
<td>-2.63</td>
<td></td>
</tr>
</tbody>
</table>
the 25°C and 70°C extraction protocols with a higher proportion for the cold extract), which was approximately tenfold higher than effect of time (0.49%) and more than two fold that of the effect of ratio (-1.86%). The interactions between parameters gave low values except for the one between time and temperature (-0.86) which could be potentially influential. Interestingly, the interaction between the three parameters, which could not be seen from the previous figures had by far the highest effect of all interactions and might also be important for the response obtained.

Standard deviations obtained for the triplicates from each protocol were very high inducing a pretty wide confidence interval (Table 5.12). The best reproducibility was obtained for extract 2 with a standard deviation of 2.26% and the least reproducible analysis was found for protocol 8 (11.25%). The calculated interval, based on these standard deviations, was 3.93%. Due to this wide interval, only the extraction temperature could be defined as influential in determining the sulphate content in seaweed extracts.

**Table 5.12 Calculation of confidence intervals for the sulphate proportion in extracts from seaweed harvested in summer**

<table>
<thead>
<tr>
<th>Sulphates</th>
<th>EXT 1</th>
<th>EXT 2</th>
<th>EXT 3</th>
<th>EXT 4</th>
<th>EXT 5</th>
<th>EXT 6</th>
<th>EXT 7</th>
<th>EXT 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard deviations $s_i$ (%)</td>
<td>4.82</td>
<td>2.26</td>
<td>3.94</td>
<td>4.15</td>
<td>6.01</td>
<td>3.26</td>
<td>3.54</td>
<td>11.25</td>
</tr>
<tr>
<td>Variance $S^2$ (%)</td>
<td>0.008</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Confidence intervals</td>
<td>3.93</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.2.4 Effect of extraction parameters on laminaran composition

Laminaran is a β-glucan with some terminal mannitol units. This experiment aimed to investigate the influence of extraction parameters on the proportion of mannitol units in laminaran and to compare the composition between two time points of the year (early spring and summer). Inter-batch comparison revealed no significant differences in the mannitol content of laminaran in extracts from early spring (20.6%) and summer (26.7%) *A. nodosum* batches with a $p$ value of 0.052. Investigation of the effect of extraction parameters was then undertaken to figure out and predict the laminaran composition in seaweed extracts. Calculation of laminaran concentration is explained in Section 2.6.3 and free mannitol is subtracted from total mannitol in extract to approach the quantity of mannitol linked in laminaran.
Analysis of the influence of the main parameters of time, temperature and extractant/biomass ratio revealed differences between the batches (Figure 5.11). First of all, laminaran in the extract from the summer batch contained, on average, more mannitol than that from the winter seaweed batch (26.68% versus 20.91% respectively). Then, effect of parameters on laminaran-associated mannitol yields seemed to be more important in the second series (i.e. summer *A. nodosum* batch). The only similarity found between both time points was the coefficient of the slopes, i.e. negative for time and temperature and positive for the ratio. Moreover, the effects of parameters on mannitol yields for the spring batch, despite opposite slope trends, appeared to be similar in degree, which was not the case for the summer batch. Indeed, temperature had a much higher effect than ratio while time appeared to be relatively non-influential.

Concerning the 2D and 3D interactions between parameters in determining extraction yields of laminaran-associated mannitol, *Figure 5.12*, presents a very different profile of results to the findings for the individual parameters. Overall, the results suggest that all the combined effects of interactions on mannitol yields from the summer *A. nodosum* batch are probably insignificant, despite the observed individual effects for the main parameters (*Figure 5.11*). In contrast, for the early spring/February batch, despite the effects observed for each of the main parameters, all parameter interactions appeared to have an important effect on the proportion of mannitol (laminaran-associated) obtained. Interactions involving time and a low level of the other parameter (temperature or ratio), at a short extraction, gave a much higher response than that observed for the longer extraction. This
difference was significantly decreased at the higher temperature or ratio levels. Moreover, the longer extractions (6 h) gave slightly higher mannitol content than the shorter extractions. Finally, the last interaction, temperature/ratio, showed a similar response between the two temperatures at a low ratio and a higher response for the cold extractions at a high ratio.

The corresponding yield values calculated for the parameter effects were relatively high as shown in Table 5.13. For the Early spring/February batch, a minimum effect of 2.19% was calculated for the influence of ratio while the maximum effect (3.68%) was determined for the interaction of time/temperature. For the other seaweed batch (summer), temperature was by far the most important parameter, with an effect value of 10.16%. As seen from the previous figures, the effect of time and overall 2D and 3D interactions were low.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>February</th>
<th>June</th>
</tr>
</thead>
<tbody>
<tr>
<td>average</td>
<td>21.91%</td>
<td>26.68%</td>
</tr>
<tr>
<td>Time</td>
<td>-3.14%</td>
<td>-0.41%</td>
</tr>
<tr>
<td>Temperature</td>
<td>-2.95%</td>
<td>-10.16%</td>
</tr>
<tr>
<td>Ratio</td>
<td>2.19%</td>
<td>5.60%</td>
</tr>
<tr>
<td>Time/temperature</td>
<td>3.68%</td>
<td>-1.56%</td>
</tr>
<tr>
<td>time/ratio</td>
<td>2.21%</td>
<td>1.51%</td>
</tr>
<tr>
<td>temperature/ratio</td>
<td>-2.59%</td>
<td>-0.68%</td>
</tr>
<tr>
<td>time/temperature/ratio</td>
<td>-0.15%</td>
<td>-0.29%</td>
</tr>
</tbody>
</table>

Figure 5.12 Effect of interactions between time, temperature and ratio on the proportion of mannitol (laminaran associated) from the early spring and summer seaweed batches

Table 5.13 Calculation of parameters and interactions effects for the mannitol proportion in laminaran extracted
Standard deviations were somewhat high, resulting in wide confidence intervals (Table 5.14) of approx. 3% (2.89% and 3.04% for the early spring and summer batches, respectively). High standard deviations were calculated for each extraction protocol, and no trend was identified that could be used to define a more reproducible extraction.

Wide confidence intervals resulted in less significant parameters for the proportion of laminaran-associated mannitol proportion in laminaran extracted. Different parameters were identified as influential between the two batches (Table 5.15). Indeed, for the winter batch, time, temperature and the interaction between those two parameters were important with a negative coefficient for the individual parameters and positive coefficient for the 2D interaction between both parameters, which was also the most influential parameter interaction. For the summer batch, temperature was by far the most important parameter (with a negative coefficient) and contrary to winter batch, time was not influential but ratio was.

Application of the mathematical model to take into consideration the effect of parameters A (time), B (temperature), ratio C and/or the interaction between time and temperature (AB) on the yield/concentration of laminaran-associated mannitol in extracts from *A. nodosum* harvested in early spring (February) and summer (June) would result in the following total yields:

![Table 5.14 Calculation of confidence intervals for the proportion of mannitol associated with laminaran in extracts.](image)

![Table 5.15 Influential parameters and parameter interactions for the proportion of laminaran-associated mannitol in extracts](image)
The proportion of laminaran-associated mannitol in the extracts was slightly higher for the summer harvested seaweed and the extraction parameters, temperature and ratio had an important effect on the response (yield). Extraction parameters were also influential for the early spring batch but with lower effect on yield.

5.2.5 Influence of extraction parameters on the size distribution of fucoidan fractions present in extract

Previous studies have indicated that the bioactivity of fucoidan is likely to be dependent not only on the composition but also on the size. Each of the extracts was fractionated by size according to the method described in Section 2.5.6. Fucoidan content (based on monosaccharide composition) was analysed in all 10 fractions (fraction S0, permeate following filtration through a 0.2 µm cut-off filter, retentates and permeates from ultrafiltration through 300, 100, 50, 30 and 10 kDa molecular weight cut-off, MWCO membranes). In total, 7 size fractions were analysed, i.e.: above 0.2 µm, between 0.2 µm and 300 kDa, between 300 and 100 kDa, between 100 and 50 kDa, between 50 and 30 kDa, between 30 and 10 kDa and below 10 kDa. The average proportion of fucoidan (based on

\[
\% \text{Mannitol (early spring)} = 21.91\% - 3.14\%A - 2.95\% B + 3.68\%AB \\
\% \text{Mannitol (summer)} = 26.68\% - 10.16\% B + 5.60\%C
\]

Figure 5.13 Average (n=24) of reparation of fucoidan size fractions between the different protocol used using different time (from 1 to 6 hours), temperature (from 25°C to 70°C) and ratio (from 2 to 5)
total carbohydrate and monosaccharide analysis results) present per size fraction was calculated for each of the eight extraction protocols (done in triplicates). The results obtained for the size distribution are displayed in Figure 5.13. The fucoidan extracted was mainly of high molecular weight, with the quantities in the > 100 kDa retentates representing more than 80% of the total fucoidan. The distribution of the main size fraction was 0.2 µm to 300 kDa, followed by the fraction above 0.2 µm and then the one between 300 and 100 kDa, which accounted for 40.13%, 29.88% and 13.53% of the quantifiable ‘fucoidan’, respectively. The smallest fraction, below 10 kDa represented, on average, 8.10% of the total fucoidan. All other fractions were in low proportion with 1.56%, 4.28% and 2.34% for the fractions 50-100 kDa, 30-50 kDa and 10-30 kDa, respectively. Once again, differences were observed between the extraction protocols in terms of ‘fucoidan’ size fractions.

A first investigation of effect of extraction parameters on the size distribution was performed. The average proportion of each size fraction per level of extraction parameters (low and high level, for example for time, 1 hour and 6 hours) is displayed in Figure 5.14-A for time, Figure 5.14-B for temperature and Figure 5.14-C for ratio.

As observed previously, the main fractions were high molecular species (above 300 kDa) and extraction protocol seemed to have an effect of the relative proportion of each size fraction. Indeed, most of parameters looked to have an effect on all fractions. First of all, the average effect of a shorter extraction time revealed a repartition of the relative size distribution similar to that of the total average, meaning that the main fraction was the one between 0.2 µm and 300 kDa followed by the one above 0.2 µm and so on. After longer extraction, a decrease of the proportion of the size fraction between 0.2 µm and 300 kDa was noticed (from 45.52% to 35.10%) with a proportionally higher distribution of the species above 0.2 µm (from 22.10% to 37.66%). Regarding the other fractions, longer extractions decreased a proportion of ‘fucoidans’ in the 100-50 kDa and 50-30 kDa as well (from 2.32% to 0.81% and from 5.72% to 2.84%, respectively). For the other fractions, the effect of time appeared to be less important with lower variation in the respective proportions of ‘fucoidans’ across the size fractions.
Figure 5.14 Repartition of fucoidan size fractions for A) short (1hr) and long (6hrs) extractions (n=12) B) cold (25°C) and hot (70°C) extractions (n=12) and C) using small (2) and bigger (5) ratio water (ml)/fresh seaweed (g) (n=12)
In contrast to the effect of extraction time, a higher level of temperature increased the proportion of ‘fucoidan’ in the main fraction (0.2 µm – 300 kDa) and a small decrease the ‘fucoidans’ present in the fractions above 0.2 µm was observed. At low temperature, the proportion of those two fractions was similar (31.07% and 32.06%, respectively) but a significant difference was noticed after high temperature extraction (28.69% and 48.26%). Temperature had also an effect on the other size fractions. Indeed, extraction at the higher temperature yielded a proportion of the fractions between 300 and 100 kDa and the species <10 kDa. Moreover, the fraction between 50-100 kDa was higher at high temperature. The latter two fractions were similar between the two extraction conditions.

Finally, the main effect of extraction parameters on fucoidan size fraction investigated was probably the effect of the ratio on the fraction above 0.2 µm. Indeed, extractions with a small volume of water gave a very high proportion of the fraction above 0.2 µm (42.83% versus 34.34% for the ‘fucoidan species’ between 0.2 µm and 300 kDa). This proportion was considerably decreased by increasing the amount of water added under the same condition, with the proportion of the highest molecular fraction below 20% (16.93%). Proportionally, three other fractions significantly increased, from 34.34% to 46.27% for the one between 0.2 µm and 300 kDa, from 8.85% to 18.50% for the fraction between 300 and 100 kDa and from 0.14% to 2.99% from the molecules between 100 and 50 kDa. The Ratio did not appear to have a significant on the other size fractions.

5.2.6 Preliminary investigation on alginate composition

Different techniques were investigated to evaluate (semi-quantitatively, if possible), the alginate content in the seaweed extracts. Due to the complexity of the extracts studied, interactions with other compounds were systematically observed. Nevertheless, preliminary research was undertaken, using specific enzymes to degrade alginates into smaller carbohydrates, to investigate the alginate composition.
The first analysis was qualitative to determine whether the enzymes were active on the prepared seaweed extracts. Two enzymes were used: AlyA1 which was a G-lyase (G-block rich regions hydrolysed) and AlyPa, an M-lyase (M-block rich regions hydrolysed). After an overnight incubation of the two seaweed extracts (one extracted at 25°C and one at 70°C) with the enzymes was conducted. Following incubation, the samples (and controls) were applied to a C-PAGE gel (carbohydrate polyacrylamide gel electrophoresis, alcian blue stain combined with silver nitrate detection) as described in Section 2.6.5; the results are shown in Figure 5.15. For the cold extract, no oligosaccharide production (or concentrations too low for detection), was noticed using either of the two enzymes whereas, with the hot extract, oligosaccharides were observed, but only after incubation with AlyA1 or a mixture of the enzymes (which was probably due to the action of AlyA1).

By repeating this experiment on more extracts (Figure 5.16), the same observation was confirmed. Indeed, on the cold extracts (EXT 1 and 2), the enzyme AlyA1, a G-lyase, seemed to be inactive whereas oligosaccharide production was noticed after an overnight incubation on the seaweed extracts prepared at high temperature (EXT 3 and 4). Moreover, the difference between extractions 3 and 4 was the extraction duration (as well as extracts 1 and 2). Indeed, extracts 3 (and 1) was obtained following a 1 hour extraction and extract 4 (and 2) obtained after 6 hours. The intensity of the band formed looked higher in the lane.
corresponding to the treated extract 4 suggesting an effect of the extraction duration on the alginate concentration or composition.

The action of alginate lyase on alginates induces the creation of double bounds and release of oligosaccharides that have a $\lambda_{\text{max}}$ at 235 nm. The action of the enzyme could so be measured by following the increase in optical density overtime. Results of the action of
AlyA1, the G-lyase, on the four extracts tested previously are displayed in Figure 5.17. The results were compared to the results obtained for the commercial alginates. As seen previously, maximum alginate lyase activity was obtained with extracts 3 and 4, which were extracted at high temperature but no absorbance difference was noticed between extracts 1 and 2. Once again, the activity against Extract 4 was higher than on the third one, confirming an influence of extraction time. By comparison to commercial alginates, the enzyme activity on the Extract 4 was almost similar to the one observed on the commercial product at 0.3% w/v concentration and the other seaweed extracts were between the commercial product at the concentration of 0.1% and 0.2%. The enzyme was almost three times more active against extract 4 than on the cold process extracts.

The same analysis was done using the M-lyase enzyme on the same extracts and results are presented in Figure 5.18. The activity of the enzyme was much lower than the other one. Indeed, the activities observed on the extract were all lower than the activity of the enzyme against the commercial alginates (included at 0.1% w/v). One hypothesis may be that the mannuronic content, or potentially the pattern and repetition of M blocks, may be lower than the G blocks. Interestingly, the highest activities were found with extracts 1 and 2 (with no differences between them) which were cold processed extracts. The cold processed extracts probably contained more repetition of M-blocks than the alginates extracted at higher temperature.

Finally, the four samples were run through a HPLC system equipped with two Sephadex G-30 and G-300 columns installed in series, to separate oligosaccharides produced.
by the enzyme AlyA1. UV analysis of the eluate at 235 nm allowed detection of the double bonds created by the action of the AlyA1 enzyme. However, different compounds present in the extract (e.g. polyphenols or polysaccharides) absorb light in this region of the spectrum, which explains the peaks obtained in the control sample, i.e. sample of the extract before enzymatic treatment (Figure 5.19). The first peaks, eluting around 30 minutes, corresponded to high molecular weight (> 300 kDa) compounds that were excluded from the resin matrix.

![Figure 5.19 HPLC chromatogram (UV detection at 235 nm) of the four untreated seaweed extracts](image)

Those compounds appeared in higher proportion in extracts 3 and 4. Additional compounds were eluted from the matrix in order of decreasing size, with more/higher concentrations of compounds being observed in the extracts processed at 70°C. Finally, the last peaks corresponding to salts in the sample eluted after 70 min.

The same analysis was repeated for the same samples after an overnight incubation with the alginate lyase, AlyA1. The control (or baseline) absorbances were subtracted to reveal the peaks produced by the action of AlyA1. The results are displayed in Figure 5.20. The salt peaks almost disappeared as no further peaks were produced during enzymatic hydrolysis. The oligosaccharides generated eluted between retention times of 60 and 70 minutes with a higher concentration of oligosaccharides produced for the extract 4 which confirmed the results obtained by C-PAGE (Figure 5.16). The enzyme was slightly more active against extract 3 than against extracts 1 and 2 (both of which yielded no real differences between them). More interestingly, the peak(s) corresponding to the high molecular weight compounds that absorbed at 235 nm were significantly higher for the enzyme-treated extract 4 than for extract 3 (or extracts 1 and 2). No real differences were noticed between the extracts 1 and 2, in contrast to extracts 3 and 4, suggesting that duration
of extraction had a potential influence on alginate composition and/or alginate concentration, but only where the high temperature extraction processes were used. The main oligosaccharides produced were tri and tetra saccharides in all cases. The relative proportions and distribution of each oligosaccharide appeared similar for all samples.

As noted previously in the ultrafiltration experiments to profile ‘fucoidan’ polysaccharides in the extracts (Section 5.2.3), some compounds are present in the fraction

![Figure 5.20 HPLC chromatogram (UV detection at 235 nm) of four seaweed extracts after an overnight incubation with specific alginate G-lyase (AlyA1)](image)

![Figure 5.21 HPLC chromatogram (UV detection at 235 nm) of a cold process A) crude seaweed extract and B) its filtrate at 0.2 μm](image)
with a molecular size above 0.2 µm. A first investigation was done to test the hypothesis that this fraction also contains alginates by comparing the results of the action of the G-lyase enzyme on the crude extract by subsequent filtration through a 0.2 µm filter, followed by size exclusion by HPLC. *Figure 5.21*, above, shows the HPLC chromatogram, with UV detection at 235nm, of the blanks (before enzymatic hydrolysis) of the crude extract 1 (colder extraction process, i.e. 25°C) and the 0.2 µm filtrate of the same extract. No differences were noticed between the two samples. The two blanks seemed to have the same composition of compounds that absorbed at 235nm and was composed of a high molecular weight molecule (first peak) and then mainly salts (highest peak).

After an overnight enzymatic hydrolysis, differences were observed between the two samples, i.e. crude extract and pre-filtered crude extract (*Figure 5.22*). Surprisingly, different proportions of oligosaccharides were produced by the enzyme on the two extracts. Indeed, as seen previously, the tri and tetra saccharides remained the main sugars but the proportion was slightly different. After filtration at 0.2 µm, by comparison to the crude extract, the proportion of dp3 and dp2 oligosaccharides decreased, while the relative amounts of the dp4, dp5 and dp6 oligomers increased. Moreover, the hexa-saccharide was not detected in the enzyme-treated crude extract. Furthermore, a peak that might correspond to a resistant alginate fraction (M blocks or MG blocks) was much higher in the crude extract than in the filtrate. This suggested that the alginates in the filtrate were almost totally

*Figure 5.22* HPLC chromatogram (UV detection at 235 nm) of a cold process A) crude seaweed extract and B) its filtrate following filtration through a 0.2 µm filter after an overnight incubation with an alginate G-lyase (AlyaA1)
digested whereas an appreciable amount of residual alginates remained in solution in the crude extract.

The same experiment was conducted on the extract 4 to compare alginates present in extracts from cold (extract 1 previously displayed) and hot extraction processes. Blanks of crude extract from a hot process extraction process and filtrate following filtration of the crude extract through a 0.2 µm membrane gave similar HPLC profiles (Figure 5.23). In comparison with the extracts obtained using the colder (25°C) extraction process (see Figure 5.21), similar peaks were found in both chromatograms, but at a higher concentration for the hot process extracts, as evident from the scale difference between Figures 5.21 and 5.23.

After overnight enzymatic hydrolysis, differences between the two fractions were also noticed (Figure 5.24). First of all, the peaks corresponding to the salts, despite being identical in the sample controls (Figure 5.24) were different between the two enzymatically-treated samples (Figure 5.24). Indeed, the proportion of salt appeared to be higher in the crude extract than in the filtrate. Moreover, the concentrations of oligosaccharides produced were much lower for the crude extract. In this extract, oligosaccharides from dp3 to dp6 were detected with a higher proportion of tetra-saccharides followed by tri-saccharides. The other product peaks were in minor proportion. The peak corresponding to the high molecular weight compounds was slightly higher or more pronounced after enzymatic hydrolysis than in the corresponding control (compare Figure 5.24 with Figures 5.23). For the
corresponding filtered extract, only three oligosaccharides were detected (dp3, dp4 and dp5). In contrast with the crude extract, the main oligosaccharide peak was a tri-saccharide followed by the tetra-saccharide (dp5 was present in minor proportion). The response factor for the high molecular weight peak was more than four times higher in the filtered extract.

With the ‘hot’ processed extract, the alginate lyase appeared to be more active on the filtered fraction, resulting in greater production of oligosaccharides but also in a higher proportion of the high molecular fraction. Interestingly, the peak corresponding to the resistant fraction of alginates was not detected in the hot processed extracts in contrast to the cold processed extracts.

5.3 Discussion

Polysaccharide composition has been shown to vary according to many factors such as seaweed species, seasonality, localisation, seaweed pre-treatment or the extraction process used to recover specific biomolecules (Ale & Meyer 2013). For this reason, a direct comparison between the results of this study, which investigated the effects of seasonality and influence of defined extraction parameters on the composition of complex carbohydrates
in the extracts, to the results found to-date in the literature was not straight forward. Indeed, in most of the published results to-date, seaweeds were subjected to a pre-treatment (air drying or organic solvent treatment) which could induce modification of the native compounds in the seaweed (Maisuthisakul & Pongsawatmanit 2004; Li et al. 2008).

In general, fucose-containing polysaccharides from algae are heteropolysaccharides typically composed of 20-60% L-fucose (Mian & Percival 1973) Kloareg et al 1986; Duarte et al 2001; Wang et al 2008), with varying minor amounts of other sugars including the pentose sugar D-xylose, hexoses D-galactose, D-mannose, L-rhamnose, D-glucose and D-uronic acids, as well as non-sugar substituents such as sulphate. In some algal fucoidans, fucose may even be a minor component and galactose or uronic acids more abundant (e.g. ‘xylofucogalacturonans’; (Mabeau et al. 1990; Nishino et al. 1994; Li et al. 2006). Some examples of so-called sulphate ‘galactofucans’ have even been reported (Rocha et al 2005). The work reported in this chapter on A. nodosum, revealed an increase in 4.9% on average, in the fucose content of seaweed extracts (reflecting fucoidan levels) prepared using the same extraction protocols on seaweed harvested in summer in comparison to that harvested in early spring. The same trend was observed in fucoidan extracted from Costaria costata (Laminariales) with an increase of fucose of 6.1% from April to June (Imbs et al. 2010). This observation of a seasonal (summer) increase in fucose/fucoidan levels contrasts, however, with the results obtained for Undaria pinnatifida (Laminariales), in which no variation of fucose was noticed between winter and summer samples of this seaweed (Skriptsova et al. 2009). Xylose also increased by 4.8% in A. nodosum extracts between the early spring and summer time points (this work) in contrast to the study done on C. costata in which the xylose content decreased slightly (0.8%). A decrease in xylose content was similarly observed in another study on extracts from Padina pavonica (Ermakova et al. 2012). For A. nodosum (this work), the relative proportions of all other monosaccharides quantified (e.g. galactose, mannose, rhamnose) decreased between the early spring and summer extracts (4.3%, 2.1% and 3.4% decreases in the levels of galactose, rhamnose and mannose, respectively). In the literature, results obtained or the quantities of these monosaccharides were varied according to the species investigated. For example, the galactose content increased by 3.6% between April and June extracts of C. costata (Imbs et al. 2010) and increased by around 20% for U. pinnatifida (Skriptsova et al. 2009). The seasonal levels of rhamnose and mannose were similar for A. nodosum (this work) and reports for both C. costata bs et al. 2010)and U. pinnatifida (Skriptsova et al. 2009) in that a decrease was observed between winter/early summer and mid-summer seaweed samples. However, the degree of the variation was higher for Laminariales spp with a decrease of
3.8% and 9.1%, respectively, in rhamnose and mannose levels for *C. costata* (Imbs et al. 2010), whereas corresponding decreases of 2.1% and 3.4% were calculated for *A. nodosum* (this work). The higher variation in Laminariales was confirmed by the study done on *U. pinnatifida*, in which a seasonal decrease in the mannose content of around 15% was noted (Skriptsova et al. 2009).

Extraction time was also shown to have some influence on the ‘fucoidan’ composition of extracts from *A. nodosum* in this work, under the conditions used for extraction over a 24 hour extraction time course (see Section 2.2.2 and Section 3.2.5.4 as well for findings reported in this chapter). In this study, fucose levels increased slightly over time with a corresponding minor decrease in xylose and mannose concentrations. In the experimental design applied in this research (extraction for 1 and 6 hours), the extraction time was statistically defined to be insignificant for the relative levels of monosaccharides known to contribute to the composition of fucoidans extracted from the early spring seaweed sample. Similar work done on commercial *Sargassum spp* showed that longer extraction periods tended to decrease the fucose content (by around 5% within 24 hours) and increase the mannose content (by around 12%) (Ale et al. 2011a). Similar results were obtained for a fucoidan extracted from *Adenocystis utricularis* harvested in summer (increase in fucose content by 4%) (Ponce et al. 2003). The summer batch of *A. nodosum* in this research yielded an increase of the fucose content using the longer extraction time (1.7%) but the extraction time had no statistical effect on the levels of the other monosaccharides.

In this work, the extraction temperature appeared to have the most influence on the monosaccharides associated with fucoidan composition. The effect of extraction temperature (from 160°C to 200°C) was investigated on the fucoidan composition in extracts from *Fucus vesiculosus*. Those extreme temperatures gave different results to those obtained for *A. nodosum* in this study. Higher temperatures of 160°C to 200°C were in fact shown to decrease the fucose content (by 28.95% from 160°C to 200°C) and increase the galactose content (by 22.87%) for *F. vesiculosus* fucoidan (Rodríguez-Jasso et al. 2012) whereas, the application of higher extraction temperatures (70°C) reported in this chapter increased the fucose concentration (by 6%) and decreased the galactose (by 7%) in the *A. nodosum* extracts. Another study that used more comparable temperatures (from 22°C to 60°C), for the extraction of fucoidans from *F. vesiculosus*, revealed that the proportion of fucose in the extracted fucoidan was also lower at higher temperature (by 3.3%) but no significant changes were noticed for galactose and xylose contents (Rupérez et al. 2002). By comparing two extracts from *U. pinnatifida*, one derived by extraction at room temperature followed by 60°C (Skriptsova et al. 2009) and the other one following extraction at 85°C (Mak et al.
2013), the higher temperature extraction resulted in a lower proportion of fucose, even though the extraction time and ratio were different but shown to be insignificant. Regarding the sulphate content in extracted fucoidan, only extraction temperature had been shown to be influential (in this study). The same outcome was noticed on Laminaria digitata fucoidans extracted with other protocols (including enzyme and microwave assisted extractions) (Hahn et al. 2012). As time was not influential concerning the sulphate extraction yield, Hahn and co-workers suggested to minimise the duration of extraction to avoid hydrolysis of sulphate esters.

In this study on A. nodosum, temperature was also shown to significantly influence the laminaran composition in both early spring and summer seaweed batches. Indeed, an increase in temperature decreased the average proportion of mannitol in the laminaran extracted. Even though, laminarans are sometimes defined as a linear β-glucans (Klarzynski et al. 2000), ramifications and branching has been found in several extracted laminarans (Devillé et al. 2004; Kim et al. 2000). Moreover, soluble laminaran, extracted at cold temperature, was also shown to be more branched than the insoluble form (extracted only at high temperature) (Percival & McDowell 1968). Generally, ramifications consist of branch points ending in a terminal mannitol unit (Usov & Chizhov 1993). All of these previous findings could explain the lower mannitol content of laminarans extracted at higher temperature from A. nodosum in this work. In this work, in addition, the effects of the duration of extraction, and the ratio of extractant to seaweed biomass were shown to be also important factors in determining the composition of extracts with respect to potential laminaran polysaccharides in the early spring and summer batches. For example, a higher extractant to biomass ratio may increase the solubility and extractability of the laminaran by increasing the likelihood of H-bonding between the polysaccharide and solvent (water). However, isolation of laminarans in the extracts and more detailed analysis of structure and composition would be required to make more definitive conclusions on the effects of extraction parameters on laminaran composition.

Evaluating the influence of extraction parameters on the size distribution of fucoidans is a complex due to the number of inter-dependent factors that have to be taken into consideration. Indeed, effect of temperature is dependent on the solvent used for extraction. Using an acidic solvent or extractant, high temperature was shown to damage native fucoidan through partial hydrolysis (Ale & Meyer 2013). This observation was reinforced by an earlier result on the effects of acid extraction on a fucoidan of ~1,600 kDa from F. vesiculosus at 60°C (Rupérez et al. 2002). For the same seaweed species, a similar extraction protocol at higher temperature, yielded a fucoidan with a size of ~ 680 kDa
(Nishino et al. 1994). In other examples, e.g. *Adenocystis utricularis*, the extraction solvent (water, acidic and calcium chloride) did not appear to have an influence on the size of the extracted fucoidan with fucoidan sizes of between 15 and 19 kDa being observed (Ponce et al. 2003).

In this work, a fraction with a molecular size above 0.2 µm and containing fucoidan (based on monosaccharide analysis) was identified in the aqueous *A. nodosum* extracts. To the author’s best knowledge, a fucoidan of this size has not been reported so far from *A. nodosum*. This fraction was shown to be even more pronounced after longer extraction, which contradicts the findings reported in a patented process to produce high molecular fucoidans in which a longer extraction time was shown to extract fucoidan of a smaller size (Hakano et al. 2007). Furthermore, the proportion of the high molecular fraction reported in this chapter was not influenced by the extraction temperature but was affected by the ratio of extractant to biomass. Indeed, the proportion of this fraction was more than 2-fold lower when a higher ratio was used. It should be noted that a higher ratio yielded a lower percent total solids content in the final extract. The effects of time and ratio on this fraction suggest the hypothesis that fucoidan present in this fraction may be involved in some type of complex formation. Previous studies have reported on the binding properties of fucoidans to divalent cations (Paskins-Hurlburt et al. 1978). Moreover, the kinetics of complex formation between metal ions and polysaccharides has been shown to be dependent on the chemistry of the polysaccharides and salt concentration (higher concentration, faster chelation) over a time scale of hours (Rotureau & van Leeuwen 2009). In the findings reported in this chapter, when the high molecular fraction was lowest in concentration, fractions between 300 kDa-0.2 µm and 100 – 300 kDa were in higher proportions and these latter size fractions might correspond more closely to the size of native fucoidans. Indeed, fucoidan size reported in the literature, was mainly found to be between 100 and 1,000 kDa (RiouxEt al. 2007). However, the as yet poorly understood diversity of fucoidans from brown algae in terms of size distribution, chemistry and composition may be more of a contributing factor to the size distribution of fucose-containing fractions reported in this work.

In this work, the alginate content and composition in extracts from *A. nodosum* appeared to vary according to the extraction protocol used. First of all, alginate content seemed to be higher in extracts obtained using the longer extraction processes as observed from the C-PAGE results. Under different experimental conditions (stirring speed, particle size of biomass), the extraction yields of alginates from *Laminaria digitata* had been shown to reach a maximum after at least 2 hours (Vauchel et al. 2009). In addition, in the findings reported for *A. nodosum* in this chapter, the results obtained by C-PAGE suggested that the
extracts obtained using extraction protocols at 70°C contained more alginates than those obtained by the cold (25°C) extraction processes. In contrast, alginate yields from *Sargassum spp*, obtained using two extraction temperatures showed similar results after sodium carbonate extraction (Chee et al. 2010). More interestingly, the viscosity measurement of the extracts was different between the cold and hotter process suggesting a different composition of the alginates. Indeed, the M/G ratio was shown to affect the viscosity, with a positive correlation between G blocks and viscosity (more G blocks, higher viscosity) and inversely for M blocks (more M blocks, lower viscosity) (Jothisaraswathi et al. 2006; Saraswathi et al. 2003). The alginates extracted by Chee et al. had a higher viscosity at low temperature extraction, so those extracts might contained more G blocks, which is the opposite observation done in this study. Nonetheless, further investigations are required to figure out the influence of extraction parameters on the alginate composition and a plan of action is described in chapter 6. All extracts were stored at -20°C to investigate the stability of frozen fraction for consistent composition overtime.

5.4 References


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Chapter 6: Preliminary screening of the potential prebiotic activity of crude seaweed extracts and purified polysaccharide fractions
6.1 Introduction

As defined by the European food safety and authority, a prebiotic is an ingredient able to modify the microbiota balance (Food and agriculture organization of the United Nations 2007). This statement means that a prebiotic ingredient should be able to enhance the growth of beneficial bacteria more than the growth of potentially harmful ones or, reduce the growth of potentially pathogenic strains more than the growth of beneficial ones.

More than 150 strains of more than 20 species of probiotic bacteria have been identified in the human gastrointestinal tract, with the lactobacilli receiving particular attention in recent years (Salyers et al. 1977). Bacteria are classified as potentially pathogenic or beneficial, as illustrated in Figure 6.1. The study conducted in this research focused mainly on the effects of seaweed extracts and polysaccharide-rich fractions on specific Lactobacilli species and strains, which are the only species defined to-date as potentially beneficial (O’Sullivan et al. 2010). *Pseudomonas sp.* have been shown to

![Figure 6.1 Distribution of the dominant, sub-dominant and minor species of human fecal microflora (O’Sullivan et al. 2010)](image-url)
produce enzymes, including alginate lyase, that degrade polyuronic acid polymers contained in seaweed extracts (Farrell & Tipton 2012). *Lactobacilli* have been shown to provide various beneficial effects on human health, which range from production of an exopolysaccharide with prebiotic properties (findings for *Lactobacillus plantarum*) during growth (Tsuda & Miyamoto 2010) to anti-obesity effects brought about by inducing insulin resistance after oral administration of bacteria such as *Lactobacillus casei* (Naito et al. 2011). At present, *Lactobacillus* strains are found (and included) in different dairy-based consumer products including yoghurts.

In recent years, *in vivo* studies using seaweed extracts have been performed on laboratory (rats, mice...) or farm animals (mainly pigs). Alginate oligosaccharides were shown to increase the numbers of bifidobacteria and lactobacilli when the concentration used was higher than 1% for the *in vitro* test and more than 2.5% for the *in vivo* studies (Wang et al. 2006). Moreover, Kuda and co-workers evaluated the change in the profile of organic acids and putrefactive compounds in rat faeces after administration of diets enriched either with laminaran or with low and high molecular weight alginates (Kuda et al. 2005). Laminaran was shown to supress indole, cresol and sulphide production, which are considered as markers of conditions symptomatic of, or likely to promote colon cancer. *In vivo* studies in pigs were performed using whole dried seaweed (Braden et al. 2004) or uncharacterised extracts. Indeed, in most of the studies, extracts were defined as “seaweed extracts containing laminarans and fucoidans” without details being provided on the extraction process (Reilly et al. 2008; Lynch et al. 2010; McDonnell et al. 2010). In the studies conducted by these three researchers/research groups, analysis of microflora from different gastrointestinal (GIT) tissues (cecum, colon, intestine) showed a decrease of the number of potentially harmful bacteria (*E. coli, Enterococci*) as well as a decrease in beneficial bacteria (*Bifidobacteria* and *Lactobacilli*). The authors suggested that the fucoidan acted more like an antimicrobial agent than a prebiotic ingredient (McDonnell et al. 2010). Dierick et al. reported a decrease in *E. coli* numbers in the small intestine of pigs after a diet enriched with fully-characterised extract from *A. nodosum*. Furthermore, this treatment was shown to increase the balance between *Lactobacilli* and *E.coli* (Dierick et al. 2009) towards improved probiotic numbers. An opposite effect, i.e. anti-prebiotic effect, was observed after treatment with alginates with an increase of Enterococci counts and a decrease in the numbers of *Lactobacilli* in the intestine, colon and cecum of pigs; no details were provided on the origin of the alginate (Janczyk et al. 2010). However, in terms of the prebiotic potential of natural seaweed extracts and polysaccharide-rich fractions, much remains to be
investigated on the bioactivity profile of the compounds/extracts, and very little is known about the relationship between composition and bioactivity.

This chapter reports on the comparison of the potential prebiotic activity of a crude seaweed extract containing most of the water soluble compounds of the seaweed and different fractions derived from the crude seaweed extract enriched in specific polysaccharides. Eight crude extracts of *A. nodosum* were prepared using the extraction protocols outlined in Section 2.7.3, and individual enriched polysaccharides were purified from crude extracts (S0 fractions) as described in Chapter 2, Section 2.8.1. Bacterial strains were selected and grown in specified nutrient media containing the seaweed extracts and enriched polysaccharide fractions, under specific growth conditions (Section 2.8.2). Depending on whether the bacteria were probiotic organisms or potential gut pathogens, class 1 or class 2 cultivation facilities were used and the appropriate precautions taken in terms of handling of bacteria, samples and sample storage.

The study aimed to investigate the effects of the extracts/compounds on beneficial bacteria, and on two potential opportunistic pathogens, *Escherichia coli* and *Pseudomonas aeruginosa*. Bacterial growth was evaluated in a time-course bioassay system by monitoring the change in the optical density of cultures during growth of the bacteria. The difference growth phases were statistically defined and the turbidimetry variation in the culture during each phase was compared between the extracts. This approach enabled definition, first of all, of the influence of the extraction process/parameters on bioactivity but also enabled selection of the most promising seaweed extract fraction. The ultimate goal of this preliminary study was to select the best extraction protocol to produce the most promising extract in order to run a human clinical trial in the format of a dietary intervention study. In addition, by knowing the extraction parameters used and the composition of the extract, the effect of composition on bioactivity could be correlated.
6.2 results

A preliminary screen was conducted with a few selected extracts in order to optimise the bioassay system. First of all, the extract concentration was optimised in order to be able to see an effect of the extracts but also to be able to discern differences between extracts. Selection of a suitable carrier minimal medium was also essential to support growth of Lactobacilli and Bifidobacteria but provide sufficient optical quality to be able to compare the effect of extracts clearly. Normally these bacteria are grown in Man. Rogosa and Sharpe (MRS) medium, but this is a very rich medium making it difficult to assess the effects of the seaweed extracts; in addition, the medium had poor optical properties making it less suitable for the microbial bioassay. In contrast, to the pathogenic strains were grown under the optimum conditions, to provide a valid approach with which to test potential bacteriostatic or bactericidal effects.

6.2.1 Optimisation of the extract concentration

It was essential to establish the best concentration range of extract to use to obtain valid bacterial growth curves. Figure 6.2 shows a growth curve obtained for E. coli in LB media with the addition of 1% extract. The negative control contained medium but no

![Figure 6.2 Escherichia coli growth curves in LB media containing different seaweed extracts included at 1% (w/v) as well profiles obtained for negative and positive controls]
bacteria, while the positive control contained only bacteria and medium which was not supplemented with extract. Additional controls were included in the microbial bioassay. These included independent replicates cultures consisting of medium supplemented with glucose or phloroglucinol (1%). The rationale for these controls was the glucose is a readily utilized carbon source, and should yield an optimal growth profile against which other growth profiles can be compared, while phloroglucinol is a known seaweed phenolic substance that is representative of phenolic substances present in crude seaweed extracts. As phenolic substances have known antibacterial properties, the response of bacteria in the bioassay to phloroglucinol would indicate how they may respond to extracts containing such compounds. Four different seaweed extracts were tested in parallel, three S0 fractions (crude seaweed extracts) and a fucoidan fraction purified from Extraction 4 (1 h, 70°C, ratio of 5:1). Unlike glucose and phloroglucinol, the 1% (w/v) seaweed extract solutions tested were dark and gave high optical density reading from the beginning (T0). Moreover, the range of optical density obtained with the seaweed extracts were not in the accurate range of the equipment used (Tecan GENios system and BioTek Powerwave HT Microplate spectrophotometer, which were more accurate for OD readings ≤2). Consequently, analysis of bacterial responses to the seaweed extracts was difficult to determine. Therefore, a sample blank was included (sample and media without bacterial inoculum), and the concentration used of the seaweed extract was decreased.

Figure 6.3 shows the same experiment, i.e. *E. coli* growth curve in LB medium, this time with a 0.1% (w/v) concentration of each extract. The sample blank was subtracted at

![Figure 6.3 Escherichia coli growth curves in LB medium on different seaweed extracts and controls at 0.1% (w/v)](image)
each time point. Using this protocol, direct comparison between extracts and the positive control (which was considered to support optimal bacterial growth) could be easily made. Indeed, glucose, as expected, enhanced bacterial growth, whereas the crude seaweed extracts decreased or retarded growth.

Decreasing the concentration of the extracts to 0.01% (w/v) was inadequate to observe effects due to the extract, as shown in Figure 6.4, as a 0.01% concentration of each extract yielded almost identical growth curves for all the tests and the positive control.

The optimum concentration of seaweed extract appeared to be 0.1% (w/v) and subtraction of the sample blank from the test allowed comparison of the effect of the added seaweed extract on the bacterial growth. To directly compare the seaweed extracts and controls, glucose was also used at 0.1%.

![Figure 6.4 E. coli growth curves in LB media on different seaweed extracts and controls at 0.01% (w/v) of each extract.](image-url)
6.2.2 Optimisation of the culture medium

The optimum growth media for most of the probiotic strains is MRS broth which contains a lot of carbon sources like glucose, peptone or yeast extract for example. However, although being an excellent growth medium for *Lactobacilli* and most *Bifidobacteria*, the effects of the seaweed extracts cannot be determined clearly with this medium and it has poor optical properties in the bioassay system, as mentioned previously. Therefore, a ‘minimal’ medium should be used that would support growth of the *Lactobacilli* and *Bifidobacteria* strains, but enable clear visualisation of the effect of seaweed. The first step consisted of preparing a “MRS” media without those glucose, peptone and yeast extract.

*Figure 6.5* shows growth curves obtained for *Lactobacillus acidophilus* in the modified MRS medium with the different extracts included at 0.1% (w/v). The lowest growth was obtained when the medium was not supplemented with extract (or sugar). The addition of carbon/nutrients in the form of seaweed extract enhanced the growth slightly.

However, the difference between the extracts and positive control were too small (max ΔOD = 0.1). For this reason, additional media were tested, one of which was M9 medium.

M9 medium is a salt base formulation only, which only contains some essential nutrients for bacterial growth. *Figure 6.6* shows the growth of *Lactobacillus acidophilus* in (A) M9 medium and in (B) water supplemented with different carbon/nutrient sources. In
both cases, the lowest growth was obtained with the culture that did not contain any carbon source (no sugar). Ranking of extract effectiveness was conserved between the two experiments. Indeed, in both media, EXT4 S0 gave the highest growth followed by EXT2 S0. Differences between the extracts and negative controls were even higher when water was used as the carrier medium. EXT4 FUC, glucose and EXT9 S0 gave similar responses in M9 medium. The maximum variation between extracts was more noticeable in the second test set using water as carrier medium (ΔOD=0.44 in water and 0.32 in M9). Finally, the growth phases appeared to be well defined in M9 medium with an apparent lag phase from T0 to T2 and an exponential phase from T4 to T10. In water, growth was slower and the growth phases were more variable and not as well defined, especially in the cultures containing the seaweed extracts. In addition, in contrast to M9 medium, cultures in water did not reach the exponential phase at the same time. For example, during growth on glucose only (positive control) standard at 0.1%
control), the stationary phase started after 28 h of incubation, but occurred after 14 h with some of the seaweed extracts.

Water appeared to be a better carrier to compare the effectiveness of the seaweed extract to promote probiotic bacterial growth. Comparison of M9 and water as carrier media was repeated using another Lactobacillus strain (Lactobacillus plantarum) to double check the results obtained and to establish if the effects seen for *L. acidophilus* were observed for other species of *Lactobacilli*.

*Figure 6.7* shows the growth curves obtained for *L. plantarum* using the same seaweed extracts as in the previous experiment on the same carrier media: M9 (A) and water (B). In contrast to findings for *L. acidophilus*, the previous experiment, differences between effects of the carbon/nutrient sources were easier to observe in the M9 medium. Indeed, very
low growth was noticed with water as carrier, and only slight differences in absorbance could be detected between the test and control cultures. Whereas, in M9 medium (Figure 6.7-A), comparison between samples could be made readily and growth phases were well clear and well-defined.

M9 medium appeared to be a good compromise to investigate the effects of the seaweed extracts as a carbon/nutrient sources or their positive or negative effects on the growth of Lactobacilli. Despite better results being obtained with water as carrier in the case of L. acidophilus, a carrier medium containing a minimum of nutrients appeared to be required to grow other strain of Lactobacillus. In order to be able to do a direct comparison between the strains, it was important that the same medium would be used for the entire screening study.

6.2.3 Preliminary screening of seaweed extracts and polysaccharide-enriched fractions

A first screening study was conducted with the different seaweed extracts prepared as explained in Section 2.8.1. The extracts tested were: crude extract (S0), crude extract dialysed (S1), alginate-rich fraction (ALG), fucoidan-rich fraction (FUC) and laminaran-rich fraction (LAM). These 5 categories of fractions were prepared for each of the 8 different protocols (see Section 2.7.3), which results in a total of 40 samples. This first screen was more focused on identifying the effects of the extracts and polysaccharide-enriched fractions on bacterial species classed as opportunistic pathogens, which a key parameter to underpin any potential prebiotic activity against probiotic organisms. Indeed, a bactericidal (or even bacteriostatic) effect on opportunistic pathogens is essential to modify the gut microbiota in a positive way and to prevent selection of extracts that might impact negatively on gut health during the clinical trial. A Lactobacillus strain was added to this first screen to investigate a potential boosting effect on a probiotic strain in parallel with an assessment of antibacterial activity against the opportunistic pathogens. For pathogenic strains, the optimum medium (LB broth) was used while M9 medium was used for cultivation of the Lactobacillus sp.

6.2.3.1 Effects of the crude extracts (S0) on microbial growth

The S0 extracts, being crude extracts, contained many different seaweed compounds, including polyphenols and salts, in addition to the target polysaccharides. The effects of the extraction protocol on extract composition was investigated to correlate
activity to the extraction conditions and to extract composition (and has been discussed previously in Chapters 4 and 5).

Before statistical analysis of the growth, a general comparison of the extracts and the reproducibility of replicate tests was done. Figure 6.8 shows the growth curves for *E. coli* using crude extracts (S0) as a supplement in the medium (LB broth). Glucose and a culture grown on medium only (without an additional carbon source) were used as controls. The sample “no extract” was defined as the normal growth of *E. coli* in optimum medium without added carbon source (OD of the end stationary phase = 0.694). Addition of 0.1% w/v glucose to this culture increased the growth (OD = 0.862), which was in contrast to the

![Figure 6.8 Escherichia coli growth curves in LB medium containing the different seaweed crude (S0) extracts at 0.1%w/v in comparison with growth on glucose and no added carbon source.](image)

effect of every seaweed extract, all of which reduced growth. Moreover, differences were noticed between the seaweed extracts. Indeed, the best growth suppressing effect seemed to be obtained with EXT8 with an OD of 0.333 at the end of stationary phase and the lowest activity was found with the EXT 5 with a difference of only 0.06 in the OD when compared to the control (“no extract”). In general, the extracts processed at the higher temperature (70°C), which will be referred to as ‘hot process extract(s)’ in the text (i.e. EXT 3, 4, 7 and 8) appeared to give better results than those produced at the lower (25°C) extraction temperature.

Reproducibility between the triplicates was generally good as shown in Figure 6.9, which shows representative growth curves for with *E. coli*, for both controls (i.e. no extract
and glucose only) and the third S0 extract, with the standard deviation being measured at each point during the growth time series. Standard deviations were slightly higher for the controls but stayed constant over time. Growth on all extracts showed good reproducibility and especially for extracts 3, 7 and 8 (Figure 6.9-B) with an average of the standard deviation of 0.014, 0.015 and 0.019, respectively. The biggest difference between replicates was noticed for extract 5, which yielded with an average standard deviation of 0.083.

In order to define with accuracy the growth phases, the average optical density values were statistically compared pairwise be conducting a one way ANOVA. The lag phase was characterised by no significant difference in OD at the beginning of the time course. In the log phase, optical density increased faster and was significantly different between points separated by 1-2 hours. Finally, the OD remained consistent over time during the stationary phase. Figure 6.10 provides an example of statistical analysis conducted on the time course growth profiles for E. coli, with the example shown specifically...
illustrating results obtained during growth of this bacterium on glucose. Averages were compared two by two (one way ANOVA) and non statistically different results were grouped and identify with the same letter (results that do not share a letter are significantly different). The average of OD readings at T0 and T1 both were assigned the letter N, meaning that there was a non-significant difference between these time points. After an hour of incubation, the optical density started to increase and differences were observed between the time points up to 8 hours, after which turbidimetric readings stayed more constant. The statistical analysis was used to define the growth phases for \textit{E.coli} on glucose, which were as follows: lag phase from 0 to 2 hours, log phase from 2 to 8 hours and the stationary phase from 8 to 20 hours.

The same analysis was conducted for all extracts and the results are summarised in Table 6.1. No lag phase was identified in the bacterial growth profiles on extracts 2 and 3. In this instance, T1 gave an OD average significantly different than T0. For the other extracts, differences in OD were observed after 1 or 2 hours with no particular trend corresponding to the extraction protocol used. Greater differences were noticed in the log phase, the most pronounced was obtained using glucose as a carbon source (7 hours), while the shortest log phase (~4 h) was observed for extracts 4, 5 and 6. Most of the seaweed extracts tested appeared to reduce or shorten the log phase (7 hours for glucose against 4 or 5 h for the seaweed extracts).

<table>
<thead>
<tr>
<th>Glucose</th>
<th>lag phase</th>
<th>log phase</th>
<th>stationary phase</th>
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<tbody>
<tr>
<td>EXT1</td>
<td>0 - 2</td>
<td>2 - 7</td>
<td>7 - 20</td>
</tr>
<tr>
<td>EXT2</td>
<td>0 - 7</td>
<td>7 - 20</td>
<td></td>
</tr>
<tr>
<td>EXT3</td>
<td>0 - 7</td>
<td>7 - 20</td>
<td></td>
</tr>
<tr>
<td>EXT4</td>
<td>0 - 2</td>
<td>2 - 6</td>
<td>6 - 20</td>
</tr>
<tr>
<td>EXT5</td>
<td>0 - 2</td>
<td>2 - 6</td>
<td>6 - 20</td>
</tr>
<tr>
<td>EXT6</td>
<td>0 - 2</td>
<td>2 - 6</td>
<td>6 - 20</td>
</tr>
<tr>
<td>EXT7</td>
<td>0 - 1</td>
<td>1 - 6</td>
<td>6 - 20</td>
</tr>
<tr>
<td>EXT8</td>
<td>0 - 1</td>
<td>1 - 6</td>
<td>6 - 20</td>
</tr>
</tbody>
</table>

The next step of the analysis was to compare the difference in optical density between the beginning and the end of each growth phase ($\Delta$OD) and correlate the findings to bacterial cell numbers. Table 6.2 displays the average and standard deviations for the $\Delta$OD values obtained (three replicates) in the lag phase. A one way ANOVA was done to determine the statistical differences between the extracts and groups that were defined. Extracts that did not share a letter were statistically different. During the lag phase, some differences were already observed between the extracts, and especially for EXTs 5 and 6.
which gave a statistically higher OD than the other extracts (0.140 and 0.108, respectively). Other fractions started the log phase with lower optical density, e.g. around 0.06 for the EXT 4, 0.02 for glucose and EXT 1, 0.01 for EXT 7 and 8 and finally 0 for the EXTs 2 and 3.

During the log phase (Table 6.3), maximum growth was observed for glucose ($\Delta$OD = 0.762), which was significantly different from the lag phases observed for all of seaweed extracts. Maximum growth on a seaweed extract was obtained for EXT 2 ($\Delta$OD of the log phase = 0.464), which was significantly higher than for the other extracts. Minimum variation in OD was obtained for the EXTs 4 and 6 with no significant difference noted between both extracts ($\Delta$OD = 0.270). EXTs 1 and 3 were located to the same groups with both extracts yielding statistically lower growth than EXT 2 and glucose but higher growth than EXTs 4, 6 and 8.
Despite low OD differences observed throughout the duration of the stationary phase, some differences were noticed regarding the variation in optical density during this phase. In fact, the optical density continued to increase for some extracts, especially EXTs 4, 5 and 6, with an OD variation between 0.17 and 0.20 (Table 6.4). EXTs 3, 7 and 8 revealed a low change in optical density between the beginning and the end of this phase with the lowest for the last two extracts ($\Delta$OD = 0.02). Concerning the other extracts (1 and 2), the growth differences during the final phase were similar to glucose, significantly lower than EXT 4 and higher than EXTs 7 and 8.

In order to correlate the growth of *E. coli* to the extraction protocol used for each extract, the average values for optical density variation within each growth phase were calculated for each level of extraction parameter: 1 and 6 hours for time, 25°C and 70°C for temperature and 2 and 5 for extractant/biomass ratio. For each parameter, during each growth phase, *p*-values were calculated to determine if the growth was influenced by the extraction parameters and results are summarised in Table 6.5. No significant differences were noticed with the exception of the variation during the stationary phase between the cold (25°C) and hot (70°C) process extracts. Indeed, *E. coli* seemed to continue to grow on the cold process extracts during the stationary phase. As seen previously, EXTs 3, 7 and 8 (hot process extracts) gave the lowest variation in OD during this phase in contrast to EXTs 5, 6 and even EXTs 1, 2 which are cold process extracts. The average of the OD variation for the

<table>
<thead>
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<tbody>
<tr>
<td>Level</td>
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<td>EXT 1</td>
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<td>EXT 7</td>
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<td>EXT 8</td>
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</table>

Table 6.4 Average ($n=3$) and standard deviations of the optical density (@595nm) variation during the stationary phase of the growth of *E. coli* on seaweed extracts

<table>
<thead>
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<th>Parameters</th>
<th>Time</th>
<th>Temperature</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag phase</td>
<td>0.022</td>
<td>0.068</td>
<td>0.055</td>
</tr>
<tr>
<td>Log phase</td>
<td>0.354</td>
<td>0.315</td>
<td>0.129</td>
</tr>
<tr>
<td>Stationary phase</td>
<td>0.129</td>
<td>0.101</td>
<td>0.383</td>
</tr>
</tbody>
</table>

Table 6.5 Statistical analysis of the influence of extraction parameters for the variation of the optical density at 595nm of *E. coli* growth phase
cold process extracts (0.149) was significantly different to that obtained for the hot process extracts (0.081) with a \( p \) value of 0.33.

Finally, cultures were plated on the appropriate agar media to count the number of colonies formed after 20 hours incubation at 37°C and to estimate the number of live bacterial cells. The highest number (statistically higher; \( n = 9 \)) of live cells was found in the glucose culture with, on average \( 73 \times 10^6 \) cfu/mL of culture (Table 6.6). Statistical analysis of the results obtained from the plate counts revealed minimum differences between the EXTs in that the average number of colonies (\( n = 9 \)) was between 29 (EXT 2) and 42 (EXT 7). The minimal statistical differences between the extracts were probably due to the low reproducibility of the experiment with high standard deviation values, despite the number of replicates (9). Statistical analysis of the effect of extraction parameters on the number of live cells (Table 6.7) revealed no significant differences between them. Nonetheless, the average value for the colony counts showed a higher number at the high level parameter for each extraction (i.e. long extraction at high temperature and high ratio).

The same analyses were conducted on the corresponding growth profiles for *Pseudomonas aeruginosa* and the results are summarised in Table 6.8. The reproducibility of the growth was evaluated by taking the average standard deviation for all time points. Similar to the results obtained for *E. coli*, the most reproducible profiles were obtained with
EXTs 3, 7 and 8, with an average of standard deviation of 0.013, 0.006 and 0.008 respectively, and profile with greatest variance was obtained for EXT 1 with an average standard deviation value of 0.088. In general, the reproducibility appeared better for the growth on hot process extracts (protocols 3, 4, 7 and 8). Statistical analysis to define the beginning and end of the different growth phases showed some differences between the extracts. Apart from EXT 4, the log phase for *P. aeruginosa* growth seemed to start faster on hot extracts than the log phases for growth on cold extracts. Indeed, for EXTs 3, 7 and 8, the log phase started after 3 hours of incubation (like glucose) and the variation in optical density was similar between those extracts (between 0.02 and 0.03), whereas log phases on the cold extracts started after 6 hours (5 hours for EXT 1) and for EXTs 2 and 5, a low change in OD (around 0) was obtained and a slightly bigger change for EXTs 1 and 6 (between 0.07 and 0.08). Consequently, this growth phase was longer for the hot extracts, i.e. duration of 5 hours (6 hours for EXT 8) *versus* 2 hours for the cold process extracts (and EXT 4). Nevertheless, maximum growth was obtained on glucose and EXT 2 with an OD variation above 0.3, which was significantly higher than values for EXT 7 and 8 (ΔOD between 0.25 and 0.30). The lowest growth was observed mainly on cold extracts (EXTs 1, 5 and 6). The stationary phases started at the same time for all cultures and lasted between 11 and 13 hours. During this phase, optical density continued to increase for the cold process extracts (EXTs 1, 2, 5 and 6), more than for the hot extracts. Consequently, the number of

<table>
<thead>
<tr>
<th>Table 6.8 Summary of the statistical analysis of <em>P. aeruginosa</em> growth profiles on crude seaweed extracts (50) and glucose</th>
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<tbody>
<tr>
<td><strong>Average of standard deviations</strong></td>
</tr>
<tr>
<td><strong>Lag phase</strong></td>
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<tr>
<td>Duration (start–end)</td>
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<tr>
<td><strong>Log phase</strong></td>
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<td>Duration (start–end)</td>
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<tr>
<td><strong>Stationary phase</strong></td>
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<tr>
<td>Duration (start–end)</td>
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<tr>
<td><strong>Colony counts</strong> (x10^6 cfu/mL)</td>
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<td><strong>Glucose</strong></td>
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<td><strong>EXT 1</strong></td>
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<td><strong>EXT 3</strong></td>
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<td><strong>EXT 7</strong></td>
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<td><strong>EXT 8</strong></td>
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</table>
live cells in these cultures was higher even though no statistical differences were noticed between the extracts.

Despite no significant differences between the extracts regarding the number of \textit{P. aeruginosa} colonies formed on agar plate cultures of 20 hours culture samples (\textit{Table 6.8}), the trend identified previously was confirmed by calculation (\textit{Table 6.9}). Indeed, the average number of live cells from cultures grown on cold extracts (14 x 10^6 cfu/mL) was 2-fold higher than the number obtained from cultures grown on hot extracts (7 x 10^6 cfu/mL). This higher number of live cells correlated with the variation of optical density during the stationary phase. Indeed, no differences were identified between the level of each extraction parameter during the first two growth phases, temperature was significantly important during the stationary phase. As seen previously, cultures on cold extracts continued to display an increase in growth during this phase (\textit{\textit{OD}} 0.173 \textit{versus} 0.070 on hot extracts) in contrast to the cultures grown on hot extracts resulting in higher number of live cells.

Analysis of the growth of \textit{Lactobacillus plantarum} revealed that different growth responses were obtained than those noted for \textit{E. coli} and \textit{P. aeruginosa}, and the extracts appeared to enhance the growth (instead of reducing it). The same approach of statistical analysis was performed and results are combined in \textit{Table 6.10}. First of all, the reproducibility was excellent for this bacterium on all extracts. The largest average standard deviation (0.030) was obtained for EXT 5 and the lowest for the EXT 3 at 0.009. Contrary to the previous strains studied, \textit{L. plantarum} was grown in M9 medium (minimum media based on nutrients) supplemented with the seaweed extracts at 0.1% w/v (or with glucose for the control). The bacteria needed more time to adapt to the medium and, consequently, the lag phase was slightly longer. The longest lag phase (4 hours) was observed during growth of \textit{L. plantarum} on glucose and EXT 6. With EXTs 3 and 8, this phase was 2-fold shorter (2 hours) and 3 hours on all other extracts. Even though this represents an adaptation phase,

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<th>Parameters</th>
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<th>Temperature</th>
<th>Ratio</th>
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<td></td>
<td>1 hr</td>
<td>6 hrs</td>
<td>0.05</td>
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<tr>
<td>Lag phase</td>
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<td>0.036</td>
<td>0.905</td>
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<tr>
<td>Log phase</td>
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<td>0.209</td>
<td>0.958</td>
</tr>
<tr>
<td>Stationary phase</td>
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<td>0.095</td>
<td>0.154</td>
</tr>
<tr>
<td>Colony count (x10^6 cfu/mL)</td>
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<td>11</td>
<td>0.932</td>
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bacterial growth still occurred. The variation of optical density observed during this step was lower for cultures exhibiting the shorter lag phase durations and higher for the cultures with the longest lag phases.

In contrast, the duration of the log phase gave the opposite pattern of results to the lag phase. Indeed, EXTs 3 and 8, which yielded a shorter lag phase, appeared to sustain a longer log phase (10 and 9 hours, respectively). The shortest log phases were noticed during growth on glucose and EXTs 5 and 6 which had the longest lag phases. For the other extracts, the duration of the log phase was defined at 8 hours. The best extract to enhance the

<table>
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<tr>
<th>Table 6.10 Summary of the statistical analysis of L. plantarum growth on crude seaweed extracts (50) and glucose</th>
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<tr>
<td><strong>Average of standard deviations</strong></td>
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*Lactobacillus plantarum* growth was EXT 3, which yielded with an O.D increase of 0.671 which statistically higher than all other carbon sources. The lowest growth was calculated for glucose and EXTs 5 and 6 (shortest log phases) with no significant difference between these extracts and with glucose. Finally, the time series was stopped after 20 hours of incubation. The stationary phase lasted between 8 and 10 hours depending on the extracts. The main changes between the end of the log phase and stationary phase were noted for EXTs 1, 5 and 7. Indeed, the O.D kept increasing during the stationary phase in cultures growing on those extracts and was significantly higher than EXTs 3, 6, 8 and glucose.

Statistical analysis of extraction parameter effects revealed a significant correlation with the temperature used in extract preparation for most of the studied responses (Table 6.11). The lag phase had a shorter duration for cultures grown on EXTs 3 and 8, which were
hot process extracts. Consequently, the optical density variation of this phase was lower and hot process extracts gave statistically lower ΔOD during the lag phase (0.017 on average against 0.035 for cold process extracts). The opposite observation was made for the log phases. Indeed, despite a lower OD at the beginning, hot process extracts gave significantly higher average OD variations during the log phases than the cold extracts (0.601 and 0.479, respectively). Despite no significant differences in the OD variation during the stationary phase, the number of live cells remained statistically higher in the cultures grown on the hot process extracts. The significant differences between cold and hot processed extracts were most likely the consequence of higher growth on the extracts processed at the higher temperature. Time and ratio had no effects on the number of cells alive after 20 h of incubation on seaweed extracts. Unlike the analysis of the pathogenic strain growth, time was identified as influential for the growth of *L. plantarum* during the log phase. Indeed, if no significant difference was noticed during the lag phase, the extracts obtained by the short extraction process gave statistically higher optical density variations during the log phase. During this phase, the optical density increased, on average, by 0.577 for the cold extracts and 0.502 for the hot extracts. No difference had been shown for the OD variation during the stationary phase. Finally, the volume of water added for the extraction (ratio) did not appear to be influential in determining the growth of bacteria on the resulting extract.

Table 6.11 Statistical analysis of the influence of extraction parameters for the variation of the optical density at 595nm of *L. plantarum* growth phase and the number of colonies formed after 20 hours of incubation at 37°C on crude seaweed extracts (50)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Time</th>
<th>Temperature</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
<td>6 hrs</td>
<td>25°C</td>
</tr>
<tr>
<td>ΔOD</td>
<td>0.022</td>
<td>0.030</td>
<td>0.157</td>
</tr>
<tr>
<td>Lag phase</td>
<td>0.577</td>
<td>0.502</td>
<td>0.019</td>
</tr>
<tr>
<td>Log phase</td>
<td>0.033</td>
<td>0.035</td>
<td>0.854</td>
</tr>
<tr>
<td>Stationary phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colony count (x10⁶ cfu/ml)</td>
<td>26</td>
<td>25</td>
<td>0.812</td>
</tr>
</tbody>
</table>

To select the best extracts from the S0 fraction, the results obtained for the three strains were summarised to define the important extraction parameters. This summary focused on the optical density variation of the log and stationary phases and the number of live cells at the end point. Table 6.12 combines the OD variation of the log phase and shows that the extraction parameters used to prepare the extracts were statistically influential only for *L. plantarum* growth. Indeed, a shorter extraction time enhanced growth better than a longer extraction (ΔOD = 0.577 and 0.502 respectively). Temperature was also defined as influential, as cold extracts were not as effective in promoting growth of the beneficial
bacteria as hot extracts as is evident in the smaller difference of optical density between the lag and log phase (0.479 versus 0.601 for hot extracts) in Table 6.12. The best extracts for promoting the growth of *L. plantarum* were extracted at 70°C during 1 hour and these extracts did not have the same growth promoting effects on the other bacterial species, i.e. opportunistic pathogens.

On review of the variation of optical density during the stationary phase (Table 6.13), it can be seen that the growth was much slower than during the log phase but differences between the beginning and the end of the phase were observed for all bacteria tested. It should be noted that the average ΔOD values represent the differences between the average OD values for the stationary phase and the average OD values for the log phase. Interestingly, no effect of extraction parameters were noticed on the growth of *L. plantarum* (very low growth occurred during this phase), whereas temperature was influential in determining the most effective seaweed extracts against the two pathogenic strains (Table 6.13). Cultures containing hot process extracts appeared to reduce the growth of *E. coli* and *P. aeruginosa* (ΔOD = 0.081 and 0.070, respectively) while on cold extracts, the growth continued to increase with an OD amplification between the log and stationary phases of 0.149 and 0.173 for respectively, for *E. coli* and *P. aeruginosa*. 

<table>
<thead>
<tr>
<th>Log phase</th>
<th>Time</th>
<th>Temperature</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
<td>6 hrs</td>
<td>p value</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.353</td>
<td>0.315</td>
<td>0.129</td>
</tr>
<tr>
<td>ΔOD</td>
<td><em>P. aeruginosa</em></td>
<td>0.210</td>
<td>0.209</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>0.577</td>
<td>0.502</td>
<td>0.019</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stationary phase</th>
<th>Time</th>
<th>Temperature</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
<td>6 hrs</td>
<td>p value</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.129</td>
<td>0.101</td>
<td>0.383</td>
</tr>
<tr>
<td>ΔOD</td>
<td><em>P. aeruginosa</em></td>
<td>0.148</td>
<td>0.095</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>0.033</td>
<td>0.035</td>
<td>0.854</td>
</tr>
</tbody>
</table>

Table 6.12 Effect of extraction parameters on the optical density variation during the log growth phase on crude seaweed extracts (50) 

Table 6.13 Effect of extraction parameters on the optical density variation during the stationary growth phase on crude seaweed extracts (50)
Finally the number of live cells, evaluated by plate counts at the end of the growth experiment (20 hours of growth) showed an effect of temperature only (Table 6.14). After 20 hours of growth, cultures grown on hot process extracts had fewer live \textit{P. aeruginosa} cells but supported higher \textit{L. plantarum} cell viability, which corroborates the findings from the growth curve. The ability to support growth of \textit{Lactobacillus sp}, yet check or suppress growth of \textit{Pseudomonas sp} corresponds exactly to the type of activity sought. Extraction parameters seemed to have a lesser influence on cell viability and cell numbers in the case of \textit{E. coli}.

In conclusion, S0 extracts prepared at high temperature gave statistically higher growth promoting (or growth suppressing) activities on the strains investigated. Moreover, a short extraction time yielded an extract that was beneficial for the optimal growth of \textit{L. plantarum}. Taking these two parameters into consideration, extracts prepared under a short duration for extraction (1 hour) and at high temperature (70°C) were selected, i.e. extracts 3 and 4.

### 6.2.3.2 Screening of the effects of dialysed crude extracts (S1) on microbial growth

The S1 extracts were obtained by dialysis of the extracts S0 using ultrafiltration units equipped with 2 kDa MWCO polyethersulphone (PES) membranes (see Section 2.8.1). The dialysis process aimed to remove salts and small compounds like mannitol or oligosaccharides. In proportion, polysaccharides and polyphenols would be expected to be >2 kDa and should be in higher concentration than the smaller molecules (see Chapters 3 and 4). The eight S1 fractions were screened for effects on the growth of the same bacterial species (\textit{E. coli}, \textit{P. aeruginosa} and \textit{L. plantarum}) but only the summary tables to define the influence of extraction parameters are presented here. The detailed comparison between the extracts is provided in Appendix 9-A.
During the log phase, contrary to *E. coli* (no effect of extraction parameters), all parameters were calculated as influential for the growth of *L. plantarum* growth (*Table 6.15*). Similar to the S0 fractions, shorter extractions at high temperature produced an extract that yielded better growth of this *Lactobacillus* species. Moreover, the ratio was, in this case, influential for the growth promoting effects on this bacterium, but was also found to have an effect on *P. aeruginosa*. Extracts produced with a smaller volume of water (ratio 2) gave significantly lower growth of *P. aeruginosa* and higher growth of *L. plantarum*. Concerning this growth phase, an extract produced at high temperature, during an extraction period of 1 hour at an extractant/biomass ratio of 2, was significantly better for the activity sought (growth promoting for *Lactobacilli*, yet growth suppressing effects for *Pseudomonas*). This protocol corresponded to the EXT 3.

Likewise the S0 fractions, despite an effect of all extraction parameters for *L. plantarum* growth during the log phase, no significant differences were observed during the stationary phase on S1 fractions, as shown in *Table 6.16*. Temperature gave also the same trend with a reduction in growth in cultures containing a hot process extract for the two opportunistic pathogens (0.014 and 0.032 for hot extracts versus 0.136 and 0.092 for cold extracts for *E. coli* and *P. aeruginosa*, respectively). Moreover, the extractant/biomass ratio had an effect on the growth of *P. aeruginosa*, during the last or stationary growth phase. This effect was the opposite to the effect noticed during the log phase, meaning that the

<table>
<thead>
<tr>
<th>Log phase</th>
<th>Time</th>
<th>Temperature</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
<td>6 hrs</td>
<td>25°C</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.360</td>
<td>0.346</td>
<td>0.796</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>0.105</td>
<td>0.116</td>
<td>0.558</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>0.407</td>
<td>0.312</td>
<td>0.004</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stationary phase</th>
<th>Time</th>
<th>Temperature</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
<td>6 hrs</td>
<td>p value</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.110</td>
<td>0.067</td>
<td>0.206</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>0.069</td>
<td>0.054</td>
<td>0.450</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>0.034</td>
<td>0.042</td>
<td>0.567</td>
</tr>
</tbody>
</table>
growth was slower on extracts produced on the larger volume of water (ratio of 5:1). Regarding this growth phase, the best protocol to use to produce an extract with growth promoting effects on *Lactobacillus sp.* and growth suppressing properties on opportunistic pathogens was a high temperature and high ratio extraction method, which corresponded to EXTs 4 and 8.

Analysis of culture viability by plate counting revealed that the three parameters (time, temperature and ratio) had an effect on the number of live cells: ratio on *E. coli*, time and temperature on *L. plantarum*. Table 6.17 shows that extracts prepared using a larger volume of water reduced the number of live *E. coli* cells (77 x 10⁶ cfu/mL for ratio 2 and 45 x 10⁶ cfu/mL for ratio 5; p value of 0.000) but had no statistically relevant effect on the other strains. Time and temperature had an effect on *L. plantarum* with a statistically relevant growth-promoting benefit observed for extracts prepared during a 1 hour extraction period at 70°C. A longer extraction time reduced the number of live cells by 9 x 10⁶ cfu/mL and high temperature increased the cell density by 15 x 10⁶ cfu/mL. No significant effect was identified on *P. aeruginosa*. Combining these findings, the best protocol to use to generate an extract with the desired bioactivity would involve the use of a 1 hour extraction at 70°C with a ratio 5, which corresponds to EXT 4.

Table 6.17 Effect of extraction parameters on the number of live bacterial cells after 20 hours of incubation on dialysed seaweed extracts (S1)

<table>
<thead>
<tr>
<th>Colony count (x10⁶ cfu/mL)</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
<th>L. plantarum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr</td>
<td>68</td>
<td>8</td>
<td>34</td>
</tr>
<tr>
<td>6 hrs</td>
<td>54</td>
<td>8</td>
<td>25</td>
</tr>
<tr>
<td>p value</td>
<td>0.082</td>
<td>0.439</td>
<td>0.016</td>
</tr>
<tr>
<td>Temperature</td>
<td>25°C</td>
<td>8</td>
<td>22</td>
</tr>
<tr>
<td>70°C</td>
<td>67</td>
<td>8</td>
<td>37</td>
</tr>
<tr>
<td>p value</td>
<td>0.098</td>
<td>0.478</td>
<td>0.000</td>
</tr>
<tr>
<td>Ratio</td>
<td>2</td>
<td>8</td>
<td>27</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>p value</td>
<td>0.000</td>
<td>0.402</td>
<td>0.165</td>
</tr>
</tbody>
</table>

6.2.3.3 Screening of alginate rich fractions (Alg) for effects on microbial growth

The alginate fractions were obtained by acid precipitation of the S0 extracts and alginates were resuspended in sodium hydroxide prior to dialysis against distilled water using ultrafiltration concentration units equipped with PES 2 kDa MWCO membranes to neutralise the solution and remove residual salts (see Section 2.8.1). The detailed comparison between the extracts is provided in Appendix 9-B.
The influence of the extraction parameters was slightly different for the growth of bacteria on alginate rich fractions. Table 6.18, which shows the influence of extraction parameters during the log phase, reveals no apparent correlation between the extraction protocol and the growth of *P. aeruginosa*. For the other two strains, two factors were influential in generating bioactive extracts, i.e. temperature and ratio for *E. coli* and time and temperature for *L. plantarum*. The effect of temperature had the opposite effects on the OD variation during the log phase for *E. coli* and *L. plantarum*. In this case, low temperature extracts yielded extracts that reduce the growth of the opportunistic pathogens and enhance the growth of *L. plantarum* with *p*-values that confirm the significance of these findings. Concerning the effect of ratio on *E. coli* growth, preparation of an extract with a lower volume of water significantly decreased bacterial growth (ΔOD was 0.285 versus 0.502 for ratio 5). Once again, a shorter extraction time yielded an alginate fraction that was better for growth of *L. plantarum*. In general, extracts prepared using all parameters at a low level (1 Hour, 25°C and ratio 2; i.e. EXT 1).

Extraction parameters showed a low influence concerning the variation in optical density during the stationary phase. Indeed, the only significant effect was identified for temperature for in the case of Alg fractions shown bioactivity against *P. aeruginosa* growth (Table 6.19). Nevertheless, some trends were noticed between the strains. During the stationary phase, the OD variation for *E.coli* was between 0.09 and 0.10. Concerning *L. plantarum*, the growth was lower with an OD variation between 0.038 and 0.051. With *P. aeruginosa*, Alg from hot extracts reduced the growth significantly in comparison to cold...
extracts (ΔOD 0.055 and 0.126, respectively); the other parameters were not significantly influential. Regarding the growth during the stationary phase, hot extracts (ECTs 3, 4, 7 and 8) gave better results.

Regarding the number of live cells at the end of the specified growth time period, the growth during the stationary phase and effect of extraction were generally low. The only effects were shown to be extraction time on L.plantarum and ratio on E.coli (Table 6.20). Growth of E.coli on Alg prepared from extracts using a lower volume of water (ratio 2) yielded lower number of live cells in the culture after 20 hours of incubation (121 x 10^6 cfu/mL versus 180 x 10^6 cfu/mL for the ratio 5). L.plantarum grown on extracts derived

<table>
<thead>
<tr>
<th>Colony count (x10^6 cfu/ml)</th>
<th>Time</th>
<th>Temperature</th>
<th>Ratio</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli</td>
<td>1 hr</td>
<td>1.65</td>
<td>0.086</td>
<td>161</td>
</tr>
<tr>
<td></td>
<td>6 hrs</td>
<td>1.14</td>
<td>0.224</td>
<td>121</td>
</tr>
<tr>
<td>P.aeruginosa</td>
<td>9</td>
<td>1.11</td>
<td>0.806</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1.12</td>
<td>0.844</td>
<td>11</td>
</tr>
<tr>
<td>L.plantarum</td>
<td>39</td>
<td>0.000</td>
<td>0.540</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>82</td>
<td>0.58</td>
<td>0.423</td>
<td>64</td>
</tr>
</tbody>
</table>

Table 6.20 Effect of extraction parameters on the number of live bacterial cells after 20 hours of incubation on alginate rich fractions (Alg)

from a shorter extraction process (i.e. 1 hour) significantly tended to die more than extracts from the longer extraction process (39 x 10^6 cfu/mL and 82 x10^6 cfu/mL, respectively). The best extraction protocols regarding the number of live cells in culture was the ones that lasted for 1 hour at a ratio 2, which corresponded to EXTs 1 and 5.

Alg from EXT 1 appeared to be the best for the activity sought. Nevertheless, EXT 3 was considered as potentially interesting (better results during the stationary phase and was prepared by extraction for 1 hour at a ratio of 2.

6.2.3.4 screening of fucoidan rich fractions (Fuc)

After precipitation of alginates, the supernatant was subjected to another precipitation using cold ethanol to precipitate the fucoidan-rich fraction (see Section 2.8.1). The pellet was resuspended in water and dialysed using ultrafiltration concentration units equipped with PES 2 kDa MWCO membranes. The detailed comparison between the extracts is provided in Appendix 9-C.
Growth of *P. aeruginosa* on fucoidan-rich extracts was very low during the log phase (around 0.01 in OD variation) and no effect of extraction parameters were identified (*Table 6.21*). Concerning *E.coli*, the extraction ratio yielded a fucoidan fraction that influenced the growth during the log phase; fucoidan extracted with a higher ratio reduced the growth of the bacteria (ΔOD 0.411) in comparison with extracts produced over a longer duration and with a higher ratio small ratio (ΔOD 0.578). Finally, lower temperature yielded FUC fractions enhanced to support the growth of *L.plantarum* (ΔOD 0.513) Regarding the growth during the log phase, the extraction protocol using high ratio and low temperature gave better bio-activity. These parameters corresponded to EXTs 2 and 6.

During the stationary phase, the same effects were observed. Indeed, a higher ratio yielded a FUC fraction that reduced the growth of the two pathogenic strains and an extract produced under low temperature enhanced the growth of the probiotic bacterium (*Table 6.22*). Time was also an insignificant parameter in terms of production of a FUC fraction with significant growth boosting or suppressing effects during this growth phase. The best extracts for this growth phase were those extracted for 1 hour at a high extractant/biomass ratio, which corresponded to EXTs 2 and 6.

Despite no observed effect of time during the stationary growth phase, this parameter was significantly influential for the number of live cells in *P.aeruginosa* and *L.plantarum* cultures at the end of the growth period (*Table 6.23*). In both cases, a shorter extraction time resulted in significantly lower numbers of live cells (9 x 10^6 cfu/mL for...
P. aeruginosa and 31 x 10^6 cfu/mL for L. plantarum); however, a longer extraction period yielded a fucoidan with growth promoting effects on L. plantarum that gave higher numbers of live cells after 20 h growth (43 x 10^6 cfu/mL for LP). The number of live P. aeruginosa cells was also influenced by the extraction temperature, as, fucoidan derived from the extract produced at higher temperature, reduced the number of live cells by 7 x 10^6 cfu/mL. Finally, the only parameter that yielded a FUC fraction with significant influence on the growth of E. coli was the ratio. As seen previously, E. coli grew less well on fractions extracted using a higher ratio of extractant/biomass during the log and stationary phases, so consequently, the number of live cells was also lower (69 x 10^6 cfu/mL against 86 x 10^6 cfu/mL for extracts prepared using a lower ratio). FUC from extracts prepared at high temperature and ratio gave better results. The best extracts therefore to yield fucoidan fractions with the best bioactivity profile were those prepared at high temperature and ratio (at the two time durations). These protocols corresponded to EXTs 4 and 8.

Extracts 2 and 6 were identified to be the best extracts regarding the variation of optical density during the log and stationary phases. Nevertheless, regarding the number of live cells, fucoidan-rich fractions from hot extracts, and especially EXTs 4 and 8 were better. Four fucoidan-enriched extracts were therefore selected for this fraction.

### 6.2.3.5 Screening the effects of laminaran rich fractions (Lam) on microbial growth

The laminaran-rich fraction was the residual fraction after precipitation of the alginate and fucoidan from the seaweed extracts prepared using the 8 extraction protocols (see Section 2.8.1). The main polysaccharide was laminaran but the fractions contained also high levels of polyphenols. Consequently, some precipitation occurred in the culture and the growth curves were not reflecting the true growth profiles for the bacteria even though the optical density appeared to increase. Figure 6.11 shows the growth curves for L. plantarum in medium containing the laminaran-rich fractions. In the first hour of incubation, the OD increased very quickly (except on EXT 5) and the profile or shape of the growth curve was
different for each extracts. In addition, colony counting at the end of the growth incubation period revealed that the bacteria did not grow well, as the average number of live cells in culture was around $1 \times 10^5$, which more than 10 times lower than the cell density number on the other extracts. Moreover, on half of the fractions, no colonies were obtained on the agar plates. Based on the results with these fractions, it was not possible to obtain statistical data and therefore, this fraction was not evaluated as potential candidate for prebiotic activity.

### 6.2.3.6 Overall comparison of the bioactive properties of seaweed fractions

Different extracts were selected from the screening studies reported in this chapter. Selection was based on the effect of extraction parameters on the bioactivity observed against the three strains studied. A comparison was then done between the different bacteria using only the selected extracts: S0, S1, Alg and Fuc. The same factors were analysed: variation of optical density during the log and stationary phases and the number of live cells at the end of the incubation. Table 6.24 compares the variation of optical density during the log phase between the fractions for the three strains. Regarding *E. coli*, the best growth was identified on fucoidan extracts with an OD variation of 0.411, which was not significantly different from the growth on S1 fractions ($\Delta$OD was 0.400), and S0 and alginates fractions gave statistically lower growth (and were not significantly different between each other with $\Delta$OD of 0.306 and 0.277 respectively). Almost the same observation was made for *P. aeruginosa* growth, except that the fucoidan fractions were significantly higher than growth
on the other seaweed extract fractions (ΔOD 0.306); S1 fractions gave the lowest OD variation at 0.138 (was not statistically different from the result obtained during growth on the S0 fraction with a ΔOD of 0.175). Finally regarding the data for *L. plantarum*, the best growth was obtained on the S0 extracts with a delta OD of 0.625 (significantly higher than the other fractions) and the lowest growth on the S1 and alginate fractions with ΔOD values of 0.393 and 0.360, respectively.

On further analysis of the optical density variation during the stationary phase, no significant differences were noticed between the extracts for *L. plantarum* (*Table 6.25*), even though the ΔOD values obtained on the S0 fractions seemed lower (0.023 relative to approx. 0.060 for the other fractions). Evaluation of the data for the pathogenic strains has shown that growth of *E. coli* during the stationary phase was significantly higher on the S0 fractions (ΔOD = 0.143) and the lowest growth was observed on the S1 and fucoidan-rich fractions with a ΔOD of 0.018 and 0.026 (no statistical difference was obtained between the data from both batches of fractions). The alginate-rich fraction yielded the highest growth of *P. aeruginosa* during the stationary phase (not significantly different than S0 extracts), and the lowest growth was obtained on the S1 fractions with an OD variation of 0.023.

---

**Table 6.24 Average of the log phase optical density variation (@595nm) of cultures grown on the best extracts of each fraction**

<table>
<thead>
<tr>
<th>Fractions</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
<th>L. plantarum</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0</td>
<td>0.306&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.175&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.625&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>S1</td>
<td>0.400&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.138&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.393&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Alg</td>
<td>0.277&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.231&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.360&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fuc</td>
<td>0.411&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.306&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.481&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Table 6.25 Average of the stationary phase optical density variation (@595nm) of the best extracts in each fraction**

<table>
<thead>
<tr>
<th>Fractions</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
<th>L. plantarum</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0</td>
<td>0.143&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.088&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.023&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>S1</td>
<td>0.018&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.023&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.059&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Alg</td>
<td>0.084&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.118&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.061&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fuc</td>
<td>0.026&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.057&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.060&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Despite some differences in the optical density variation during the last two growth phases, no statistical differences were observed regarding the number of live \textit{P. aeruginosa} and \textit{L. plantarum} cells in culture after 20 hours of incubation on the different fractions (\textit{Table 6.26}). The only significant differences were noted for the \textit{E. coli} cultures, with a minimum of live cells obtained with the S0 extracts (35 \times 10^6 \text{ cfu/mL}) and the maximum for the growth on alginate-rich fractions at 97 \times 10^6 \text{ cfu/mL}.

\textit{Table 6.26 Average of number of live cells in culture (x10^6 \text{ cfu/mL}) between the best extracts in each fraction}

<table>
<thead>
<tr>
<th>Fractions</th>
<th>\textit{E. coli}</th>
<th>\textit{P. aeruginosa}</th>
<th>\textit{L. plantarum}</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0</td>
<td>34.8\textsuperscript{c}</td>
<td>7.8\textsuperscript{a}</td>
<td>36.2\textsuperscript{a}</td>
</tr>
<tr>
<td>S1</td>
<td>71.6\textsuperscript{b}</td>
<td>8.5\textsuperscript{a}</td>
<td>38.8\textsuperscript{a}</td>
</tr>
<tr>
<td>Alg</td>
<td>96.6\textsuperscript{a}</td>
<td>6.9\textsuperscript{a}</td>
<td>29.7\textsuperscript{a}</td>
</tr>
<tr>
<td>Fuc</td>
<td>34.8\textsuperscript{b}</td>
<td>10.8\textsuperscript{a}</td>
<td>41.9\textsuperscript{a}</td>
</tr>
</tbody>
</table>

To summarise those results, despite a higher growth of the pathogenic strains during the stationary phase, the optical density variations during the log phase on the S0 fractions were more in correlation with the target bioactivity under investigation. The growth of \textit{E. coli} and \textit{P. aeruginosa} during the log phase was significantly lower and statistically higher for \textit{L. plantarum}. Moreover, the number of live cells of \textit{E. coli} was significantly lower in the cultures containing this fraction.

\textbf{6.3 Discussion}

The working concentration was a key parameter to screen the different seaweed extracts. Indeed, due to the brownish colour of the extracts, a high concentration (1\% \text{ w/v}) resulted in a high optical density (even at T0) and the OD increase due to the bacterial growth was minimal or impossible to determine. By contrast, if the extract concentration was too low (0.01\% \text{ w/v}), effects on the bacteria were reduced and no differences between the extracts could be determined. As explained before, and confirmed by O’Sullivan et al., the effect of extraction parameters on seaweed extracts being evaluated for prebiotic activity has not been investigated, to the author’s best knowledge. Moreover, most of the experiments to evaluate prebiotic potential has been done with poor characterisation of the extracts (O’Sullivan et al. 2010).
Comparison of the extraction protocols was done initially based on the type of extracts (S0, S1, Fuc, Alg and Lam). The first fractions investigated was the S0 extracts, which was a crude extract that basically contained a mixture of the water-soluble compounds from the seaweed but in different proportion according to the extraction protocol. With this fraction, interestingly, the duration of extraction was shown to be influential but only on *L. plantarum* during the log phase, and the extraction temperature had the most potent growth suppressing effects on *E. coli* and *P. aeruginosa*, especially during the stationary phase. Indeed, fractions extracted with the shortest extraction time (1 hour) significantly enhanced the growth of *L. plantarum* only, during the log phase with no significant effect during the stationary phase or on the number of live cells at the end of the growth cycle. Extracts processed at shorter durations, as seen in Chapter 4, were shown to contain more mannitol and less fucoidans. Mannitol is known to be fermented by bacteria and especially by *Lactobacilli* (Chen et al. 1983). Higher temperature extracts decreased the growth of potential pathogenic strains but only during the stationary phase resulting in a lower number of live cells in culture. Surprisingly, the extraction temperature appeared to yield extracts from some seaweed species that did not affect the growth of *L. plantarum* as shown by Gupta *et al.* Indeed, comparison between fresh and heated seaweeds showed differences in *L. plantarum* growth according to the seaweed used (Gupta *et al.* 2011). Those differences were mainly noticed with extracts from *Laminariales* species (where a higher growth of *Lactobacilli* was noted after heat treatment) and no growth was observed on *Himantalia elongata* extracts. The main difference in the seaweed composition was the content of phenolic substances, which were more than 10 times higher in *H. elongata* than in the Laminariales (Rodríguez-Bernaldo de Quirós *et al.* 2010). Consequently, *A. nodosum* extracts composition would be closer to *H. elongata* than the *Laminariales*. But contrary to the experiment done on *H. elongata*, a higher growth was noticed on *A. nodosum* extracts prepared at higher temperature. The effect of temperature on extract composition was mainly reflected in the fucoidan fraction (Chapters 4 and 5) with a higher percentage obtained using high temperature. A high concentration of sulphated fucans was shown to induce resistance to bacterial degradation (Michel *et al.* 1996). Moreover, a study on different bacterial strains with purified polysaccharides showed that no bacteria grew on fucoidan extracts, especially *E. coli* and *P. aeruginosa*, but no data were displayed for *Lactobacilli* strains (Fujii *et al.* 1992).

The S1 extracts were obtained after dialysis of the S0 extracts. Consequently, the compositions would be expected to be similar except that all small molecules like salts and mannitol should be removed. Proportionally, the polysaccharides and polyphenols content
should have increased. The effect of time and temperature were similar that the findings observed on the S0 fractions. However, the ratio between volume of water used for the extraction and the quantity of biomass, appeared to be an important parameter as well. Indeed, during the log phase, extracts prepared with a smaller ratio reduced the growth of *P. aeruginosa*. In terms of composition, using a smaller extraction ratio induced a higher polyphenol content which can explain a lower growth due to their antimicrobial properties (Daglia 2012; Segovia Bravo et al. 2007).

Preliminary work to determine the alginate composition in the *A. nodosum* extracts was done using characterised alginate lyases (Chapter 5). In terms of bacterial growth on alginate-rich fractions, the low temperature process extract appeared to decrease the *E.coli* growth and promote the *L. plantarum*. Alginate extracted at low temperature appeared to contain a M/G ratio higher than hot extracts. No results on the effect of the alginate M/G ratio on the prebiotic activity have been published to date, to the best author knowledge.

On the fucoidan rich fractions, the ratio had been shown to influence bacterial growth, especially that of *E. coli*. Indeed, purified fucoidans extracted using higher ratio appeared to decrease the growth of *E.coli* during the log and stationary phases. Ratio had been shown not to influence the monosaccharide composition of fucoidan but affected the size partition (Chapter 5). Using a higher ratio, the predominant fucoidan size fraction was between 100 kDa and 0.2 µm, which might correspond to the native fucoidans (Rioux et al. 2007; Foley et al. 2011). A smaller ratio induced a higher proportion of the fraction above 0.2 µm which probably consists of some complexed molecules. Native fucoidans appeared so to be more active on pathogenic strains. The effect of extraction temperature was mainly significant to promote *L. plantarum* growth during the log and stationary phases (higher growth on fucoidans extracted at low temperature). Temperature was shown to influence the fucoidan size (a higher proportion of low molecular weight fucoidans) and also the monosaccharide composition (lower content of fucose and xylose). Oligosaccharides have been shown to have a good prebiotic activity (Maathuis et al. 2012). The *Lactobacilli* might use the lower fucoidan size fraction whereas the higher sizes acted on the pathogenics to reduce their growth. Sufficient quantities of each fraction can be prepared and used to grow the bacteria to define the exact activity of fucoidans as a future study.

By comparing the best extracts in the different fractions, and the best compromise between inhibiting the potential pathogenic strains growth and promoting the growth of *Lactobacillus sp*, a candidate emerged, which is the crude S0 extract. The higher activity of crude extracts might be explained by a synergy between all the compounds (polysaccharides
between them or polysaccharides/polyphenols...) in the extracts. By purifying some compounds (therefore removing others), some activity were lost, either inhibiting the growth of potentially pathogenic strains or enhancing the growth of beneficial ones.

6.4 References


Chapter 7: Concluding remarks
The first important step of this study was to define the most suitable seaweed species to initiate a detailed analysis on the effect of extraction and purification processes on the yield and composition of seaweed extracts and polysaccharide-enriched fractions. Initially, the first target was to isolate the water-soluble polysaccharides from brown seaweeds (fucoidans, alginates and laminarans). The choice to continue the investigation with *Ascophyllum nodosum* was based on the quantity of fucoidans extracted (higher than the other species investigated), but also on the easy access to this biomass as it was (and still is) the main seaweed that is industrially processed in Ireland.

A first investigation on different aspects of the methods for the extraction and purification of seaweed compounds extracted from *Ascophyllum nodosum* was thus initiated. The analysis of all fractions obtained during a multi-step extraction (Chapter 3) that was previously developed in our group (O’Connell, 2008) allowed the determination of the purification steps required to obtain enriched and more purified polysaccharides. The phenolic compounds were also extracted in water as the extraction solvent in non-negligible quantities, using this method (O’Connell, 2008). In Chapter 3 of this thesis, a first screening was conducted to compare different hydrophobic resins able to bind or adsorb these compounds from different seaweed extracts, but the level of total sugars was also shown to decrease. A more detailed knowledge about the phenolic substances and pigments extracted will be required ultimately to define the best protocol to isolate/remove them from the crude extracts prior to recovery of the target polysaccharides. From the work conducted, information like the exact types of compound present appeared to be essential (generally conducted by LC-MS analysis) as well as the potential interactions between polyphenols and polysaccharides (NMR analysis), and this could be an objective for future research. The problem of interactions between molecules is an important and complex phenomenon. This work has shown (Chapters 3, 4 and 5), that some interactions were probably at play between polysaccharides, as highlighted by the experiment to remove the alginates from the crude extract by precipitation (Chapter 3) and later in the size fractionation conducted in Chapter 5. Indeed, in using acid or calcium chloride (to generate the two insoluble forms of alginates), other compounds, and especially some fucoidans were also precipitated. More interestingly, the quantity of other compounds precipitated and even their composition were different according to the chemical used.

From these observations, different questions could be asked that should form the basis of future research. Are the fucoidans linked to the alginates? Are insoluble forms of fucoidans generated? Are linkages broken by acidifying the solution? Are fucoidans and alginates (alginates only, or fucoidans only) binding to divalent cations, such as calcium? And given that aqueous-based extraction methods have been used, what, if any, implications
do these observations have for the natural interactions of these molecules, in the as yet poorly understood composition and ultrastructure of the cell walls of brown seaweeds? To answer these questions, a more detailed analysis of the precipitation process has to be investigated in the future, including some HPLC and LC-MS and NMR analysis.

Based on all of the findings, it became clear that a good understanding of the extraction process was essential before investigating further purification steps and prebiotic evaluation. A preliminary investigation on the extraction time showed that different compounds were extracted to varying extents (Chapter 3). To find out more about how to control the extraction process, a design optimisation study based on a single step extraction was done (Chapter 4) to investigate the effects of different parameters such as extraction time, temperature, liquid/biomass ratio, seasonality and biomass particle size on the yield and composition of the extracts. The idea was to control, as much as possible, the extraction process in order to be able to scale-up the extraction (ideally with similar trends with the lab scale) to produce enough material to run a clinical based on the bioactivity results obtained in Chapter 6, but also to determine if extraction could be controlled to generate more reproducible and less variable end-products. All of the parameters investigated are essential to determine in a consistent manner, the relationship between structure/composition and bioactivity.

The investigation of the different extraction parameters was compared to similar published work. Moreover, the initial time course extraction reported in the third chapter, showed that the fucoidan concentration in solution increases at longer extraction times, whereas the mannitol, laminaran and polyphenol concentration remained constant after one hour. By investigating in more detail the influence of extraction duration on the extraction yields and the proportion of each compound in solution, results of the preliminary and more detail experiments were shown to correlate well. Indeed, the fucoidan concentration increased over time and time was shown to statistically influence the extraction yields and the fucoidan content in solution (Chapter 4). Furthermore, for the other compounds mentioned previously, their proportion in solution was generally not statistically influenced by the extraction time. Generally speaking, all extraction yields of the compounds investigated in this work (primarily mannitol, fucoidans, laminarans, alginates and phenolics) showed an increase in eater-extractable levels at longer extraction times, higher temperature and higher ratio of extractant to seaweed biomass.

The statistical analysis of the influence of the extraction parameters on the extraction yields allowed the development of mathematical models to predict, as much as possible, the likely result of the extraction. Nevertheless, differences were noticed between the batches
and a universal mathematical model could not be applied (one model is valid for only one set of experimental conditions: if one parameter changes, even seasonality, another mathematical model should be applied). An extraction is a complex process that can yield a lot of variation between batches, so the parameters have to be controlled as much as possible. For example, the cutting process used should be always the same to avoid variation (Chapter 3 in this work highlighted the difference between industrially cut and manually cut biomass). Seasonality variations were also noticed. The composition of the seaweed changes over time, consequently, so do the extracts. Knowledge of the influence of extraction parameters can compensate to some degree for those natural variations. However, the mathematical models proposed were established for batches using seaweed harvested at a precise time of the year. The next step would be to determine the period of validity of the models. Indeed, is the model established still valid after a week? A month? Two months, etc? Furthermore, will the model established June 2012 still be valid in June 2013? To answer to those questions, the same experiment should be done using seaweed harvested at regular intervals (every week, month) for a period of at least 3 years. Using monthly seaweed harvests during a three year period, this project would deal with 864 extractions which represent a large amount of samples and significant analysis time, but it would answer many questions, and be of special relevance to industry, including companies like OGT who are producing seaweed extracts. Notwithstanding the long term value to industry, the study conducted is to the author’s best knowledge, the first scientific data from a multi-dimensional evaluation of a one-step, aqueous-based extraction process.

The investigation of fucoidan composition has been extensively studied in many different species. Nevertheless, conclusions concerning the real composition/structure/size of fucoidan have still not been elucidated and many differences have been highlighted in terms of fucoidan fractions obtained in these studies. Indeed, fucoidan composition/size is critically depending on many factors like seaweed species, seasonality, localisation, extraction process and even the technique/approach used for the analysis. By combining all of these important parameters, relevant experiments to compare the results to the previous reports was in general very difficult due to the variability that exists in seaweeds, seasonality, and methods used for harvesting, processing and extraction of biomolecules. However, trends could be observed especially in the effect of temperature on fucoidan composition. Indeed, in different published work to-date, the fucose content of fucoidan has been shown to be lower when the extraction was done at higher temperature. This finding was the opposite of the observation made in this research (Chapter 5). This difference could be explained by the number of factors previously cited that would not necessarily have been evaluated in previous reports. Furthermore, the extraction time yielded an increase in the fucose content in extracted fucoidan in this research, but only in the summer batch (Chapter
3, during the time course extraction). Similarly to the approach taken to predict extraction yields, mathematical models have been proposed from this research to “predict” the fucoidan composition but the period of the validity has also to be checked in the further by repeating this experiment at different time points of the year.

The fucoidan sizes reported in this research were also shown to vary (Chapter 5). In this study, extracts were fractionated using an ultrafiltration (UF) system with different MWCO PES membranes. Results obtained could only be considered as trends, as some compounds were retained on the membrane (recoveries between 70 and 80% according the molecular weight cut off used). Nevertheless, the main fraction was shown to be higher than 100 kDa, which corresponded to the most reported sizes in the literature. Furthermore, a fraction above 0.2 µm was also identified for the first time. Such a high molecular weight fucoidan-rich fraction has never been reported, so a hypothesis of the formation of complexes between polysaccharides and polyphenols can be made, as mentioned earlier. The linkage types between polysaccharides or between polysaccharides and polyphenols could be investigated by NMR analysis after isolating each polymer individually. This fraction was more abundant when the extraction time increased and also when the ratio liquid/biomass was lower. This observation could reflect the kinetics of complex formation over time, or more intriguingly, it could reflect the time-dependent extraction of inherent cell wall complexes. The low ratio induced a higher concentration of all compounds in solution, and this too could facilitate complex formation, possibly in a concentration-dependent manner. Interaction between compounds in seaweed extracts is poorly reported so further investigation is required to validate (or not) this hypothesis. Once again, NMR and LC-MS analyses could provide more details on the structure of those complexes. Moreover, the use of specific monoclonal antibodies against fucoidans could yield important information, and this approach is being used in more and more studies on the investigation of seaweed cell wall structure, but as long as the structure is not well defined, some issues could exist regarding the specificity of an antibody. The use of these antibodies, however, could also help understand if the small molecular weight fucoidan extracted is actually present in the seaweed, or if it is the result of degradation of a larger polysaccharide during the extraction process.

The precise quantification of alginates was not achieved in this study despite some trials done to quantify uronic acids in plant extracts, including for example the adaptation of the sulfamic acid method (Melton & Smith, 2001). This protocol included a reagent that contain concentrated sulphuric acid and an incubation step at 100°C, which converted monosaccharides to their furfural forms and a resulted in the formation of a strong brown colour that interfered with subsequent colorimetric detection. Yet, this method appears to
have been used in previous report for the detection of acidic polysaccharides (including pectins). An alternative protocol was investigated that involved use of specific alginate lyase enzymes, isolated and characterised by a French research group in the CNRS “Station biologique de Roscoff”, France. A collaboration was initiated to treat the seaweed extracts produced in Chapters 4 and 5 with the different alginate lyase enzymes. The hypothesis made previously on large molecular weight complex formation was reinforced by the tests using the alginate lyases. Indeed, different fractions were observed after the enzyme digestion of the crude extract and the filtrate at 0.2 µm suggesting the presence of alginates in the fraction larger than the 0.2 µm MWCO. Unfortunately, the final answer (quantification and composition of alginates) could not be completely achieved. However, some interesting results were obtained suggesting a difference of alginate composition according to the extraction protocol used. To investigate more in details the effect of extraction parameters on the alginate composition, a plan for future research has been decided, in collaboration with the group in Roscoff. A series of new experiments will be initiated, including the collection of all fractions separated by HPLC (Chapter 6) for NMR analysis to determine the M/G (mannuronic and guluronic acids) ratio in each fraction.

Finally, the activity of the different seaweed extracts on the growth of specific bacteria of relevance to gastrointestinal-tract mediated human health gave promising results for a prebiotic activity (Chapter 6). In fact, the extraction protocol was shown to be very important in terms of extraction yields, extract and polysaccharides composition. Different compositions induced different bioactivity, and the results have emphasised the importance of being able to control the outcome of the extraction to target specific bioactivities, as well as to be able to reproduce the same sample. The activity of the alginate rich fractions (Alg) was more effective when the extraction was done for a short time at the low ratio of extractant to seaweed biomass. The main parameter identified as influencing the alginate composition (Chapter 5) was the extraction temperature (high temperature appeared to yield alginates with more G blocks than the cold extracted counterparts, which appeared to contain more M blocks). In that case, further investigation is required to make conclusion on the effect of alginate composition on the bacterial growth. Concerning the fucoidan fraction, two different observations were made. First of all, fucoidans extracted at low ratio had been shown to be more effective in selectively promoting bacterial growth (reduction in the growth of potential pathogens and enhanced growth of ‘probiotic’ strain) and the fucoidans extracted at high temperature were shown to significantly reduce the number of live cells in pathogen cultures. The effect of temperature on fucoidan composition influenced all components of the polysaccharide. The cold extracts contained more galactose, rhamnose and sulphate groups than the hot extracts. The fucoidans have been
described to be fucose-rich polymers (backbone) so a higher proportion of the other monosaccharides might reveal more branching on the polymer backbone, which may influence its effect on the bacterial growth (more branching may make the polysaccharide more accessible to the action of bacterial enzymes, thus it could be used as a nutrient source, but increased branching could also cause steric effects that might reduce colony formation). Again, a more detailed analysis of the fucoidan structure is required to validate these hypotheses. Some fractions appeared to act more like an antimicrobial agent than a prebiotic by reducing the growth of all bacteria. Statistical analysis was a key factor to define the best candidate for the clinical trial/dietary intervention study on humans (ongoing). To run the clinical trial, production of the extract had to be scaled up so the extraction yields were also very important. Indeed, some extracts showed very interesting results, especially the fucoidan fractions extracted at low temperature, but the yields were too low to be sustainable for scale up. This preliminary screening showed that a potential interaction occurred between the seaweed extract and the bacteria. More investigations concerning this potential interaction have been initiated by the analysis of the culture at different time points during the incubation. The parameters being studied are the enzyme profile produced by the bacteria to degrade/fragment the seaweed component. In parallel, a detailed analysis of the residual seaweed extract is being done. The next step of this project will be to investigate the 2-D and 3-D interactions between the prebiotic (seaweed extract), probiotic (bacteria)/pathogen and gut cells. Two collaborations have been started with international groups in Germany and Italy that will commence the investigation of these aspects into the future.

References


O’Connell, E. (2008). *Purification and characterisation of an algal polysaccharide (fucoidan) and enzymes involved in modification of this polysaccharide.* National University of Ireland, Galway.
Appendices
Appendix 1: List of materials

All the chemicals used in the laboratory were of standard analytical grade. De-ionised water from the Milli-RX 20, Millipore system (Department of Biochemistry, NUI Galway), was used to prepare solutions.

*Chemicals and research materials were purchased from the following companies:*

**Becton Dickinson (Oxford, UK):** 1 mL syringe

**Dionex, USA:** Anion eluent concentrate; anion standards; CarboPac PA100 column; CarboPac PA100 guard column; CarboPac SA10 column; CarboPac SA10 guard column; IonPac AS22 column; IonPac AS22 guard column

**Hickeys fabric shop (Galway, Ireland):** Muslin

**Lennox Laboratory Supplies, Dublin, Ireland:** Ethanol

**Oxoid Ltd. (Hants, UK):** LB-agar; LB-broth; MRS-agar; MRS-broth

**Sarstedt (Wexford, Ireland):** General laboratory consumables: All pipette tips; 96 well micro test plates used for colorimetric assays; microcentrifuge tube (0.5, 1.5 and 2.0 mL); gloves; sterile centrifuge tubes (15 mL and 50 mL); 0.22 µm PDVF filters

**Sartorius:** vivacon-2 centrifugal units

**Sigma-Aldrich Chemical Co. Ltd. (Dublin, Ireland):** A: Acetic acid, Alcian blue; amberlite resins IRA900, IRC50, XAD2, XAD4, XAD7, XAD16; ammonium chloride C: calcium chloride; carboxymethyl-cellulose; D(+)-cellobiose D: dialysis tubing 10 kDa MWCO; E: ethylcellulose; F: folin-ciocalteu reagent; formic acid; fucoidan; L(+)-fucose; G: D(+)-galactose; D(+)-galacturonic acid; D(+)-glucose; glycerol; H: hydrochloric acid L: lactose; laminaran (*Laminaria digitata*); M: magnesium chloride; magnesium sulphate; manganese sulphate; mannitol; D(+)-mannose, 0.22 µm PVDF syringe filter units; N: neutral red dye P: phenol; phloroglucinol; polygalacturonic acid; potassium hydroxide (50% solution) R: L(+)-rhamnose; S: sodium alginates; sodium carbonate; sodium chloride; sodium hydroxide (pellet form); sodium phosphate dibasic; sodium phosphate monobasic; sulphuric acid; T: trifluoroacetic acid (TFA); X: D-xylose; xylene
Thermo-Fischer: dialysis cassette 2 kDa MWCO; temperature controller; ultrafiltration units (400 mL capacity); ultrafiltration membrane 1, 3, 10, 50 and 100 kDa MWCO
Appendix 2: Monosaccharide standard curves determined by HPAEC

- **Mannitol**
  - Equation: $y = 0.4295x$
  - $R^2 = 0.9899$

- **Fucose**
  - Equation: $y = 0.3374x$
  - $R^2 = 0.9927$

- **Galactose**
  - Equation: $y = 0.5282x$
  - $R^2 = 0.9916$
Rhamnose

$$y = 0.3364x$$
$$R^2 = 0.9928$$

Glucose

$$y = 0.6356x$$
$$R^2 = 0.9882$$

Xylose

$$y = 0.6961x$$
$$R^2 = 0.9934$$
Mannose

\[ y = 0.4806x \]
\[ R^2 = 0.99 \]
Appendix 3: Media used for the bacterial growth

Man, Rogosa and Sharpe media (MRS broth)

MRS is the optimum media to grow lactobacilli strains. In this study, it was used to keep the bacterial culture “alive” before use to inoculate the prebiotic plates. The MRS broth composition is as follow:

<table>
<thead>
<tr>
<th>Typical Formula</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10.0</td>
</tr>
<tr>
<td>‘Lab-Lemco’ powder</td>
<td>8.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>4.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>20.0</td>
</tr>
<tr>
<td>Sorbitan mono-oleate</td>
<td>1 ml</td>
</tr>
<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>2.0</td>
</tr>
<tr>
<td>Sodium acetate 3H2O</td>
<td>5.0</td>
</tr>
<tr>
<td>Triammonium citrate</td>
<td>2.0</td>
</tr>
<tr>
<td>Magnesium sulphate 7H2O</td>
<td>0.2</td>
</tr>
<tr>
<td>Manganese sulphate 4H2O</td>
<td>0.05</td>
</tr>
<tr>
<td>pH 6.2 ± 0.2 @ 25°C</td>
<td></td>
</tr>
</tbody>
</table>

Lysogeny broth (LB)

LB media was used to grow E.coli and P.aeruginosa before inoculating the test plates. The LB composition is:

<table>
<thead>
<tr>
<th>Typical Formula</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>10.0</td>
</tr>
</tbody>
</table>
Minimum media M9

M9 media was used for the prebiotic test. It contains only salts as the minimum requirement to grow bacteria, the carbon source was then only given by the seaweed extracts.

<table>
<thead>
<tr>
<th>Typical Formula</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄, 7H₂O</td>
<td>12.8</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>1.0</td>
</tr>
<tr>
<td>MgSO₄ 1M solution</td>
<td>2 ml</td>
</tr>
<tr>
<td>CaCl₂ 1M solution</td>
<td>1 ml</td>
</tr>
</tbody>
</table>
# Appendix 4: Template of the prebiotic plate layout

<table>
<thead>
<tr>
<th>H</th>
<th>G</th>
<th>F</th>
<th>E</th>
<th>D</th>
<th>C</th>
<th>B</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>water instead of bacteria</td>
<td>114(\mu)l mix (Media + water) + 66(\mu)l Sample\textsuperscript{11} 0.3% + 20(\mu)l bacteria</td>
<td>water instead of bacteria</td>
<td>114(\mu)l mix (Media + water) + 86(\mu)l water</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>water instead of bacteria</td>
<td>114(\mu)l mix (Media + water) + 66(\mu)l Sample\textsuperscript{12} 0.3% + 20(\mu)l bacteria</td>
<td>water instead of bacteria</td>
<td>114(\mu)l mix (Media + water) + 66(\mu)l water + 20(\mu)l bacteria</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>water instead of bacteria</td>
<td>114(\mu)l mix (Media + water) + 66(\mu)l Sample\textsuperscript{13} 0.3% + 20(\mu)l bacteria</td>
<td>water instead of bacteria</td>
<td>114(\mu)l mix (Media + water) + 66(\mu)l Sample\textsuperscript{1} 0.3% + 20(\mu)l bacteria</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>water instead of bacteria</td>
<td>114(\mu)l mix (Media + water) + 66(\mu)l Sample\textsuperscript{14} 0.3% + 20(\mu)l bacteria</td>
<td>water instead of bacteria</td>
<td>114(\mu)l mix (Media + water) + 66(\mu)l Sample\textsuperscript{2} 0.3% + 20(\mu)l bacteria</td>
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**Comments:** Mix media + water = 1.1ml, 2x media + 1.5ml water final extract concentration 0.1% (0.3% extract solutions were used).

Plate number: 

Date: 

| Strain: D (625) bacteria at T0 = |

---

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Appendix 5: Calculation of polysaccharide composition

After the run of a sample through the HPLC system, the software (chromeleon) creates a sample analysis containing the information concerning the sample (name, sequence) and the analytic protocol (program ran, quantification method used, volume injected, dilution…). The chromatogram shows the separation of the different compounds detected and a summary table of the results is displayed. The retention time of the peaks allow identifying the compounds according to the standard ran in the same sequence. The
peak area is used to quantify the concentration of the compounds according to the standard. The relative shows the proportion of each peak according to the sum of all area. Finally, the amount is calculated taking into consideration the peak area and the dilution factor. The final result is expressed as the compound concentration (in µg/ml) in the extract.

To calculate the fucoidan concentration, the concentration of fucose, galactose, rhamnose, xylose and mannose is summed.
### Appendix 6: Complete dataset for the influence of extraction parameters on extraction yields and crude extract compositions

#### Appendix 6-A: Effect of extraction parameters on compound extraction yields in the first batch and calculation of confidence intervals

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| variance $s^2$ | 0.004 | 0.023 | 0.004 | 0.000 | 0.002 |
| interval       | 0.28  | 0.66  | 0.26  | 0.08  | 0.21  |
### EFFECT OF INDIVIDUAL PARAMETERS AND INTERACTIONS THEREOF (% seaweed DW) vs Compound extraction yields (% seaweed DW)

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</table>

| Ash           | 2.63 | 0.25 | 0.41 | 0.14 | 0.16 | 0.10 | -0.07 | -0.02 |
| Phenolics     | 1.85 | 0.12 | 0.16 | 0.13 | -0.05 | 0.03 | 0.06 | 0.02 |
| Total sugars  | 4.90 | 0.09 | 0.83 | 0.50 | 0.36 | 0.13 | -0.07 | 0.31 |
| Mannitol      | 1.52 | -0.23 | 0.26 | 0.45 | -0.19 | -0.04 | -0.04 | -0.02 |
| Fucoidans     | 0.75 | 0.19 | 0.29 | 0.06 | 0.10 | 0.03 | 0.00 | -0.01 |
| Laminarans    | 2.28 | 0.06 | 0.26 | 0.30 | -0.07 | 0.06 | -0.06 | -0.01 |

### Standard deviation $s$ and confidence intervals (% seaweed DW)

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<th>Total sugars</th>
<th>Mannitol</th>
<th>Fucoidans</th>
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variance $s^2$ 0.002 0.000 0.005 0.002 0.000 0.003

interval 0.19 0.05 0.30 0.19 0.06 0.24
Appendix 6-C: Effect of extraction parameters on the compound extraction yields in the third batch and calculation of confidence intervals

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| Ash (%)       | 3.41 | 0.22 | 0.23 | 0.25 | 0.07 | 0.00 | -0.05 | 0.05  |
| Phenolics (%) | 3.10 | 0.15 | 0.15 | 0.25 | -0.07 | 0.00 | 0.07   | 0.00  |
| Total sugars (%) | 5.26 | 0.66 | 1.22 | 0.56 | 0.36 | 0.24 | 0.20   | 0.21  |
| Mannitol (%)  | 1.96 | -0.08 | 0.55 | 0.17 | -0.02 | 0.05 | 0.24   | 0.01  |
| Fucoidans (%) | 0.99 | 0.19 | 0.38 | 0.07 | 0.13 | -0.01 | 0.03   | 0.03  |
| Laminarans (%) | 2.37 | -0.02 | -0.40 | 0.52 | -0.12 | 0.02 | -0.17  | -0.04 |

<table>
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<tr>
<th>Extraction N°</th>
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<th>Phenolics (%)</th>
<th>Total sugars (%)</th>
<th>Mannitol (%)</th>
<th>Fucoidans (%)</th>
<th>Laminarans (%)</th>
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| variance s²   | 0.001   | 0.001         | 0.010            | 0.000        | 0.000         | 0.001         |
| interval      | 0.16    | 0.12          | 0.43             | 0.09         | 0.09          | 0.16          |
## Appendix 6-D: Effect of extraction parameters on the extract composition in the first batch and calculation of confidence intervals

### EFFECT OF INDIVIDUAL PARAMETERS AND INTERACTIONS THEREOF (% extract DM)

<table>
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<tr>
<th>Extraction Nº</th>
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<th>A</th>
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<th>C</th>
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<th>Laminaranas</th>
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### Standard deviation $s$ and confidence intervals (% extract DM)

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<th>Extraction Nº</th>
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<th>Phenolics</th>
<th>Total sugars</th>
<th>Mannitol</th>
<th>Fucoidans</th>
<th>Laminaranas</th>
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Variance $s^2$:

| Interval     | 1.08 | 2.68 | 1.33 | 0.27 | 1.09 |


Appendix 6-E: Effect of extraction parameters on the extract composition in the second batch and calculation of confidence intervals

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<th>Ash (%)</th>
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<th>Total Sugars (%)</th>
<th>Mannitol (%)</th>
<th>Fucose (N)</th>
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Standard deviation $\sigma$, and confidence intervals (% extract DMI)

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Table: Effect of individual parameters and interactions thereof (% extract DMI)

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<th>% extract DMI</th>
<th>% extract DMI</th>
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</tr>
<tr>
<td>Total Sugars</td>
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<td>-0.2</td>
</tr>
<tr>
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<td>-0.2</td>
</tr>
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<td>Fucose (N)</td>
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<td>Laminaran</td>
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EFFECT OF INDIVIDUAL PARAMETERS AND INTERACTIONS THEROF (% extract DMI)

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<th>Mannitol</th>
<th>Fucose (N)</th>
<th>Laminaran</th>
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### EFFECT OF INDIVIDUAL PARAMETERS AND INTERACTIONS THEREOF (% extract DM)

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<th>B</th>
<th>C</th>
<th>AB</th>
<th>AC</th>
<th>BC</th>
<th>ABC</th>
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<td>+</td>
</tr>
</tbody>
</table>

| Ash  | 16.11 | 0.26 | -0.09 | 0.04 | 0.32 | 0.02 | -0.49 | 0.21 |
| Phenolics | 14.63 | 0.08 | -0.36 | 0.12 | -0.29 | 0.02 | 0.06 | 0.05 |
| Total sugars | 24.46 | 1.85 | 3.82 | 0.72 | 1.34 | 0.37 | 0.12 | 0.82 |
| Mannitol | 9.12 | -0.81 | 1.88 | 0.03 | -0.12 | 0.34 | 0.05 | 0.03 |
| Fucoi dans | 4.50 | 0.66 | 1.46 | 0.00 | 0.49 | -0.09 | -0.06 | 0.11 |
| Lam ina rans | 11.29 | -0.58 | -2.68 | 1.74 | -0.39 | 0.11 | -0.92 | -0.10 |

### Compound percentage in extract (% extract DM)

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<th>Mannitol</th>
<th>Fucoi dans</th>
<th>Laminar ans</th>
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</table>

### Standard deviation $s$ and confidence intervals (% extract DM)

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<th>Total sugars</th>
<th>Mannitol</th>
<th>Fucoi dans</th>
<th>Laminar ans</th>
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<td>0.71</td>
</tr>
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</tr>
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<td>4.74</td>
<td>0.39</td>
<td>0.66</td>
<td>1.03</td>
</tr>
</tbody>
</table>

| Variance $s^2$ | 0.022 | 0.017 | 0.238 | 0.024 | 0.004 | 0.020 |
| Interval       | 0.64  | 0.56  | 2.11  | 0.67  | 0.27  | 0.61  |
### Proposition of equation model for the extraction yields (% seaweed dry weight)

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| Fucose        | 38.35| 0.64| 3.60| -0.72| 1.19| 0.11| 0.28| 0.03|       |           |          |         |         |
| Galactose     | 17.85| 0.12| -3.47| 1.03| -0.11| 0.08| 0.05| -0.06|       |           |          |         |         |
| Rhamnose      | 4.56 | -0.18| -1.94| -0.17| -0.28| -0.68| -0.41| 0.20|       |           |          |         |         |
| Xylose        | 19.87| 0.26| 5.30| -0.81| 0.27| 0.06| 0.14| -0.02|       |           |          |         |         |
| Mannose       | 19.37| -0.84| -3.49| 0.66| -1.06| 0.43| -0.05| -0.15|       |           |          |         |         |

### Effect of extraction parameters on monosaccharide composition of June batch fucoidan (% w/w total fucoidan)

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| Fucose        | 43.10| 1.70| 2.64| 0.27| 0.75| -0.13| 0.23| 0.24|       |           |          |         |         |
| Galactose     | 13.46| -0.06| -2.04| -0.26| -0.57| -0.14| 0.06| 0.20|       |           |          |         |         |
| Rhamnose      | 2.52 | -0.32| -0.94| 0.13| 0.11| -0.09| -0.11| 0.12|       |           |          |         |         |
| Xylose        | 24.72| -0.10| 3.32| -0.18| -0.29| -0.42| -0.09| -0.41|       |           |          |         |         |
| Mannose       | 15.97| -1.44| -3.20| -0.18| -0.23| 0.55| -0.32| -0.38|       |           |          |         |         |
Appendix 9: Growth curves and complete dataset for the statistical analysis of bacterial growth on seaweed extracts

Appendix 9-A: data on bacterial growth on the S1 fractions
Pseudomonas aeruginosa

**Graph:**
- O.D. at 595nm vs Time (hrs)
- Lines represent different conditions:
  - Ctl
  - No extract
  - Glucose
  - EXT1 S1
  - EXT2 S1
  - EXT3 S1
  - EXT4 S1
  - EXT5 S1
  - EXT6 S1
  - EXT7 S1
  - EXT8 S1

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<td>2 (6.8)</td>
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<td>12 (8-20)</td>
<td>12 (8-20)</td>
<td>12 (8-20)</td>
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**Notes:**
- Average of standard deviations
- Column titles indicate phases:
  - Lag phase
  - Log phase
  - Stationary phase
- Colony counts (x10^6 cfu/mL)
Lactobacillus plantarum

![Graph showing growth curves of different conditions over time]

### Lactobacillus plantarum

<table>
<thead>
<tr>
<th>Glucose</th>
<th>EXT 1</th>
<th>EXT 2</th>
<th>EXT 3</th>
<th>EXT 4</th>
<th>EXT 5</th>
<th>EXT 6</th>
<th>EXT 7</th>
<th>EXT 8</th>
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<td>Average of standard deviations</td>
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<td>Log phase</td>
<td>Stationary phase</td>
<td>Colony counts (x10^6 cfu/mL)</td>
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<td></td>
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<tr>
<td>Duration (start - end)</td>
<td>Duration (start - end)</td>
<td>Duration (start - end)</td>
<td>ΔOD</td>
<td>ΔOD</td>
<td>ΔOD</td>
<td>ΔOD</td>
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<td>7 (4 - 11)</td>
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<td>7 (3 - 10)</td>
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<td>8b,c</td>
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<td>0.000</td>
<td>0.077</td>
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<td>7 (3 - 11)</td>
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<td>0.053</td>
<td>5 (5 - 10)</td>
<td>0.346</td>
<td>10 (10 - 20)</td>
<td>0.026</td>
<td>3e</td>
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<tr>
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<td>6 (0 - 6)</td>
<td>0.023</td>
<td>5 (6 - 11)</td>
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Appendix 9-B: data on bacterial growth on the alginate rich fractions (Alg)
**Lactobacillus plantarum**

![Graph showing growth curves for Lactobacillus plantarum with different conditions: ctI, no extract, glucose, EXT1 Alg, EXT2 Alg, EXT3 Alg, EXT4 Alg, EXT5 Alg, EXT6 Alg, EXT7 Alg, and EXT8 Alg.](image)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Average of standard deviations</th>
<th>Lag phase</th>
<th>Log phase</th>
<th>Stationary phase</th>
<th>Colony counts (x10^6 cfu/mL)</th>
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</thead>
<tbody>
<tr>
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<td>Duration (start – end)</td>
<td>ΔOD</td>
<td>Duration (start – end)</td>
<td>ΔOD</td>
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<td>0.049abc</td>
<td>7 (4.11)</td>
<td>0.501a</td>
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<td>0.007d</td>
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<td>0.008d</td>
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<td>0.006d</td>
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<td>6 (3.9)</td>
<td>0.361cd</td>
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</table>
Appendix 8-C: data on bacterial growth on the fucoidan rich fractions (Fuc)

![Graph of bacterial growth over time](image)

<table>
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<th>Glucose</th>
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<th>EXT 2</th>
<th>EXT 3</th>
<th>EXT 4</th>
<th>EXT 5</th>
<th>EXT 6</th>
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<th>EXT 8</th>
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<td>0 - 1</td>
<td>0 - 1</td>
<td>0 - 1</td>
<td>0 - 1</td>
<td>0 - 1</td>
<td>0 - 1</td>
<td>0 - 1</td>
</tr>
<tr>
<td>0.028&lt;sup&gt;c,d&lt;/sup&gt;&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.024&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.037&lt;sup&gt;c,d&lt;/sup&gt;&lt;sup&gt;;&lt;/sup&gt;</td>
<td>0.045&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>0.052&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;;&lt;/sup&gt;</td>
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<td>0.018&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.054&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<td>1 - 10</td>
<td>1 - 6</td>
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<td>0.932&lt;sup*&lt;/sup&gt;</td>
<td>0.489&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.516&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>0.448&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.524&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.403&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.522&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.420&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>0.573&lt;sup&gt;d&lt;/sup&gt;</td>
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*Note: The values are given as average of standard deviations. The letters indicate statistical significance across the different fractions.
Lactobacillus plantarum

![Graph showing growth curves for different conditions.

<table>
<thead>
<tr>
<th>Glucose</th>
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<th>EXT 2</th>
<th>EXT 3</th>
<th>EXT 4</th>
<th>EXT 5</th>
<th>EXT 6</th>
<th>EXT 7</th>
<th>EXT 8</th>
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<td>ΔOD</td>
<td>ΔOD</td>
<td>ΔOD</td>
<td>ΔOD</td>
<td>ΔOD</td>
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<td>0.563</td>
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</tr>
<tr>
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<td>0.003</td>
<td>0.041</td>
<td>0.004</td>
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<td>41.0</td>
<td>28.7</td>
<td>23.9</td>
<td>77.8</td>
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Average of standard deviations

Colony counts (x10^6 cfu/mL)