Thesis for the Degree of Doctor of Engineering

Optimization and Characterization of Bioactive Components from *Saccharina japonica* using High Pressure Separation Technologies

by

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The Graduate School

Pukyong National University

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고압분리공정을 이용한 다시마로부터 유용물질의 추출공정 최적화 및 생리활성

Advisor: Prof. Byung-Soo Chun

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Abstract

Saccharina japonica (*Laminaria japonica*), a popular marine vegetable, has been used as a popular therapeutic agent for phlegm elimination, detumescence and weight loss for more than one thousand years. These brown seaweeds are rich in bio-functional materials. Many studies have suggested that fucoxathin and fucoidan were the main active components in *S. japonica*, exhibiting a wide range of pharmacological properties, such as anti-inflammation, anti-virus, anti-hypertensive, anti-coagulant, anti-cancer, anti-oxidant and anti-atherosclerosis etc. For these purposes, green technology process were used to extract the above mentioned valuable materials.

In the first study, Sunflower oil (SFO), soybean oil, canola oil, ethanol, and water were utilized as co-solvents to support supercritical carbon dioxide (SC-CO_2) extraction of total carotenoid (TC), fucoxanthin (FX), and phlorotannin (PT) from brown seaweed (*S. japonica*). A steepest ascent method with various temperatures [45–55°C], pressures [200–300 bar], and co-solvent flow rates [0.50–2.00 (% of CO_2, w/w)] was used to define the best operative co-solvents to enhance the extraction yields followed by response surface methodology (RSM) to optimize the extraction conditions. The best conditions for the yield of TC and FX were 50.62°C, 300 bar, and 2.00% of SFO, while for PT it was 48.98°C, 300 bar, and 2.00% of water, respectively. Overall extraction curves were determined for the optimized conditions, and experimental data were used to estimate the kinetic parameters. SFO as co-solvent showed higher fatty acid content, antioxidant activity, and oil stability than the control (SC-CO_2 only).

In the second study, *S. japonica* was treated with pressurized hot water extraction (PHWE) at a temperature of 180°C–420°C and pressure between 13 bar and 520 bar. The obtained hydrolysate were investigated for their yield, total organic carbon (TOC), pH, and Maillard reaction products, and viscosity, color, amino acid, mineral, and monosaccharide content. The extraction yield increased with an increase in temperature and varied from 72.21% to 98.91%. TOC, pH, and potassium and sodium content increased, whereas viscosity decreased, with an increase in temperature. Essential amino acids such as valine and lysine and non-

essential amino acids such as aspartic acid, glutamic acid, glycine, and tyrosine recovered well at low temperature. The content of heavy metals such as arsenic, cadmium, mercury, and lead was very low in the obtained hydrolysate. The maximum amount of total amino acids was recovered at 180°C/13 bar (761.95 ± 14.54 mg/g). The level of main monosaccharides such as glucose (6.70 g/L), fructose (8.40 g/L), and mannitol (17.50 g/L) was found to be very high at 180°C/13 bar. The results indicated that the pressurized hot water extract of S. japonica has good potential for use in the fermentation industry and can be used as human food.

In the third study, Pressurized liquid extraction (PLE) was utilized to extract sulfated polysaccharides (fucoidan) from brown seaweed *S. japonica*. Various conditions of temperature (80–200 °C), pressure (5–100 bar), and solvents (water, 0.1% sodium hydroxide, 0.1% formic acid, 70% ethanol, 50% ethanol, and 25% ethanol) were assessed; the best crude fucoidan (CF) yield was 8.23%, obtained from 140 °C and 50 bar (sodium hydroxide). Compositional analysis, FT-IR, molecular weight, monosaccharides, TGA, UV–Vis, XRD, and elemental analysis confirm that extracted polysaccharides revealed the features of fucoidan. Fucose was the main monosaccharide present in CF obtained by various solvent systems. All CF showed moderate antioxidant, antimitotic and anticancer activities. This study demonstrates that PLE is an efficacious method for enhancing the yield of polysaccharides from *S. japonica* and that it could be a potential source of natural antioxidants and anticancer agent.

Chapter 1

General Introduction

1.1. Background of Seaweed

Seaweeds (marine macroalgae) are sessile multicellular photosynthetic eukaryotes that are differentiated from plants by their lack of specialized tissues (i.e. root system and vascular structure) (S. Egan et al., 2013). Seaweed can be collected from the wild but is now increasingly cultivated also (McHugh, 2003). On a global scale 23.8 million tonnes of seaweed was cultivated in aquaculture in 2012 according to the United Nations Food and Agriculture Organization (FAO) Fisheries and Aquaculture (2014). Almost all of the production was from Asia, in which China contributed 12.8 million tonnes of the total. China is by far the largest seaweed producer followed by the Republic of Korea and Japan but seaweeds today are produced in all continents (McHugh, 2003). The industry is in rapid growth with 10.4% production growth from 2010 to 2011. Use of seaweed as food is a common tradition in Asian countries such as China, Japan and the Republic of Korea (McHugh, 2003). It is also an ingredient for the global food and cosmetics industries. Today, approximately 1 million tonnes of wet seaweed are harvested and extracted to produce about 55,000 tonnes of hydrocolloids, valued at almost US$ 600 million (McHugh, 2003). Phycocolloids as alginate, agar and carrageenan extracted from brown and red seaweeds are being used as thickening and gelling agents in various products (Jensen, 1993).

Seaweeds can also be used as soil fertilizer and in animal feed and its application in the production of biofuel is also being explored (Kraan, 2013). Seaweed falls into three broad groups based on pigmentation; brown, red and green (McHugh, 2003). Among these group the common brown seaweed (Kelp) *Saccharina japonica* is the number one aquaculture species worldwide. *S. japonica* inhabits rocky coasts in Far East Asia, including republic of Korea, China, and Japan (E.-Y. Kim et al., 2011). It has been cultured in these countries since the 1970s and thalli of the seaweed are a popular health food. FAO has reported that about 4 million tons of *S. japonica* were produced worldwide in 2007 and 98 % were generated by aquaculture (JianSan & JiaXin, 2001). The amount of *S. japonica* produced by farming in 2013 amounted to 373,264 t (wet weight); an additional 4 t (wet weight) were collected from natural populations in Korea.

1.2. Seaweed production and value worldwide

According to the Food and Agriculture Organization (2002), a review of the global production and value of brown, red and green seaweeds used in aquaculture from 1991-2000 showed brown seaweeds consistently remaining as the most voluminous and profitable seaweeds produced in aquaculture (Fig. 1.1). Brown seaweeds comprised approximately double the tonnage and value of red seaweeds, while green seaweeds had the least production in comparison. The volume of brown seaweed production increased dramatically from 1991-1993, and remained relatively constant through to 1998, with a tonnage of around 5-

5.5 million (wet tonnes) at the end of 2000. However, the commercial value of brown seaweed remained constant at around US$ 1.8 billion. The volume and value of these seaweeds are largely constituted of *Saccharina japonica* followed by *U. pinnatifida* from China (FAO, 2002).

Fung (2012) reported that brown seaweed production remained the highest among cultured seaweeds; *Saccharina japonica* (4.8 million tonnes) followed by *U.pinnatifida* (1.8 million tonnes). The second highest production of cultured seaweeds are red seaweeds; *Eucheuma* seaweeds (*Kappaphycus alvarezii and Eucheuma spp*., 3.8 million tonnes), *Porphyra spp*., (1.4 million tonnes) and *Gracilaria spp*. (1.4 million tonnes). It is apparent that the demand for brown seaweeds remained high and profitable over the past 20 years (1991-2008) and would most likely continue to do so in the following years. Algae can achieve high productivity with no agricultural inputs of arable land, freshwater and fertilizers, amenability for efficient depolymerisation, etc. There is a worldwide research effort being directed to develop macroalgae as a biofuels feedstock by making required technological innovations in both cultivation technology and biomass conversion process technology for biofuel production at the needed scale. Earlier studies on production of biofuels from marine macroalgal resources have followed typical conventional practices of hydrolysis of biomass combining both chemical pretreatment and saccharification of biomass to sugars and their fermentation.

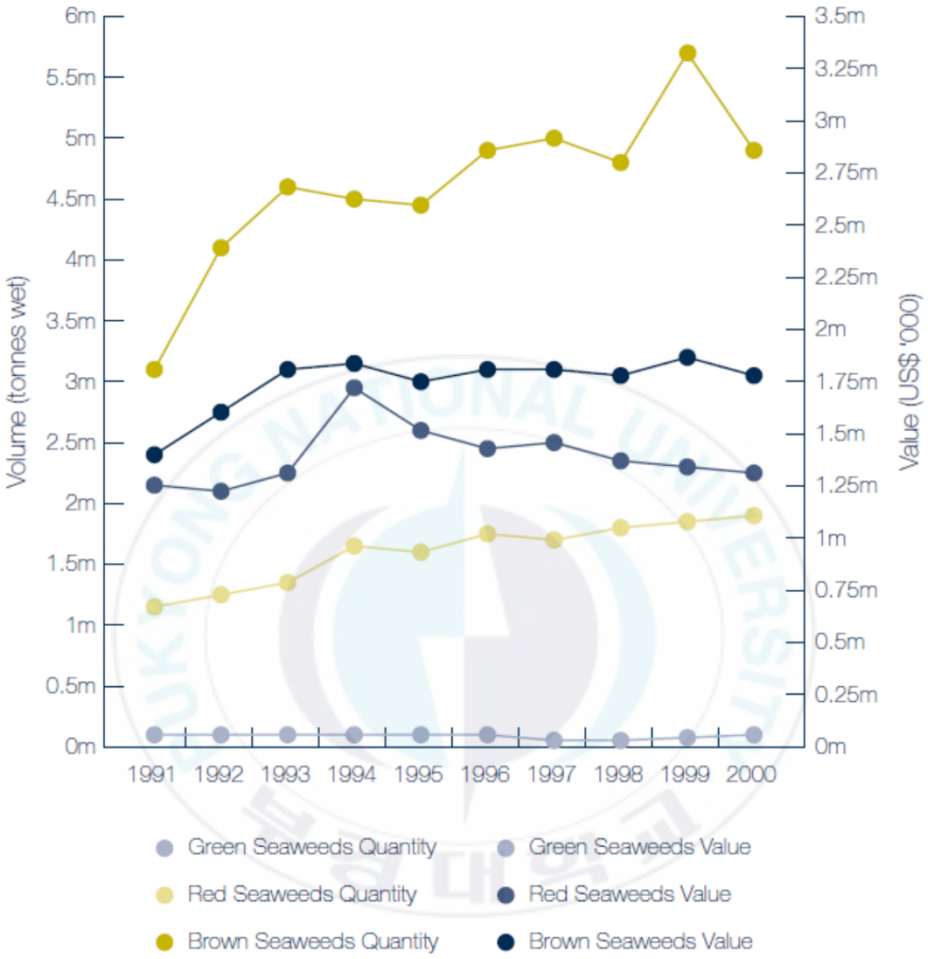

Fig. 1.1. Graph depicting brown, red and green seaweed global production and values in aquaculture in 1991-2000. Green seaweed value is minimal, so the data is not shown in this graph (adapted from FAO, 2002).

1.3. *Saccharina japonica*

1.3.1. The biology and classification of the kelp *Saccharina japonica*

Kelp of the class phaeophyceae, order laminariales contains several commercially important species within seven different families. All of the seaweeds within the order have the same basic life cycle and can be cultivated using the same techniques. There are different species of *Saccharina*; sugar kelp (*Saccharina latissima*), the horsetail kelp (*Laminaria digitata*), the winged kelp (*Alaria esculenta*) and *S. japonica*. *S. japonica* is cold-water seaweed, found in cold temperate and polar climates worldwide with a classification of family mentioned in Table. 1.1 (Redmond et al., 2014). Kelps inhabit the upper, mid, lower sublittoral zones down to depths of up to 50 meters, given sufficient light penetration (Redmond, Green, Yarish, Kim, & Neefus, 2014). *Saccharina japonica* is able to withstand strong currents and high energy environments with a branched holdfast structure. This structure superficially resembles the root structure of a land plant, but functions only to secure the plant. A strong and flexible stipe (i.e. stems for land plants) allows the blades to move with wave currents and lay flat during very low tides.

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Table 1.1. Classification of *Saccharina japonica*

Kingdom:	Chromalveolata
Phylum:	Heterokontophyta
Class:	Phaeophyceae
Order:	Laminariales
Family:	Laminariaceae
Genus:	*Saccharina*
Species:	*S. japonica*

the water, and are able to transport nutrients and the products of photosynthesis up and down of the blade. New growth of the blade occurs at the base of the blade, directly above the stipe, an area called the intercalary meristem (Fig. 1.2.) (Redmond et al., 2014).

S. japonica has a seasonal cycle of growth and reproduction, in which the combined influences of light, photoperiod, and temperature control its growth and development (Yarish & Kirkman, 1990). It has a winter growing season with much of the growth occurring in spring with increasing temperatures and light levels. The growing optimum temperature ranges between 10-15 °C (B. Egan & Yarish, 1990). All kelps including *S. japonica* have a two part life cycle, called a heteromorphic life cycle, consisting of a large visible stage (i.e. sporophyte phase), and a microscopic stage (i.e. gametophyte phase) (Fig. 1.3). The large fronds that are found in subtidal zones are called sporophytes, because when they are mature, a reproductive section of their blade (sorus tissue) (Figure 1.2) will produce and release microscopic spores into the water column (Van Patten & Yarish, 1993). Sorus tissue is a darkened, thickened portion that forms on the blade (*Saccharina*) (Fig. 1.2). Spore producing cells within the sorus tissue undergo meiosis to produce genetically distinct spores. When mature sorus tissue release billions of haploid, biflagellated meiospores (zoospores) usually settle within 48 hours. Settled spores will adhere to a substratum and initiate the formation of the microscopic stage. This is known as the gametophyte stage because the spores will develop into microscopic male or female gametophytes, which represents

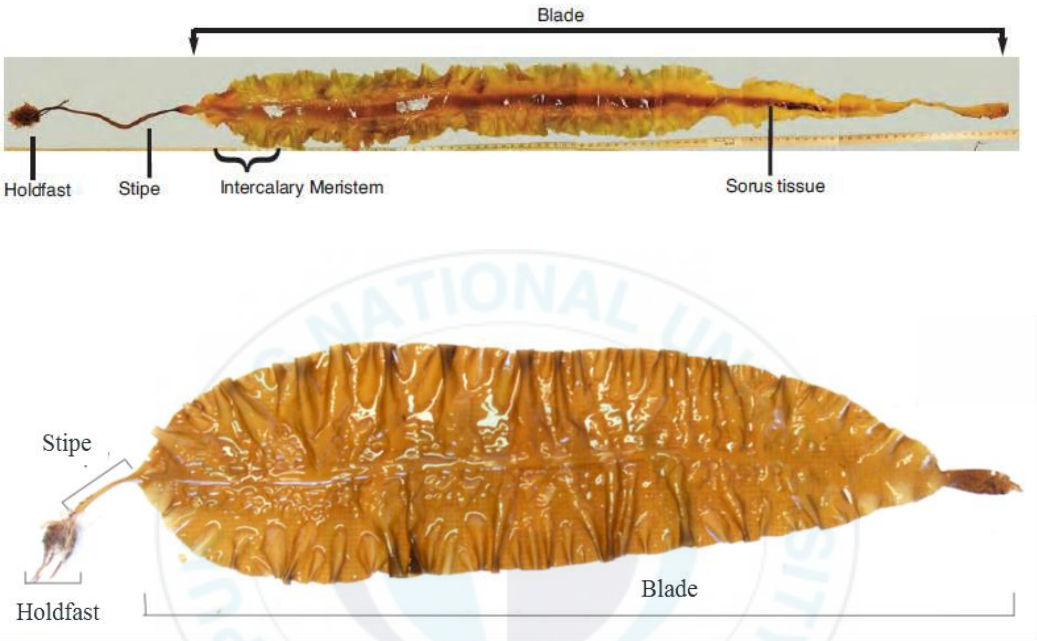

Fig. 1.2. Names of tissue parts of the kelp *Saccharina japonica*.

the sexual phase (Redmond et al., 2014).

The attached meiospore develops a germ tube and pushes all of the contents of the original spore into the first cell, called the primary cell. The vegetative growth phase then increases the primary cell size and new cells are added to form microscopic filaments that can be from 13 cells to hundreds of cells in size (Redmond et al., 2014). Then, gametophytes mature and the reproductive phase will produce oogonia and antheridia potentially in any cell of the separate female and male gametophytes, respectively (Van Patten & Yarish, 1993; Yarish & Kirkman, 1990). After the egg is fertilized, the diploid zygote divides to develop into the small juvenile sporophyte, usually developing directly on the female gametophyte. Small rhizoids are produced basally as the blade expands, and these will develop into the holdfast that anchors the blade to the substrate (Figure 1.3) (Redmond et al., 2014). Kelps grow naturally in littoral zones where they experience periodical tide and ebb, leading to distinct environmental variations in salinity, moisture and light. The light spectral distribution changes at different depths in seawater. At low tide, kelps grow under water surface where the light spectrum is similar as in air. At high tide, kelps grow under seawater of 2–5 m in depth, where blue light becomes predominant. It is logically deduced that kelps have evolved a sophisticated mechanism to adapt to the environmental light changes, especially in their photosynthetic metabolism and stress-induced responses. It has been well documented that several aspects in the *Saccharina/Laminaria* lives are affected by BL, such as photosynthesis of sporophytes and reproduction of gametophytes.

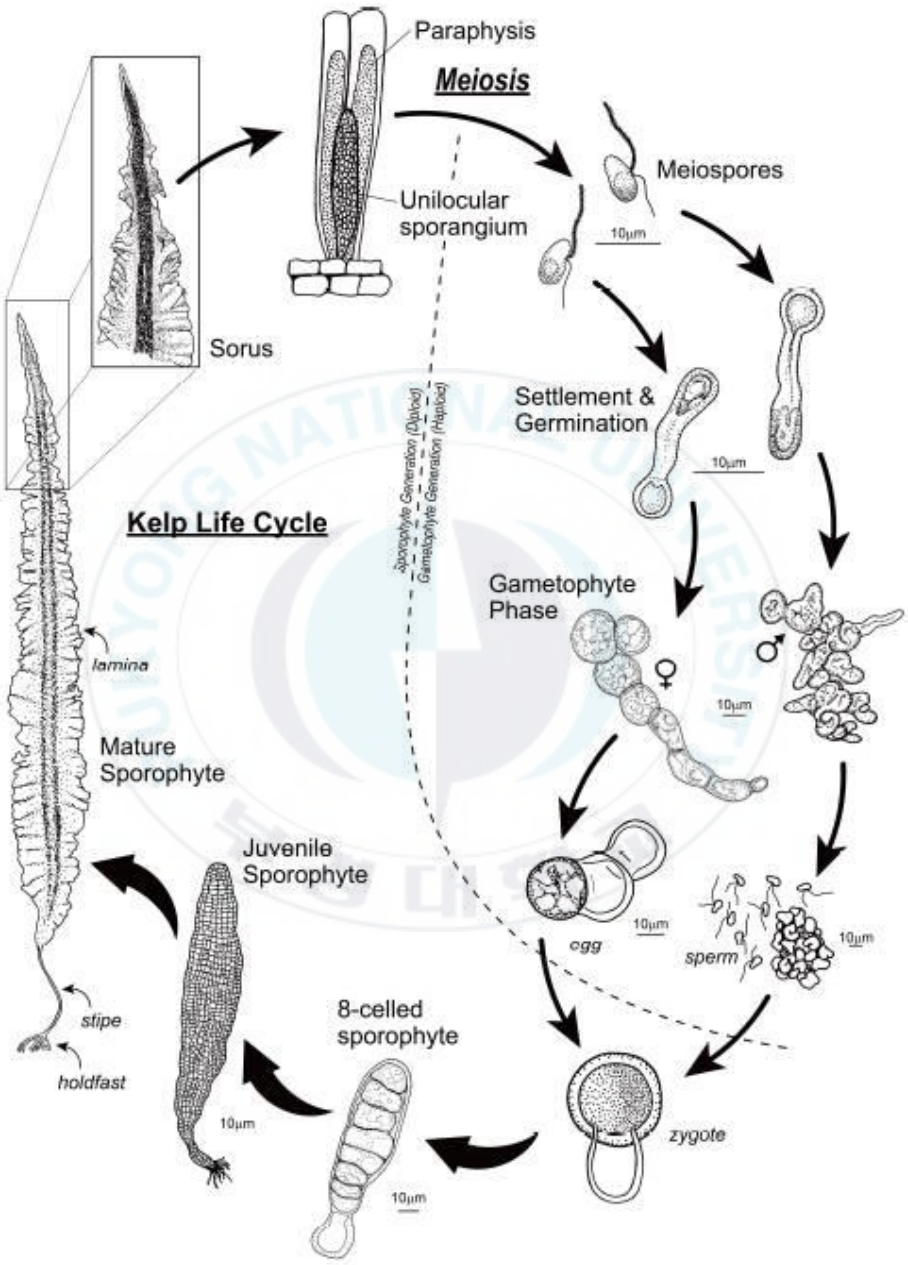

Fig. 1.3. The kelp life cycle (Redmond et al., 2014).

1.3.2. Global production of *S. japonica*

S. japonica has a potential economic value both as a food source and to the pharmaceutical industry. According to FAO data, China was responsible for 89 percent of global farmed Japanese kelp in 2002 (Fig.1.4.). The next largest producer was DPR Korea (9.4 percent). Over the decade 1993-2002, global production peaked in 1999 (4,917,788 tonnes). Current production in DPR Korea in 2002 was less than half that estimated in 1993. For example, China produced 22.3 tonnes of Japanese kelp in 1952 but by 1958 it had jumped to 6253 tonnes (dry weight). By 2000 Chinese production had reached over 4 million tonnes/yr (wet weight). At present, about 50 percent of the dried kelp in China is used for processing into algin, mannitol, and iodine; the rest is mostly destined for the domestic market, but a small amount of processed kelp is exported to Japan and Taiwan Province of China as a marine vegetable (D.-S. Kim, Lee, Cho, Kim, & Pyeun, 1995)

In Japan, the emphasis has been on increasing the production of Laminaria which grow naturally, as this is sufficient to satisfy domestic consumer demand. There are some obstacles that inhibit a Laminaria cultivation in Japan, especially the fact that the plant takes 2 years to grow into a desirable market product for Japanese consumers (Andresen, Skipnes, Smidsrod, Ostgaard, & Hemmer, 1977). DPR Korea is the second largest producer in the world. There, farmed kelp is ground into powder for nutrient supplements. Apart from China, Japan, DPR Korea, RO Korea, and Russia are net consumers of Japanese kelp (FAO, 2011).

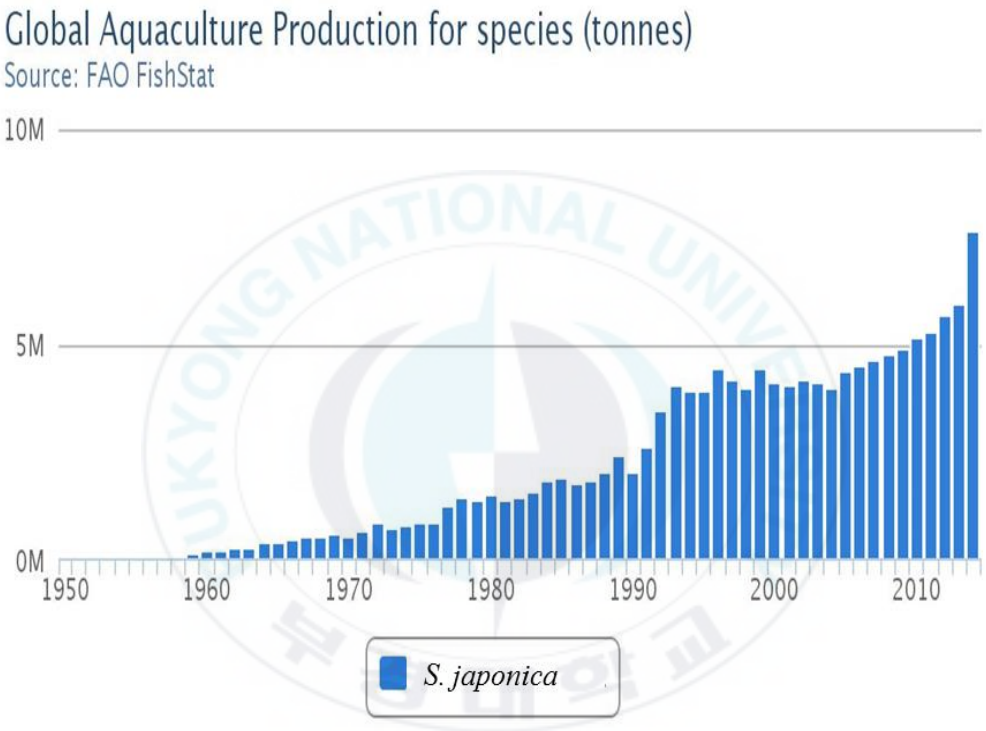

Fig. 1.4. Global production for *S. japonica* from 1950-2014 (adapted from FAO, 2014).

1.4. Fatty acids

Fatty acids are another class of compounds found in seaweed. Fig. 1.5.1. shows a typical fatty acid found in seaweed in the form of a triglyceride. Fatty acids of seaweeds generally have chain lengths of between 14-20 carbon atoms with an even number of carbon atoms and usually contain one or two double bonds (Miyashita, Mikami, & Hosokawa, 2013; Shameel, 1990). In the commonly used system of nomenclature for fatty acids, the position of the double bond is counted from the terminal end of the chains, denoted by the Greek symbol omega (ω), as shown in Fig. 1.5.2. Hence, the term 'omega-3', written as ω3.

Seaweeds have been found to contain high proportions of ω3 and as a result are of value as a nutritional supplement (Sánchez-Machado, López-Cervantes, López-Hernández, & Paseiro-Losada, 2004). The total lipid content of seaweeds is generally 1-5 % dry wt (Arao & Yamada, 1989). However, some studies have reported considerably higher values, such as the *Sargassu. horneri* and *Cystoseira. hakodatensis*, to have a total lipid content of 15 % dry wt (Nomura et al., 2013). The seaweed lipid content has been found to vary by species, geographical location, season, temperature, salinity and light intensity (Nelson, Phleger, & Nichols, 2002). This may be because the cold water species have greater need for a higher lipid content, which serves an energy reserve throughout the colder months. There is increasing interest in the potential for seaweed lipids to be used as a source of biofuel, which is driving investment in seaweed farming technology (Wei, Quarterman, & Jin, 2013).

Seaweeds have been found to contain polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (Fig. 1.5.3) (Khotimchenko, Vaskovsky, & Titlyanova, 2002). The double bonds are cis and are separated by a methylene (CH2) group unless stated otherwise. This can be written as C20:5ω3, which means a chain of 20 carbon atoms, with 5 double bonds, with the first double bond at the ω3 position (Pullen & Saeed, 2012).These PUFA are essential for human and animal nutrition. Seaweeds have a higher proportion of PUFA than terrestrial plants.51 The two classes of PUFA (ω3 and ω6) have opposing physiological functions and their balance is important for normal growth and development (Balboa, Conde, Moure, Falqué, & Domínguez, 2013). In a healthy human diet the ratio of ω6/ω3 should not exceed 10:1. Brown algae typically have a ω6/ω3 ratio of 0.6–5.1:1 (Kamat et al., 1992).

As well as nutritional value, fatty acids of certain seaweeds have also been reported to have antiviral properties (Frost & Gunstone, 1975). The most common method for quantitative determination of fatty acids is by extraction with a polar solvent system such as 2:1 chloroform/methanol followed by analysis with GC-MS. To aid in analysis they are first transesterified using methanolic HCl to form fatty acid methyl esters (FAME). Proton and carbon nuclear magnetic resonance can also assist in characterisation, especially with regards to identifying double bond configuration (Gunstone, Jie, & Wall, 1969).

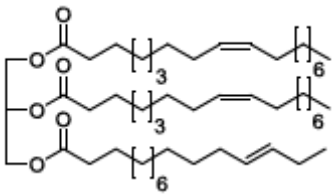

Fig. 1.5.A. A typical fatty acid triglyceride found in seaweed.

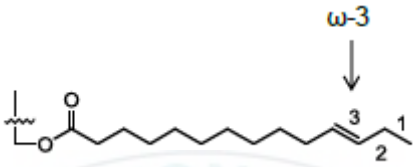

Fig. 1.5.B. The numbering system for describing the position of the double bond, showing in this instance an ω3.

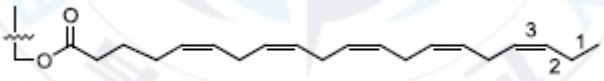

Fig. 1.5.C. An example of a PUFA, eicosapentaenoic acid (C20:5ω3), containing 5 double bonds, with the first at ω3.

1.5. Fucoxanthin

Fucoxanthin is an abundant marine xanthophyll that contains an allelic bond and two epoxy groups (Fig. 1.6.). It is estimated to account for more than 10% of total carotenoid produced in nature (Miyashita & Hosokawa, 2007; Nakazawa, Sashima, Hosokawa, & Miyashita, 2009; Terasaki et al., 2009). This characteristic lipid component of brown seaweeds is bound to several proteins, together with chlorophyll a, to form fucox-Chl a-protein complexes in the thylakoid, where it acts as a light harvesting and energy transferring pigment (S. M. Kim, Shang, & Um, 2011). Fucoxanthin in particular has been extensively investigated with respect to its strong antioxidant activity. Yan et al. (1999) demonstrated that the major active compound isolated from the carotenoid extract in *Hijikia fusiformis* was fucoxanthin that showed strong DPPH radical scavenging activity. The electron spin resonance method employed to investigate the quenching ability of fucoxanthin against the organic radicals DPPH, radical adduct of nitrobenzene with linoleic acids (NB-L) and 12-doxyl-steric acid (12-DS) indicated that in the presence of fucoxanthin, the ESR signals for these radicals are significantly decreased by 28%, 57%, and 66% respectively (Sachindra et al., 2007). From the structural point of view, it is suggested that the presence of the unique double allenic carbon (c-7', 201.84ppm) and two hydroxyl groups in fucoxanthin confer additional stability and resonance stabilisation within the conjugated double bond structure are responsible for the higher antioxidant activities (Sachindra et al., 2007; Yan et

al., 1999). Although fucoxanthin is known for its strong antioxidant activities, investigations on its involvement in the antioxidant system are limited and vague (Airanthi, Hosokawa, & Miyashita, 2011). In principle, the double bond in the polyene chain of a carotenoid enables it to exist in two configurations, trans and cis, that describe the disposition of substituent groups. All-trans fucoxanthin (~88%) was the major isomer of fucoxanthin found in fresh *Undaria pinnatifida* (Holdt & Kraan, 2011) and in most natural sources, followed by a mixture of 13-cis and 13' cis isomers (~9%) and 9'-cis isomer when stored in dark (Nakazawa et al., 2009). Nakazawa (2009) reported that the trans form of fucoxanthin had a faster uptake and incorporation into cellular lipids than its cis counterparts. Fucoxanthin also exists in another form; fucoxanthinol, which is found in human intestinal cells and mice after consumption of fucoxanthin (Maeda, Hosokawa, Sashima, Funayama, & Miyashita, 2005; Miyashita et al., 2011). This suggests that fucoxanthional is the active form of fucoxanthin in biological systems. Fucoxanthin in its pure form is vulnerable to oxidation. Nonetheless it is fairly stable in the presence of co-existing antioxidants such as polyphenol. The content of fucoxanthin was also reported to vary significantly with season and the life cycle of the algae, peaking between the winter and spring (mature phase of sporophyte) and lowest during summer (senescence phase) (Terasaki et al., 2009). Studies involving the quantification of fucoxanthin both in wild and cultured algae are limited. Quantification of fucoxathin content in various seaweeds are given in Table 1.2.

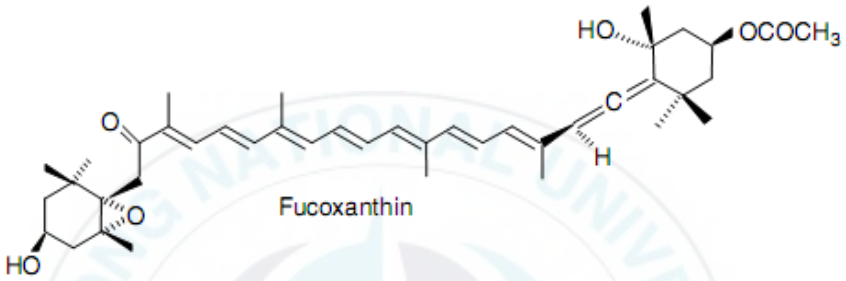

Fig. 1.6. Structure of fucoxanthin.

Table 1.2. Reported fucoxanthin contents of the Phaeophyceae class

Phaeophyceae species	Fucoxanthin content (mg/g)	References
U.pinnatifida		
young thallus	0.32	
commercial-dried	0.33	
female gametophyte	1.64	
male gametophyte	2.67	
Scytosiphon lamentaria		
young thallus	0.24	(Mori et al., 2004)
germlings	0.56	
Petalonia binghamiae		
young thallus	0.43	
germlings	0.58	
Laminaria religiosa		
young thallus	0.24	
Ecklonia radiate	1.65	
Carphophyllum mashalocarpum	1.17	
C. plumosum	1.44	(Czeczuga & Taylor, 1987)
Cystophora retroflexa	0.46	
Sargassum sinclairii	0.54	
Fucus serratus	0.56	(Haugan, 1994)
Cytoseira hakodatensis		
Lateral branch	1.90	
Fusiform branch	0.50	
Main axis	0.50	
Sargassum confusum		
Main branch (young)	1.70	Terasaki et al., 2009
Vesicle	2.90	
Leaf	2.80	
Lateral branch	2.10	
Main branch	1.60	
Main axis	0.70	
Laminaria digitata	0.46	
Laminaria japonica	0.17-0.21	
Ascophyllum nodosum	0.17-0.27	(Holdt & Kraan, 2011)
Fucus serratus	0.49-0.72	
F. vesiculosus	0.34	
Laminaria japonica (raw)	0.18	
Undiara pinnatifida		
Raw	0.11	
dry	0.08	(Kanazawa et al., 2008)
Eisenia bicycles (raw)	0.07	
Sargassum fulvellum (raw)	0.06	
Hizikia fusiformis (raw)	0.02	

1.6. Phlorotannin

Phlorotannins are a class of polyphenols found only in brown algae and typically make up 1-10 % dry wt. of the thallus (Imbs et al., 2009). They are secondary metabolites derived from phloroglucinol subunits to give a large, structurally varying polymer with a weight ranging from 126 Da to over 650 kDa, although they are more commonly observed between 10 and 100 kDa (K-W Glombitza & Pauli, 2003). There are several different types of linkages found in phlorotannins, as illustrated by the examples in Figure 1.7.1 of simple phlorotannins isolated from between the rings, as in trifucol. Phlorethols are another type on linkage which contain diaryl ether bonds (Ph-O-Ph). A range of more complex structures have also been observed such as the eckol structural component as in fucofuroeckol B (T. Shibata et al., 2004; Singh & Bharate, 2006). Around 150 algal polyphenolic structures have been isolated and characterised to date, with the Glombitza group achieving a large portion of this work (Karl-Werner Glombitza & Schmidt, 1999).

The biosynthetic pathway that produces phlorotannins is only partly understood. The formation of the phloroglucinol monomer is known to happen via the acetate-malonate pathway and involves a type III polyketide synthase enzyme (Martínez & Castañeda, 2013). The oxidative polymerisation processes by which the monomers are coupled are still unknown, although radical mechanisms have been proposed (Ito et al., 2013). There are reported examples elsewhere in nature of enzymes that couple phenols, such as the cytochrome P450 enzymes found in some bacteria, (Figure 1.7.2) (Schmartz et al., 2012).

The primary function of the phlorotannins is believed to be as a feature of the cell wall, in which it helps regulate osmotic pressure (Connan, Delisle, Deslandes, & Ar Gall, 2006). Phlorotannins have a strong UV absorbance between 190-240 nm leading to the theory that they serve as way of protecting from potentially damaging UV solar radiation (Arnold & Targett, 2003). *In vitro* tests of phlorotannins have found them to be strong antioxidants and radical scavengers due to their hydroxyl groups and conjugated aromatic systems (Balboa et al., 2013). Sub tidal species were found to have greater phenolic contents than intertidal species, which may be due to the increased need of intertidal species to protect against oxidative damage (Fairhead, Amsler, McClintock, & Baker, 2005). *In vitro* tests have shown phlorotannins to have antibacterial properties (Nagayama, Iwamura, Shibata, Hirayama, & Nakamura, 2002). Phlorotannins are made up of phloroglucinol (1,3,5-trihydroxybenzene) units with varying degrees of polymerization that may be linked through different bonds forming several structures and types, namely: fuhalols and phlorethols, which contain ether linkages; fucols, with phenyl linkages; fucophlorethols in which both ether and phenyl linkages are present; and eckols, that possess a benzodioxin linkage. Phlorotannins have been shown to be good at binding to metals and proteins (Stern, Hagerman, Steinberg, & Mason, 1996). A study showed that protein extraction yield was inversely proportional to the total phenolic content, suggesting the protein affinity of phlorotannin to be responsible (Wong & Cheung, 2001; Thomas & Kim, 2013).

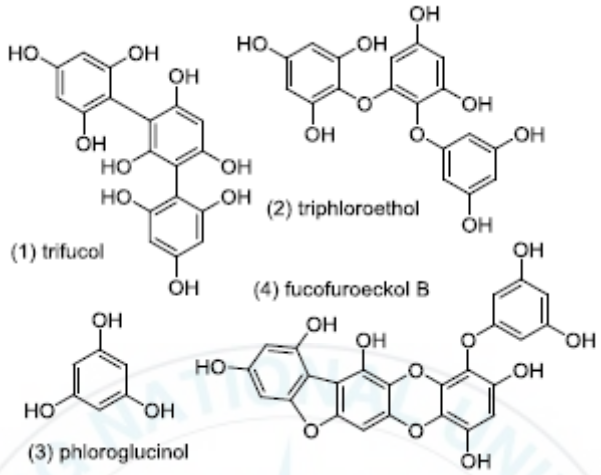

Fig. 1.7.A. A selection of simple phlorotannins extracted from brown algae feature its key structural linkages.

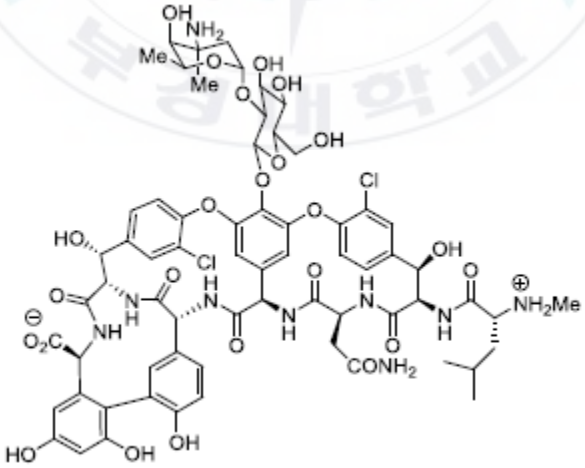

Fig. 1.7.B. The natural product vancomycin, isolated from bacteria that features both Ar-Ar and Ar-O-Ar linkages that are coupled by P450 enzymes.

1.7. Fucoidan

S. japonica and many other types of seaweed are known to be rich sources of bioactive compounds. The term 'bioactive compound' is defined as a substance, which at low concentrations, may be harmful or beneficial to living organisms (Arunkumar, Sivakumar, & Rengasamy, 2010). Bioactive compounds from seaweed polysaccharides usually have one or more of the following properties: anticoagulant, antithrombotic, antivirus, antitumor, immunomodulatory, antioxidant, and anti-inflammatory (Li, Lu, Wei, & Zhao, 2008). These properties give seaweed great potential as a food supplement or for the extraction of these bioactive compounds. Many of these properties are generated by the polysaccharide fucoidan (Li et al., 2008).

The number of published articles on fucoidan-related topics has increased dramatically since the first publication in 1913 (Kylin, 1913). Specifically, this increase took place in the last 5-10 years. Recent interest on fucoidan had focused primarily on the antitumour, anticoagulant, and antioxidant activities, as well as activities against liver and urinary system failures (Ale, Mikkelsen, & Meyer, 2011). As more scientists continue to explore this unique polysaccharide, more of its biological health benefits are being discovered (Hayashi, Nakano, Hashimoto, Kanekiyo, & Hayashi, 2008).

The nomenclature of fucoidan has evolved through several steps. In 1918 Kylin (Kylin, 1913) baptised his sulphated polysaccharide 'fucoidin'. This name was changed by McNeely (McNeely, 1959) in 1959 to 'fucoidan' to follow the usual polysaccharide nomenclature. As the extracted polysaccharides differed in their composition due to seasonal variations, local climate conditions

and algal species, it was very difficult to determine if the other mono sugars such as xylose, mannose, galactose and uronic acids were part of the fucoidan or if they were just contaminants. The term ‘fucoidan’ was even suppressed by some authors due to this uncertainty (Larsen, Haug, & Painter, 1966). To circumvent these problems, the new term ‘fucans’ was used to describe all polysaccharides rich in L-fucose. New techniques for separation and analysis made it possible to distinguish between the different types of sulphated polysaccharides, limiting the term ‘fucoidan’ to sulphated polysaccharides containing a homofucose backbone. However, not all authors stick to this terminology and some still use the outdated ‘fucoidin’ or even worse, create their own nomenclature such as ‘fucansulfate’ (Duarte, Cardoso, Noseda, & Cerezo, 2001). Berteau and Mulloy (Berteau & Mulloy, 2003) recommended the use of ‘sulfated fucan’ to describe a polysaccharide mainly based on sulphated fucose, with less than 10% other monosaccharides. This term was applied to the sulphated fucans of marine invertebrates (Vilela-Silva, Alves, Valente, Vacquier, & Mourão, 1999).

Fucoidan is a natural polysaccharide made essentially of sulphated L-fucose residues (Fig. 1.8.). Also known as sulphated fucan, it was first extracted in 1913 from brown algae (Kylin, 1913). Fucoidan is present in the cell walls of brown algae and other animal species, including the sea cucumber and sea urchin. Particularly high amounts of fucoidan are found in *U. pinnatifida* (Irhimeh, Fitton, Lowenthal, & Kongtawelert, 2005). Though many studies on identifying the structural properties of fucoidan have been carried out, the

structure still remains uncertain due to the absence of strict regularity and the numerous components that make up fucoidan as a whole (Zvyagintseva et al., 2003). Figure 1.8 shows the general structure of fucoidan but the chemical composition and structure of fucoidan varies with species (Hayashi et al., 2008). Most fucoidans have very complex chemical composition and only little regularity in the structural components is known present (Rioux, Turgeon, & Beaulieu, 2007). Fucoidan largely contains sulphated L-fucose residues. Hence fucose is the primary sugar in fucoidan. Sulphate groups also represent a large component of fucoidan and the biological activities of fucoidan is strongly related to its sulphate content (Yang et al., 2008). Besides fucose and sulphate, other monosaccharides (glucose, mannose, galactose, xylose, etc), uronic acids, and even protein are present in detectable amounts. All these compounds have increased the difficulty in structural elucidation of fucoidan (Li et al., 2008).

Research on fucoidan has so far been carried out in Japan, Korea, France, Australia, China, and the United States. Studies have indicated that fucoidan is non-toxic, non-allergenic, and has no negative effects on the human body once consumed (H. Shibata et al., 2000). No toxicological changes were observed when rats were orally administered with up to 1000 mg/Kg body weight per day of fucoidan for 28 days, but when the dose was increased to 2000 mg/Kg body weight per day of fucoidan, the plasma ALT level, a biomarker of liver injury was increased indicating that the consumption of fucoidan up to 1000 mg/Kg body weight per day was safe in rodents (Chung et al., 2010).

Fucoidan is known to exhibit a wide variety of biological activities. Among them are: anticoagulant, antioxidant, antiviral, antithrombic, and anticancer activities (Li et al., 2008). Many researchers have targeted the anticoagulant, anticancer, and antioxidant activities of fucoidan as being the most important activities in fucoidan (Synytsya et al., 2010). Seaweed polysaccharides are usually heterogeneous and branched; it may contain monosaccharide components with acetyl groups and the amount of sulfation is irregular (Bilan et al., 2002). As mentioned earlier, the structural complexity of fucoidan may vary from species to species, depending on the extraction method. For that reason, each type of fucoidan that may have unique structural features and possess varied bioactivities, could potentially be a new drug (Eluvakkal, Sivakumar, & Arunkumar, 2010). Fucoidan extracted from *S. japonica* is an acidic sulfated polysaccharide, mainly made of fucose, galactose and sulfate, with smaller amounts of mannoses, glucuronic acid, glucose, rhamnose, arabinose and xylose. Recently, substantial pharmaceutical research has been conducted on fucoidan. This research has demonstrated a strong antioxidant activity of fucoidan, which is now being marketed as a nutraceutical and food supplement Crude fucoidan is a polysaccharide made up of a complex mixture of fucose, sulphate, and low uronic acid, to a low sulphated fucan polysaccharide with high uronic acid content (Marais & Joseleau, 2001).

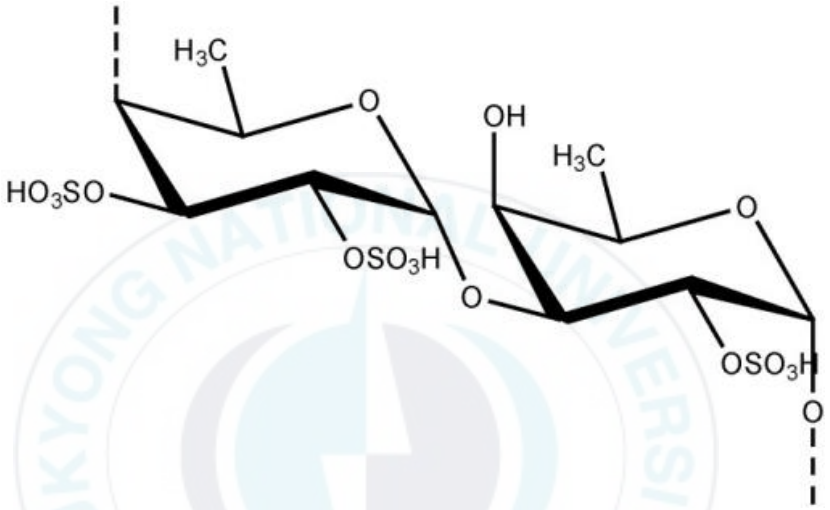

Fig.1.8. Chemical structure of fucoidan.

1.8. Supercritical fluid technology

A gas becomes a liquid if it is compressed to a high pressure. On the other hand, "if a gas is heated beyond a specific temperature and the heated gas is compressed, it becomes a fluid with intermediate properties between a gas and a liquid". This specific temperature is the critical temperature (Tc), while the specific pressure is the critical pressure (Pc). Critical temperature and pressure define the critical point, which is unique for each fluid. When values of pressure and temperature exceed the critical point, the fluid is referred to as a supercritical fluid (Brunner, 2013). This phenomenon is illustrated in Figure 1.9. In the supercritical region, the fluid can be considered as an expanded liquid or a compressed gas (Rosa & Meireles, 2009).

A fluid reaches its supercritical state above its critical pressure and temperature, as shown in Figure 1.9. Under supercritical conditions, there is only one phase with properties in between those of liquid and gas. Above the critical temperature, there is no phase transition, that is, the fluid does not become a liquid with a change in pressure. Diffusivity is intermediate between those of a gas and a liquid. Therefore, these properties allow good solvation power of the fluid and easier penetration and diffusion into the solid matrix. As a consequence, supercritical fluids present good properties to be used as solvents in extraction processes (Brunner, 2013).

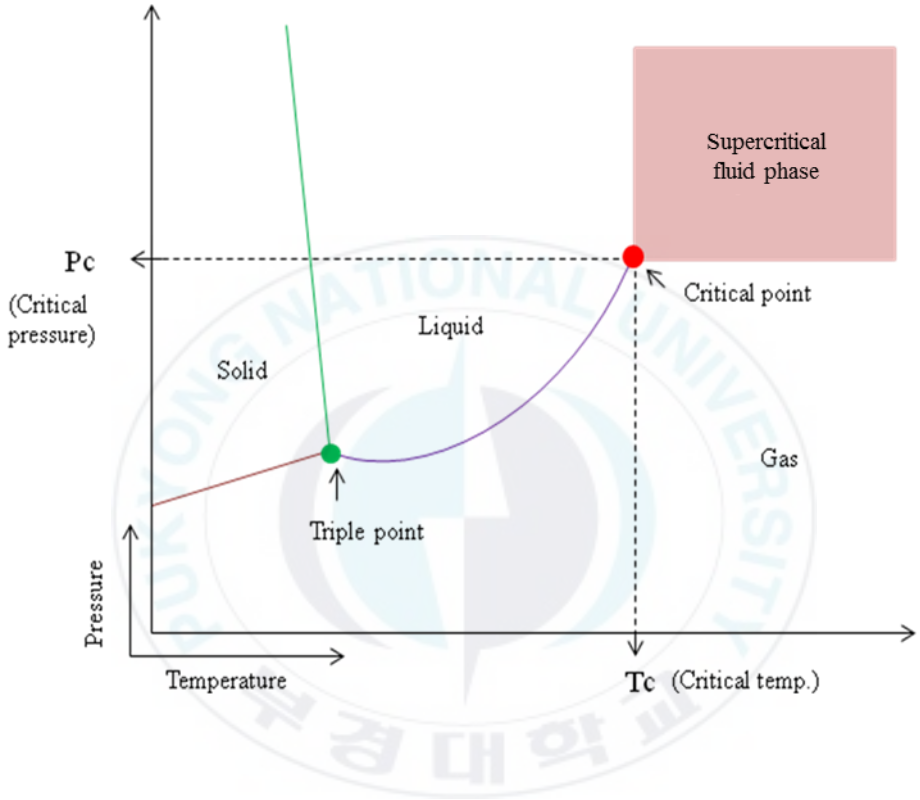

Fig. 1.9. Phase diagram of a substance with critical temperature (Tc) and pressure (Pc).

Table 1.3.A. Physical properties of gases, compressed gases and liquids

	Density (g/mL)	Dynamic viscosity (g/cm-sec)	Diffusion coefficient (cm^2/sec)
Gas (ambient)	0.0006-0.002	0.0001-0.003	0.1-0.4
Supercritical fluid (Tc, Pc)	0.2-0.5	0.0001-0.0003	0.0007
Liquid (ambient)	0.6-1.6	0.002-0.03	0.000002-0.00002

Table 1.3.B. Critical properties of various solvents

Solvent	Molecular weight (g/mol)	Critical temperature (K)	Critical pressure (MPa)	Critical density (g/cm^3)
Carbon dioxide	44.01	304.1	7.38	0.469
Water	18.02	647.3	22.12	0.348
Methane	16.04	190.4	4.60	0.162
Ethane	30.07	305.3	4.87	0.203
Propane	44.09	369.8	4.25	0.217
Ethylene	28.05	282.4	5.04	0.215
Propylene	42.08	364.9	4.60	0.232
Methanol	32.04	512.6	8.09	0.272
Ethanol	46.07	513.9	6.14	0.276
Acetone	58.08	508.1	4.70	0.278

The solvent most often used in supercritical fluid extraction is carbon dioxide because of its mild critical properties (see Table 1.3.1) and critical properties of various solvent were shown in Table 1.3.2, low cost, and high availability (Brunner, 2013).

The first supercritical fluid extraction industrial application process started in the 1930s with the deasphalting of heavy mineral oil fractions by means of dense propane in the petrochemical industry, even though the first supercritical literature report appeared in 1822. The first industrial plant (Hag AG Corporation) was installed in Germany in 1976 for decaffeination of coffee using SC-CO_2. Then, in 1982, a hops extraction plant was built in Munchester and later a tea decaffeination plant was also installed. SC-CO_2 extraction industrial plants started to be built in USA only in the year 1988 and they were also coffee decaffeination and hops extraction plants (Phelps, Smart, & Wai, 1996).

Nowadays, industrial plants are located in Europe, USA, Canada and Asia and all of them utilize SC-CO_2 as the solvent (Phelps et al., 1996). Particularly, supercritical fluid extraction used nowadays related to food processing are tea and coffee decaffeination, hops extraction, obtaining extracts from flavoring and condiments plants, extraction and fractionation of edible oils, fat removal from food, alcohol removal from wine and beer, removal of contaminants, etc (Brunner, 2005). Other industrial plants different from foods are removal of lipids from bones to be further used in orthopedic surgeries as

grafting material, removal of oil from long quartz rods for further production of fibers for fiber optical transmission and denicotinization of tobacco (Phelps et al., 1996). Although extraction is the most commonly used application of supercritical fluids, there are other applications that have been studied and investigated. Such applications involve reactions in supercritical media, biodiesel production, impregnation, fractionation, chromatography and particle formation (Caputo, Fernández, Saldaña, & Galia, 2013).

The utilization of supercritical fluids has greatly increased in the past years. Many high value products require several processing steps to achieve a high purity final product, which usually consume high amounts of energy and time. Supercritical fluids have attractive physical properties when compared to conventional solvents. The possibility of changing the solvent power by changing its density when temperature and pressure are varied is a great advantage over conventional extraction and separation methods (Brunner, 2013).

Supercritical fluid applications in particle design have been a very attractive area of research in recent years. The supercritical encapsulation processes are based on creating a high degree of solution supersaturation that leads to a great number of nucleation sites and very small crystals. Several components participate during the encapsulation process, the encapsulating material, the solute to be encapsulated, the solvent and a co-solvent. In this context, the equilibrium between phases, solid-liquid, liquid-liquid (LL), and vapor-liquid (VL) is important information needed to design a process.

A multicomponent fluid mixture can have heterogeneous phases (VL or LL) or a homogeneous phase at supercritical conditions. The pressure-temperature diagram is useful to show the bubble and dew point phase transition curves and the mixture critical point. The region under the curve is heterogeneous, while a single phase is present above the maximum pressure at any temperature. Therefore, information on the properties and behaviours of pure components and their mixture is very important for design, simulation and optimization of chemical processes.

Supercritical fluid extraction can be considered as a potential technology for extraction of desirable compounds, such as carotenoids, essential oils, caffeine and others from vegetable matrices (M. A. A. Meireles, 2003).

Supercritical fluid extraction can be defined as a unit operation by contact, where the fundamentals are based on equilibrium and physico-chemical properties of supercritical fluids, such as high solvation power, high diffusion coefficient, low surface tension and low viscosity (Velasco, Villada, & Carrera, 2007). Supercritical fluid extraction is advantageous if the supercritical fluid has low temperature, low vaporization enthalpy, high volatility and it is also inert, non-toxic and non-flammable. Furthermore, CO_2 is the most used solvent in supercritical fluid extraction (M. Meireles, 2013). CO_2 has limited capacity to dissolve polar molecules. In some cases, it is necessary to have co-solvent addition. Co-solvent has the objective to modify solvent characteristics like polarity. Thus, the use of co-solvent results in extracts with high content of polar compounds. However, when a co-solvent is used, it is necessary that pressure

and temperature ensures that the binary mixture (co-solvent + supercritical fluid) is in the supercritical state (M. A. A. Meireles, 2003; Reverchon & De Marco, 2006). Nevertheless, co-solvents used in supercritical fluid extraction should be GRAS to guarantee the advantages of supercritical fluid extraction. Some examples of co-solvents are ethanol, isopropanol, and water. Water presents some advantages compared to other organic solvents. This is because it has lower cost, less environmental issues, and it is easier to use. However, the vegetable matrix should be very dry, as there are strong interactions between water and cellulosic materials (M. Meireles, 2013).

One of the most important characteristics of supercritical fluid extraction is that small variations in pressure and temperature alter solvent selectivity. Thus, considering phase equilibrium between the solvent under supercritical conditions and the components of the solid matrix, the extracts have different compositions depending of temperature and pressure conditions (M. A. A. Meireles, 2003). According to Quispe-Condori, (2005) supercritical fluid extraction presents advantages and disadvantages compared to other extraction techniques. The main advantages are highlighted as follows:

- Because CO_2 has mild critical temperature, the use of mild temperatures in the process results in extracts close to the initial characteristics, preserving thermal labile compounds
- Higher selectivity with small variations in pressure and temperature that allows extraction of different compound classes

- The low surface tension of supercritical CO_2 allows quick penetration of the solvent into the pores of the vegetable matrix which increases extraction efficiency
- Supercritical CO_2 has higher diffusion coefficient and lower viscosity than liquids
- CO_2 is easily separated from the product through pressure reduction

Some disadvantages are:

- Different vegetable matrices have unique structure with different chemical composition and physiological structure, therefore, it behaves differently at the same process conditions, thus, there is not a universal mathematical model to represent all matrices
- Common scale-up methods are not easily applicable for industrial process
- Extracts may have different characteristics such as color, odor and chemical composition when compared to those obtained by traditional extraction methods, which can influence consumer preferences
- High initial capital investment cost due to high pressure operation

1.9. Subcritical water

Subcritical water is liquid water which is maintained in the subcritical state between its boiling point 100 ^oC at 1 bar and critical point 374 ^oC at 220 bar. Water is remained as liquid in the subcritical state due to apply the high pressure (King, 2000) shown in Fig. 1.6. Water in the subcritical states provides unique

properties over water at ambient conditions. The two distinct advantages of subcritical water are lower dielectric constant as well as higher ion product shown in Fig. 1.10. The modification in the dielectric constant of subcritical water makes it a suitable solvent for dissolving organic compounds. The ions produced in the subcritical state are three orders of magnitude higher than ions in water at ambient conditions and therefore, it has potential to act as an acid or base like catalyst. When water is used as a solvent other names have also been used: superheated water extraction (SHWE), subcritical water extraction (SWE), hot water extraction (HWE), pressurized hot water extraction (PHWE) or high temperature water extraction (HTWE).

The solvent property of water changes significantly in the subcritical region (Fig. 1.10.2). The organic solvents become partially soluble in the SCW due to the low polarity of the water at the subcritical state. The decrease in the polarity is due to the decrease in the dielectric constant of water, which decreases with increase in temperature. For example, water at 25°C and atmospheric pressure has a dielectric constant (ε) of 80, mostly due to hydrogen bonds (Yesodharan, 2002), but at 100°C, the dielectric constant (ε) reduces to 55, which is near to formic acid (ε = 58), and as the temperature reaches 200°C, the dielectric constant (ε) becomes 35, similar to that of methanol at 25°C. High temperature and high pressure reduce the surface tension and viscosity of water, which results in enhanced solubility, allowing better penetration into the matrix and resulting in better extraction. Increasing the temperature breaks the hydrogen bonds and the density and viscosity of water decreases rapidly. As the density

decreased from 960 kg/m3 at 100°C to 870 kg/m3 at 200°C the diffusivity of the water increased, enhancing its penetrating power.

The ionic strength (pKw) of water varies with the density and temperature. At pKw< 14, water is more suitable for heterolytic reaction while at pKw>14, it is suitable for hemolytic reaction (Bröll et al., 1999). The physicochemical properties of subcritical and supercritical water are summarized in Table 1.4. Here, the SCW was selected due to its relatively low density, dielectric constant and ionic strength that could facilitate the extraction of carbohydrates and phenolics at much lower temperature conditions compared to supercritical water (over 374°C) or superheated steam (>400°C), preventing degradation of the extracts. Furthermore, autocatalytic ionic reactions through water-soluble reaction products are found. For industrial chemistry, the use of SCW could be meaningful for a series of reactions that require economical as well as ecological improvement. One example is the hydrolysis of esters. Until now, this reaction only took place in the presence of strong mineral acids or bases producing considerable amounts of waste acid and salt. The enzymatic hydrolysis proceeds only with low space–time yields and the enzyme production itself is very time-consuming. With SCW, it seems very likely that ester hydrolysis will be possible without the addition of further support material.

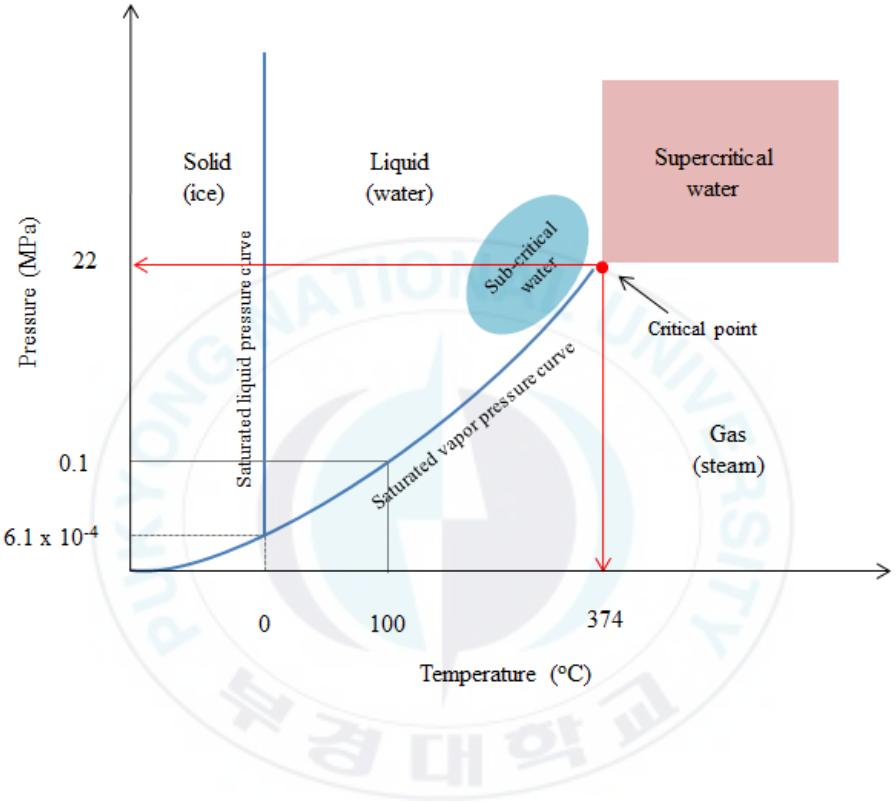

Fig. 1.10.A. Phase diagram of subcritical water with temperature and pressure changes.

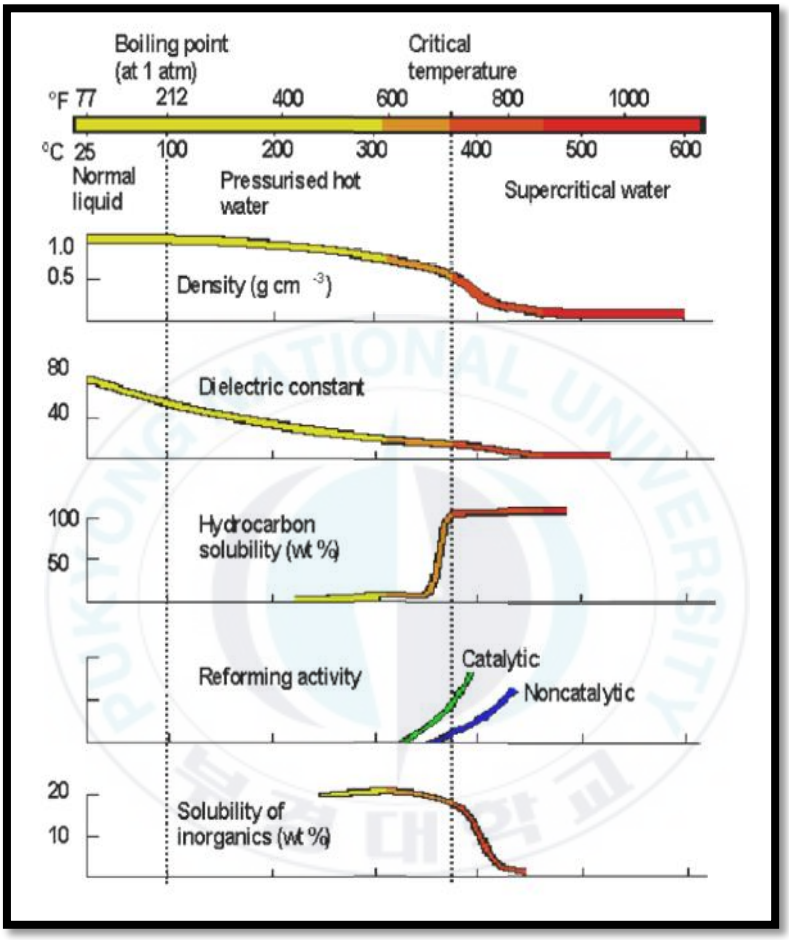

Fig. 1.10.B. Properties of normal, sub- and supercritical water (Laboratory of analytical chemistry, Finland).

Table 1.4. Properties of water

Parameters	Normal water	Subcritical water	Supercritical water	Superheated steam
Temperature (°C)	25	100-374	374-400	400
Pressure (bar)	1	2-250	250-500	1
Density (kg/m3)	1000	170-800	58-170	3
Dielectric constant (ε)	79	6-58	6-11	1
Ionic strength (pKw)	14	11-12	11-19	-

1.10. Pressurized liquid extraction

Several other names have been used for this technique (Carabias-Martínez, Rodríguez-Gonzalo, Revilla-Ruiz, & Hernández-Méndez, 2005), including accelerated solvent extraction (ASE), pressurized fluid extraction (PFE), high-pressure solvent extraction (HPSE), high-pressure, high temperature solvent extraction (HPHTSE), pressurized hot solvent extraction (PHSE) and subcritical solvent extraction (SSE). The use of water at higher temperatures has specific advantages such as being cleaner, cheaper and more environmentally friendly than the organic solvents that are usually employed in PFE (Ridgway, Lalljie, & Smith, 2007). But PHWE is essentially a variant of PLE.

PLE is an analyse and matrix independent technique which provides cleaner extracts relative to the time-consuming classical procedures used for the extraction of compounds from complex matrices. The process is based on applying increased temperatures, elevated pressures, and keeping the solvent below its boiling point, thus accelerating the extraction kinetics and enabling safe and rapid extractions. Since the first instruments became commercially available in the mid 90's, this technique gained widespread acceptance for extraction of organic micro-pollutants such as pesticides, polycyclic aromatic hydrocarbons (PAH's), polychlorinated biphenyls (PCB's) and dioxins from a great variety of environmental and clinical matrices (Björklund, Nilsson, & Bøwadt, 2000; Giergielewicz-Możajska, Dąbrowski, & Namieśnik, 2001).

PLE can be performed in both static and dynamic (flow-through) modes, or a combination of both. In static mode, the samples are enclosed in a stainless steel vessel filled with an extraction solvent, and following extraction the remaining solvent is purged with N_2 into a collection vial. Flow-through systems continuously pump solvent through the sample, but this has the disadvantage of using larger volumes of solvent and of diluting the extract. A desiccant, such as sodium sulphate, diatomaceous earth or cellulose can be added directly to the extraction cell or sorbent materials such as MIPs can be used to provide in situ clean-up. The latter has been employed in some of the work that will be discussed in this thesis under the experimental chapter. The extraction conditions must be optimized and this can be done using statistical 'experimental design' procedures to minimize the number of experiments (Pallaroni & Von Holst, 2003; Von Holst, Müller, Serano, Sporring, & Björklund, 2005). Modifiers can be added to the extraction solvent, for example water modified with a surfactant (sodium dodecyl sulphate) was used to extract PAHs from fish tissues (Morales-Munoz, Luque-Garcıa, & De Castro, 2002). For lipid containing samples, further clean-up is usually required and Gomez et al. (2002) investigated the use of several sorbents and concluded that florisil produced the cleanest extracts for their samples. An alternative approach is to perform a preliminary PLE with a non-polar solvent to eliminate the hydrophobic compounds prior to extraction of the analytes of interest (known as selective PLE).

Pressurized liquid extraction (PLE) is carried out at an elevated pressure and temperature, allowing liquid extraction above the boiling point of the solvent. The analytes solubility is therefore enhanced and the desorption kinetics are accelerated. The extraction is generally completed within a few minutes. Liquids under pressure act as solvents; therefore, higher extraction efficiency is expected at higher pressure which may be accelerated further with increasing temperature. PSE is reported for reduced solvent consumption, as well as comparable solute recoveries. Temperature increases the solvent potential of a solvent (Richter et al., 1996) by accelerating diffusion rates (Denery, Dragull, Tang, & Li, 2004). The thermal energy helps overcome the cohesive (solute-solute i.e. lipids-lipids) interactions and adhesive (solute-matrix, i.e. lipids-cell matrix) interactions (Cooney, Young, & Nagle, 2009; Richter et al., 1996). Increase in thermal energy increases molecular motion of the molecules and thereby decreasing their molecular interactions of hydrogen bonds, van der Waals forces, and dipole interactions (Cooney et al., 2009). Similarly, pressure facilitates increased transport of solvent to hard-to-reach corners, pores, surfaces and matrices. Pressure also increases the penetration power of the solvents through the cell wall to contact the lipids inside microalgal cells. Elevated pressure is reported to reduce the dielectric constant of immiscible solvents to values that better match the polarity of the lipids (Herrero, Jaime, Martín-Álvarez, Cifuentes, & Ibáñez, 2006). Pressurized solvents at elevated temperature hence improve the efficiency of traditional extraction

systems resulting in shorter extraction time and lower solvent consumption. The mass transfer rates are thereby increased (Mustafa & Turner, 2011).

1.11. Objectives of the thesis

The present study was undertaken to study the extraction of functional materials from *S. japonica* using SC-CO_2, PHWE and PLE process. *S. japonica* is rich in functional materials such as fucoxanthin and fucoidan. Green technologies have emerged as a promising/alternative technology to conventional methodologies, due the environmental hazards and other limitations. Mostly used classical process such as soxhlet extraction, maceration, solid liquid extractions, etc., has several drawbacks like the presence of high shear forces, high temperatures and also contamination of the final product with undesirable organic solvents.

Therefore, to obtain functional material from brown seaweed using sub and supercritical fluids, the following tasks have been carried out:

- Extraction of fucoxanthin and phlorotannin from *S. japonica* using SC-CO_2 using various co-solvents (Chapter 2).
- Useful materials production and characterization from de-oiled *S. japonica* using PHWE (Chapter 3).
- Extraction and characterization of fucoidan from de-oiled *S. japonica* using PLE (Chapter 4).

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Chapter 2

Influence of co-solvents on fucoxanthin and phlorotannin recovery from brown seaweed using supercritical CO_2

2.1. Introduction

Saccharina japonica (*Laminaria japonica*), a brown algae, is the most important economic seaweed cultured in the temperate seaside areas of the northwest Pacific. It is commonly consumed as a popular marine vegetable in Pacific and Asian countries, particularly in Korea, Japan, and China (Islam et al., 2013). *S. japonica* has a high nutritional value as a source of minerals, vitamins, and non-caloric dietary fiber, so it has been widely used as a health food as well as a traditional herbal medicine (Shan, Pang, Zhang, Yakovleva, & Skriptsova, 2011).

Fucoxanthin (FX) is a marine carotenoid found in macroalgae with a unique structure, including an allenic bond and oxygenic functional groups such as epoxy, hydroxyl, carbonyl, and carboxyl groups in the polyene hydrocarbon chain (Pádua, Rocha, Gargiulo, & Ramos, 2015). Recently, several studies have demonstrated the anti-inflammatory, antioxidant, and anticancer activity of FX (Gammone & D'Orazio, 2015).

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The antioxidant activity of FX involves free radical scavenging and is one of the main mechanisms underlying its anticancer effect (Zorofchian Moghadamtousi et al., 2014).

Brown seaweed accumulates a variety of phloroglucinol-based polyphenols, as phlorotannin (PT), formed from polymerization of phloroglucinol (1,3,5-trihydroxybenzene) monomer units resulting in compounds with different molecular weights (Pádua et al., 2015). PT is present in many marine organisms, especially in brown seaweed. It also has antioxidant, anti-bacterial, anti-inflammatory, and anti-allergic properties, contributing to the reputation of brown seaweed as a healthy food (Jeong-Ha et al., 2014).

Recently, there is wide interest in environment friendly technologies such as supercritical carbon dioxide (SC-CO_2) extraction, which confer advantages over conventional techniques (Gao et al., 2010). Latest works have chiefly concentrated on the extraction of oil containing FX using SC-CO_2 from some brown seaweeds. For example Roh et al. (2008) reported a method to extract FX from *Undaria pinnatifida* by SC-CO_2 with 3.0% (v/v) ethanol as co-solvent. Conde et al. (2015) found that the use of SC-CO_2 with ethanol extraction slightly improved the yield of FX from *Sargassum muticum*. However, from the previous studies, it has been found that the widely used co-solvent ethanol is not very effective for SC-CO_2 extraction of TC, FX, and PT due to their different polarities. Numerous reports have used vegetable oils as co-solvents for extracting carotenoids and water as co-solvents for extracting

tannins by SC-CO_2 with good results. Vasapollo et al. (2004) showed that with hazelnut oil as co-solvent for SC-CO_2 extraction of lycopene from sun-dried tomato, the yield could be increased to 60% of the total amount of extractable lycopene. Moreover, the presence of hazelnut oil was found to help stabilize the pigment. Sun and Temelli (2006) reported that in the presence of canola oil as a continuous co-solvent, the yield of carotenoid extracted from carrot was more than four times higher compared with SC-CO_2 extraction without any co-solvent. In the case of tannins, Pansera et al. (2004) reported that with water as co-solvent, the extraction efficiency of tannins from *Acacia mearnsii* was improved.

The purpose of this work was to compare the effects of sunflower oil (SFO), soybean oil, canola oil, 96% ethanol, and water as continuous co-solvents on the SC-CO_2 extraction of TC, FX, and PT from *S. japonica* under various extraction conditions. The effect of extraction variables (temperature, pressure, and concentration of the co-solvent) on the extraction efficiency was investigated, and a Box–Behnken design (BBD) was used to find the best conditions.

2.2. Materials and methods

In June 2015, *S. japonica*, J.E. Areschoug, 1851, Lane, Mayes, Druehl, and Saunders, was collected from Guemil-eup, Wando-gun, Jeollanam-do, Republic of Korea. The obtained samples were prepared as reported in our previous work (Sivagnanam et al., 2015). High-purity CO_2 gas (99%) was supplied by KOSEM (Yangsan, Republic of Korea). High oleic SFO, soybean

oil, and canola oil were purchased from a local supermarket. Ninety-six percent ethanol, fucoxanthin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethyl benzothiazoline-6-sulphonic acid ($ABTS^{+}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), gallic acid, and phloroglucinol were purchased from Sigma-Aldrich Chemical Co. (Missouri, USA). All of the reagents used in this study were analytical or high-performance liquid chromatography (HPLC) grade.

2.2.1. Supercritical carbon dioxide extraction

A laboratory scale setup for the SC-CO_2 process was used in the work. Exactly 100 g of freeze-dried seaweed powder was placed in a 200 mL stainless steel extraction vessel (Figure 2.1.). The instrument setup and extraction procedures were the same as those in our previous work (Sivagnanam et al., 2015).

2.2.2. Total carotenoid

Determination of the TC was carried out according to Lu et al. (2014) by measuring the absorbance of the different samples using UV mini 1240, Shimadzu Co., Japan. The following equations were used in the analysis:

$$C_a\ (\mu g\ mL^{-1}) = 16.72 \times A_{665.2} - 9.16 \times A_{652.4}, \quad (1)$$

where C_a is the concentration of chlorophyll a, $A_{665.2}$ is the absorbance at 665.2 nm, and $A_{652.4}$ is the absorbance at 652.4 nm.

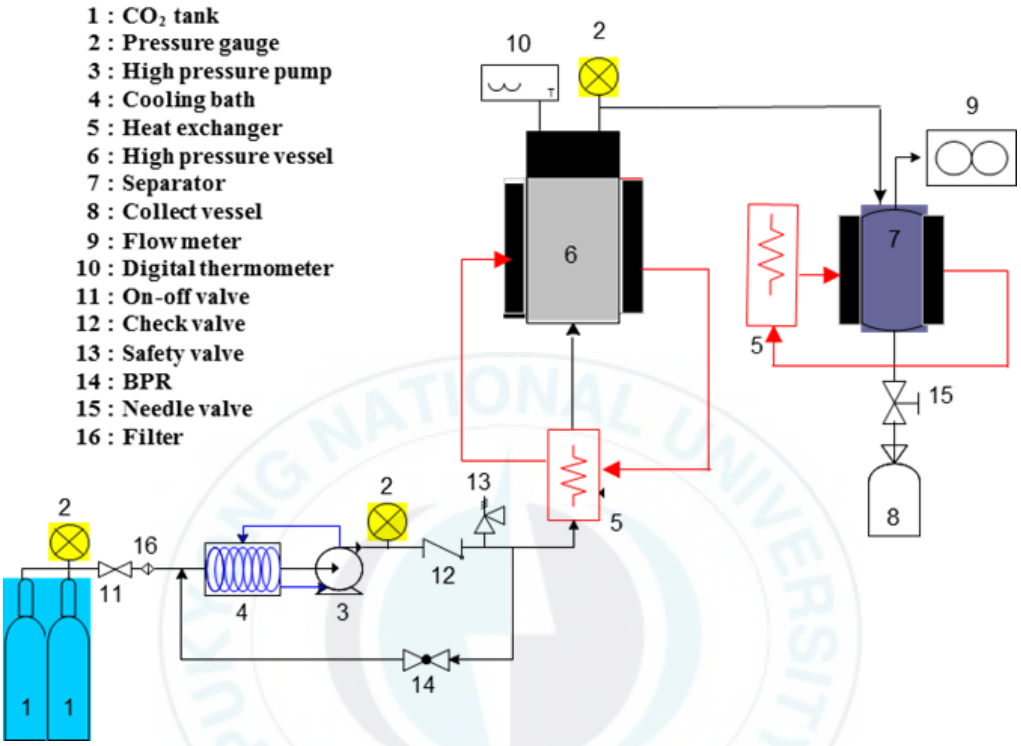

Fig. 2.1. Schematic diagram of supercritical fluid extraction with co-solvent system.

$$C_b\ (\mu g\ mL^{-1}) = 34.09\times A_{652.4} - 15.28 \times A_{665.2}, \quad (2)$$

where C_b represents the concentration of chlorophyll b.

$$C_{TC\ (x+c)} = \frac{1000.A_{470} - 1.63.C_a - 104.96.C_b}{221}, \quad (3)$$

where $C_{TC\ (x+c)}$ is the concentration of TC and A_{470} is the absorbance at 470 nm.

2.2.3. Fucoxanthin analysis

The HPLC analysis was conducted using a Waters 600 E HPLC system (Waters, Massachusetts, USA) equipped with a tunable absorbance detector (Waters 484). FX was analyzed following the method of Sivagnanam et al. (2015) The fucoxanthin content in the seaweed extracts was determined by reversed-phase HPLC (RP-HPLC) with methanol–acetonitrile (7:3, v/v) as the mobile phase at a flow rate of 1.0 mL/min. All RP-HPLC analysis was conducted at ambient temperature by using a RP column (XTerra® MS C18) with a 5.0 μm particle size and 250 mm × 4.6 mm inner diameter (id; Waters, Milford, MA, USA) protected by a guard column of 10 mm × 4.6 mm id with the same stationary phase. Briefly, an aliquot of seaweed extract was dissolved in a mobile phase and was then filtered with a 0.22 μm membrane filter. The fucoxanthin detection wavelength was set at 450 nm. FX content in the seaweed samples was expressed as milligrams per gram.

2.2.4. Quantification of phlorotannin

PT quantification followed the colorimetric method (Cuong, Boi, & Van, 2016), using phloroglucinol as standard: 0.1 mL of 10% Folin–Ciocalteu phenol

reagent was added to 300 µL of seaweed extract. After 5 min, 2.0 mL of 10% Na_2CO_3 was added to the solution. Then, the solution was vortexed for 30 s and kept in the dark for 90 min. The absorbance was measured at 750 nm. The PT content was expressed in milligrams per gram.

2.2.5. Fatty acid composition analysis

Fatty acid (FA) compositions of seaweed extracts/SFO were analyzed by gas chromatography following the method and conditions of Sivagnanam et al. (2015) A 6890 Agilent Technologies (Wilmington, Delaware, USA) gas chromatograph was used with a fused silica capillary column 100 m in length, 0.25 mm in i.d., and 0.2 µm in film length (Supelco, Pennsylvania, USA).

2.2.6. Determination of antioxidant activity

The antioxidant activity was evaluated using DPPH and $ABTS^+$, as described by Khanam et al. (2012) Various concentrations of seaweed extracts/SFO (500 µl) and 500 µl of freshly prepared DPPH solution (0.05 mg/mL methanol) were mixed together, and it was incubated in the dark for 30 min at room temperature. The absorbance was measured against methanol at 517 nm. Gallic acid was used as reference standard.

Seaweed extracts/SFO samples (150 µL) of various concentrations were mixed to react with 2.85 mL of $ABTS^+$ solution (1 mL $ABTS^+$ solution mixed with 60 mL methanol) for 2 h in the dark. The absorbance was taken against methanol at 734 nm. Trolox was used as the reference standard.

The percentage of inhibition of DPPH and $ABTS^{+}$ from the extracted seaweed extracts/oils was measured as follows:

$$\text{Inhibition\%} = (1 - A_{\text{test sample}}/A_{\text{blank}}) \times 100, \quad (4)$$

where A_{blank} is the absorbance of the methanolic blank and $A_{\text{test sample}}$ is the absorbance of the extracts/oils.

The IC_{50} value (μg/mL) is the concentration of the sample or standard required to scavenge 50% of the free radicals. The IC_{50} concentration was calculated by using BioToolKit 320 trial version.

2.2.7. Oxidation stability

The obtained seaweed extracts/SFO were stored in open vials at room temperature (20 ± 2°C), and the samples were analyzed every 7 days for 35 days. The acid value (AV; AOAC, Standard Method 969.1) and peroxide value (POV; AOAC, Standard Method 920.160) were measured.

The oxidative stability of the seaweed extracts/SFO was measured using the Rancimat method (743 Rancimat, Metrohm Co., Basel, Switzerland). A steam of filtered, cleaned, and dried air at flow rate 20 L/h is bubbled into oils (3 g) contained in reaction vessel. These vessels are placed in an electric heating block which is set at 120°C and an effluent air containing volatile organic acids from the oil samples are collected in a measuring vessel with 60 mL of distilled water. The conductivity of the water is continuously recorded and the oxidative stability index of the oil samples were automatically recorded at 120°C for

acceleration test. The conductivity of the samples was measured as induction period (h) (Jung, Kang, & Chun, 2012).

2.2.8. Physical properties of oil

The pH of the seaweed extracts/SFO was measured using a Mettler Toledo Five Easy Plus pH meter at 20°C.

A Brookfield DVII + Pro viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA 02346 USA) was used to measure the viscosity of seaweed extracts/SFO. A SC4-18 spindle with agitation at 150 rpm was then used to measure viscosity, which was expressed in centipoise (cP).

The color of the seaweed extracts/SFO was studied in the CIELab color space using a Minolta CM-2600d (Minolta Camera Co., Osaka, Japan). The following color coordinates were determined: lightness (L*), redness (a*, ± red–green), and yellowness (b*, ± yellow–blue). From these coordinates, hue (H*) and chroma (C*) were calculated as follows:

$$\text{Hue} = \frac{\tan^{-1} b^*}{a^*} \qquad \text{Chroma} = (a^{*2}+b^{*2})^{1/2}. \qquad (5)$$

2.2.9. Experimental design

SFO, soybean oil, canola oil, water (distilled water), and 96% ethanol were used as co-solvents to compare their effectiveness in improving SC-CO_2 extraction of TC, FX, and PT. The steepest experimental design was conducted under the following three sets of conditions: (a) 45°C, 200 bar, and 0.50% co-solvent concentration; (b) 50°C, 250 bar, and 1.25% co-solvent concentration;

and (c) 55°C, 300 bar, and 2.00% co-solvent concentration. The CO_2 flow rate was kept constant at 27 g/min during the entire extraction period of 4 h. The yield of TC, FX, and PT were compared (Fig. 1) with those extracted in the control experiment (SC-CO_2 only).

On the basis of earlier results acquired from the steepest ascent experimental design, the optimization of the extraction conditions with SFO and water as the continuous co-solvent was carried out using RSM. The coded and uncoded independent variables in the RSM design are listed in Table 1. A three-factor and three-level second-order regression for BBD, which gave 17 experimental runs with five central points, was generated. In this work, BBD was used to predict the levels of the factors of temperature (A), pressure (B), and co-solvent [% of CO_2, w/w] (C) (Table 2.1), and this was used to evaluate the combination of extraction variables for the maximal yield of TC, FX, and PT. The experimental data were fitted to a second-order polynomial model, as shown in the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i + \sum \sum_{i<j=1}^{3} \beta_{ij} X_i X_j \qquad (6)$$

Table 2.1. Box–Behnken experimental design with natural and coded SC-CO_2 conditions and experimentally obtained values of total carotenoids (TC), fucoxanthin (FX), and phlorotannin (PT).

Run	Temperature (°C)	Pressure (°bar)	Co-solvent (% of CO_2, w/w)	Total carotenoids (mg/g)		Fucoxanthin (mg/g)		Phlorotannins (mg/g)	
				Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	45.00	200.00	1.25	1.29	1.30	0.40	0.38	0.31	0.31
2	55.00	200.00	1.25	1.31	1.31	0.43	0.41	0.35	0.36
3	45.00	300.00	1.25	1.98	1.98	1.06	1.08	0.80	0.79
4	55.00	300.00	1.25	2.28	2.27	1.24	1.25	0.85	0.84
5	45.00	250.00	0.50	1.39	1.38	0.50	0.49	0.42	0.42
6	55.00	250.00	0.50	1.51	1.51	0.60	0.59	0.44	0.44
7	45.00	250.00	2.00	1.64	1.64	0.77	0.78	0.55	0.55
8	55.00	250.00	2.00	1.81	1.82	0.85	0.87	0.62	0.62
9	50.00	200.00	0.50	1.30	1.30	0.35	0.38	0.30	0.29
10	50.00	300.00	0.50	1.90	1.90	0.98	0.97	0.71	0.71
11	50.00	200.00	2.00	1.36	1.36	0.48	0.49	0.38	0.38
12	50.00	300.00	2.00	2.40	2.40	1.46	1.43	0.92	0.92
13	50.00	250.00	1.25	1.58	1.58	0.67	0.65	0.49	0.50
14	50.00	250.00	1.25	1.58	1.58	0.66	0.65	0.50	0.50
15	50.00	250.00	1.25	1.57	1.58	0.64	0.65	0.50	0.50
16	50.00	250.00	1.25	1.59	1.58	0.65	0.65	0.49	0.50
17	50.00	250.00	1.25	1.59	1.58	0.61	0.65	0.49	0.50

where Y represents the response variable; X_i and X_j are the independent variables affecting the response; and β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for the intercept, linear, quadratic, and interaction terms, respectively. Analysis of variance (ANOVA) was performed to evaluate significant differences between independent variables ($p < 0.05$). To visualize the relationships between the response and the independent variables, the fitted polynomial regression equations were expressed graphically using Design-Expert (version 7.1.3) statistical software (Stat-Ease, Minneapolis, MN, USA).

2.2.10. Kinetic model validation for extraction yield

A simplified mass transfer model based on Brunner's equation was used for mathematical explanation of the extraction curves (Brunner, 1994). The SC-CO_2 extraction kinetics equation for TC, FX, and PT is established as:

$$E = E_\infty(1 - e^{-kt}) \quad (7)$$

where E_∞ is the extraction yield of TC, FX, and PT when time tends to infinity (mg/g); k is the ratio of the solute concentration gradient descending rate; t is the extraction time (min); and E is the extraction yield of TC, FX, and PT when the extraction time is t (mg/g). The analysis of variance (ANOVA) was performed using origin 7.0, release 10 (OriginLab Corp., Northampton, MA, USA). Multiple range tests were used to compare means of the estimated kinetic parameters. Evaluations were based on the $p < 0.05$ significance level.

2.3. Results and discussion

2.3.1. Effects of co-solvents on the yield of various key compounds

Figure 2.2 a, b, and c shows the yields of TC, FX, and PT obtained using SC-CO_2 with various co-solvents under three sets of trial conditions using the steepest ascent method. The yield improved significantly with the addition of any of the co-solvents studied. In general, the yield of TC, FX, and PT increased with increase in the pressure, temperature, and co-solvent concentration. It was therefore concluded that the extraction conditions used (300 bar, 55°C, and 2.0% co-solvent) approached the optimal conditions for most of the co-solvents. These results were in general agreement with the findings of Krichnavaruk et al. (2008), who reported that addition of soybean oil, ethanol, and olive oil as co-solvents significantly improved the yield of astaxanthin from *Haematococcus pluvialis*. These improvements were mainly attributed to an increase in solvent density and swelling of the matrix as a result of co-solvent additions (Krichnavaruk et al., 2008).

Of the five co-solvents examined, SFO was found to be the most effective in improving the extraction yield of TC and FX, followed by canola oil, soybean oil, and ethanol. The TC content was almost equal for all the vegetable oils, while the FX content was higher with SFO as co-solvent; for PT, water as co-solvent showed high content. With SFO as co-solvent, the yield of FX was nearly 2.72–3.38 times higher than the control (SC-CO_2 only). The high effectiveness of SFO on FX could be due to two reasons. First, the solubility of

the co-solvent in SC-CO_2 may play an important role in the extraction process. SFO has less viscosity and good solubility when compared with other vegetable oils, so it makes a good mix with SC-CO_2 (Gao et al., 2010). In contrast, most of the other vegetable oils are poorly miscible with SC-CO_2. Nodar et al. (2002) reported that the solubility of soybean oil in SC-CO_2 was higher than that of SFO at 27.5 MPa and 40°C. However, the trend was reversed in the higher pressure range of 35 to 50 MPa and temperature range of 50 to 70°C. This could be the reason why soybean oil showed lower efficiency than SFO, this was in agreement with the previous works (Gao et al., 2010). Maeda et al. (2007) supported that FX was difficult to dissolve in soybean oil, while it could easily dissolve in fish oil and medium-chain triacylglycerols, and this could be a reason why the yield of FX was higher in SFO. Second, we used high oleic SFO, which is rich in monounsaturated fatty acid (MUFA) (Roman, Heyd, Broyart, Castillo, & Maillard, 2013), and thus the solubility of FX would be enhanced by the presence of more MUFAs in the system, and hence the yield was improved. However, soybean and canola oils, which are less in MUFA and contain longer chains and less volatile FAs, would be the reason for low efficient on the yield of FX and TC (Gao et al., 2010). Previous research also reported that SFO was one of the best vegetable oils for the extraction of TC (Li, Fabiano-Tixier, Tomao, Cravotto, & Chemat, 2013; Sachindra & Mahendrakar, 2005).

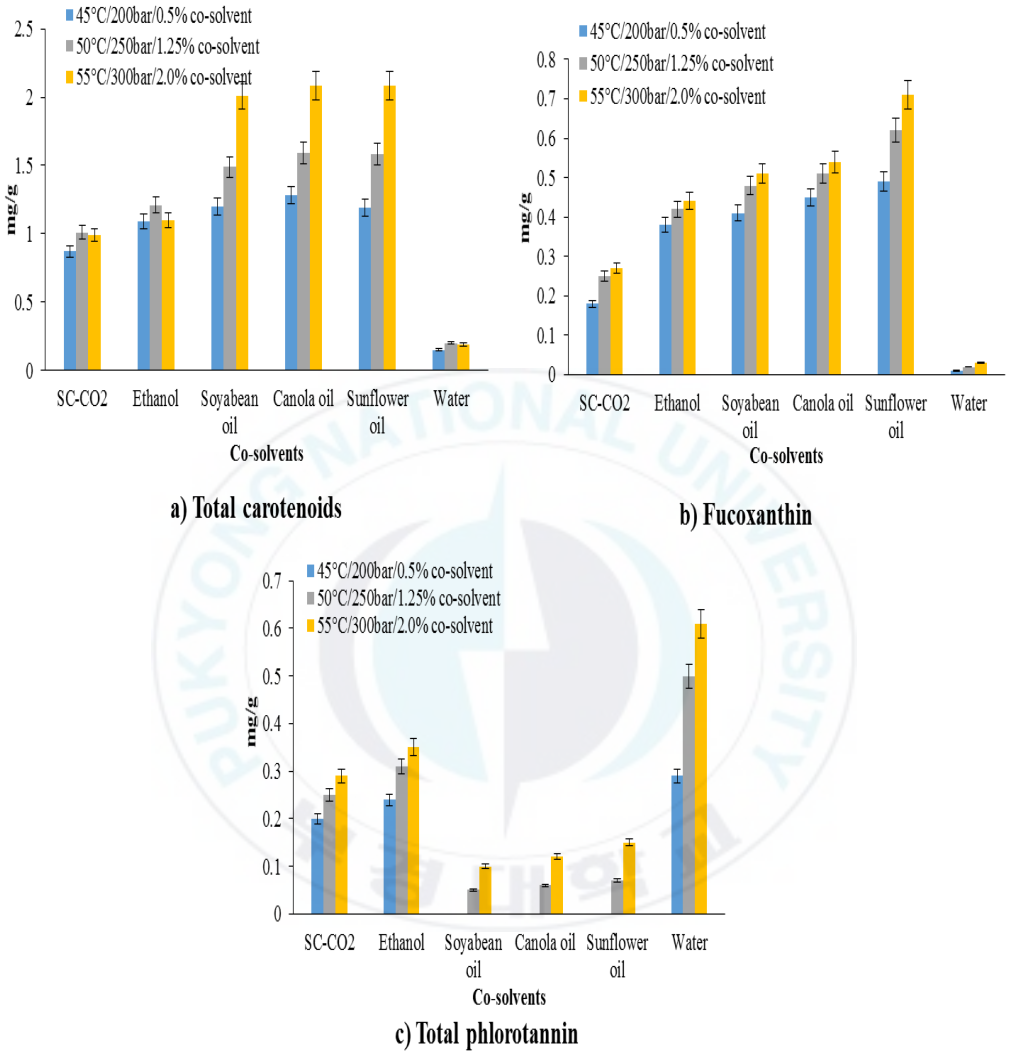

Fig 2.2. Yields of a) total carotenoids, b) fucoxanthin and c) phlorotannin obtained from experiments of the steepest ascent design.

The PT content was found to be higher when water was used as co-solvent. The content was increased as the pressure, temperature, and co-solvent flow rate increased. With water as co-solvent, the yield of PT was nearly 1.45–2.10 times higher than the control (SC-CO_2 only). The high efficiency of water for PT extraction could be due to several reasons. Tannins are an extremely chemically heterogeneous group. Water has a higher dielectric constant than ethanol, thus presenting higher polarity. Moreover, the presence of water can increase the density of the fluid mixture and cause swelling of the solid particles, improving the diffusion process and thereby the solubilization of tannin compounds. These features may have contributed to the higher extraction yield when using water as co-solvent, which has also been reported in several other studies. Pansera et al. (2004) extracted tannins from *Acacia mearnsii* bark using SC-CO_2 and found that the addition of 5% water as co-solvent gave higher yields than the addition of other co-solvents. Markom et al. (2007) showed that the efficiency of SC-CO_2 with water (50%) was higher when compared to Soxhlet and pressurized liquid extraction for the extraction of tannins from *Phyllanthus niruri* L.

2.3.2. Optimization, statistical model, and analysis of response surface

The experimental results of TC, FX, and PT from *S. japonica* using SFO and water as co-solvent are shown in Table 1. ANOVA was used to analyze the experimental data to study the statistical significance of the model terms, and the results are listed in Table 2.2. The highest model *F*-values for TC (2373.32),

FX (218.07), and PT (1277.76) were obtained, and the associated lower *p*-value was $p < 0.0001$ for all three experiments. The lack of a fit *F*-value for TC (1.52), FX (2.13), and PT (1.25) with a low *p*-value ($p < 0.10$) indicated that the model could be used to predict the responses. The R^2 value for TC (0.9996), FX (0.9964), and PT (0.9993) clearly indicated that the selected model accurately represented the actual relationship between the response and independent variables, which were well correlated (Zeković et al., 2014). In this study, the signal-to-noise ratios were found to be 160.550, 49.925, and 119.744 for TC, FX, and PT, respectively, which indicates that the developed models were best fit. A second- order polynomial equation was used to build a mathematical model to find the optimum conditions that maximize the yields of TC, FX, and PT and to study the combined relationships between the process variables and response. The developed second-order models in terms of coded variables are given as follows:

$$\text{Total carotenoids} = 1.58 + 0.077A + 0.41B + 0.14C + 0.069AB + 0.013AC + 0.11BC - 0.010A^2 + 0.14B^2 + 0.013C^2 \quad (8)$$

$$\text{Fucoxanthin} = 0.65 + 0.047A + 0.39B + 0.14C + 0.037AB - 0.00385AC + 0.087BC - 0.001647735A^2 + 0.13B^2 + 0.034C^2 \quad (9)$$

$$\text{Phlorotannin} = 0.50 + 0.023A + 0.24B + 0.074C + 0.002613AB + 0.012AC + 0.031BC + 0.00435A^2 + 0.076B^2 + 0.0048C^2 \quad (10)$$

Table 2.2. Analysis of variance (ANOVA) of the fitted second-order polynomial model for total carotenoids (TC), fucoxanthin (FX), and phlorotannin (PT)

	Sum of	DF	Mean square	F-value	*p*-Value
Total carotenoids[a]					
Model	1.71	9	0.19	2373.32	<0.0001
Residual	0.0005	7	0.00008		
Lack of fit	0.0003	3	0.0001	1.52	0.03
Pure error	0.0002	4	0.00006		
Total	1.715	16			
Fucoxanthin[b]					
Model	1.48	9	0.16	218.07	<0.0001
Residual	0.01	7	0.0007		
Lack of fit	0.003	3	0.001	2.13	0.04
Pure error	0.002	4	0.0005		
Total	1.489	16			
Total phlorotannins[c]					
Model	0.544	9	0.06	1277.76	<0.0001
Residual	0.0003	7	0.00004		
Lack of fit	0.0001	3	0.00005	1.25	0.05
Pure error	0.01559	4	0.00312		
Total	40.41742	16			

[a] S.D.: 0.008; R^2: 0.9996; mean: 1.65; adj R^2: 0.9992; C.V.%: 0.54; pred R^2: 0.9969; adeq precision: 160.550.
[b] S.D.: 0.027; R^2: 0.9964; mean: 0.72; adj R^2: 0.9918; C.V.%: 3.78; pred R^2: 0.9628; adeq precision: 49.925.
[c] S.D.: 0.006; R^2: 0.9993; mean: 0.53; adj R^2: 0.9986; C.V.%: 1.28; pred R^2: 0.9947; adeq precision: 119.744.

The experimental values were fitted to a second-order polynomial model (Eqs. 8, 9, and 10), and multiple regression coefficients were generated for all the responses using a method of least squares (Zeković et al., 2014).

Using Eqs. 8, 9, and 10, it is possible to generate responses of the surfaces shown in Fig. 2. The data in Fig. 2 show an increase in the yield of TC, FX, and PT in the final extract with an increase in the pressure, a decrease in the temperature of the supercritical fluid, and an increase in the co-solvent flow rate. Thus, the best conditions for extracting TC and FX from *S. japonica* were found to be 300 bar, 50.62°C, and 2.00 (% of CO_2, w/w), and the best conditions for extracting PT were found to be 300 bar, 48.98°C, and 2.00 (% of CO_2, w/w). The surfaces of Fig. 2.3 also show that a point of maximum yield of TC, FX, and PT had not yet been reached in the conditions analyzed, which is justified by the limitations of the extraction equipment used since it is not possible for it to operate at pressures above 300 bar.

Considering the possible conditions and substituting these values in Eqs. 8, 9, and 10, the values estimated by the model for the amount of TC, FX, and PT in the extract were 2.405, 1.432, and 0.928 mg/g, respectively. For the optimum conditions, extractions were performed in triplicate, yielding average concentrations of TC, FX, and PT of 2.391 ± 0.419, 1.421 ± 0.181, and 0.927 ± 0.026 mg/g. The FX content in the control (SC-CO_2 only) under the optimized conditions was found to be 0.351 ± 0.027 mg/g; thus, it was found

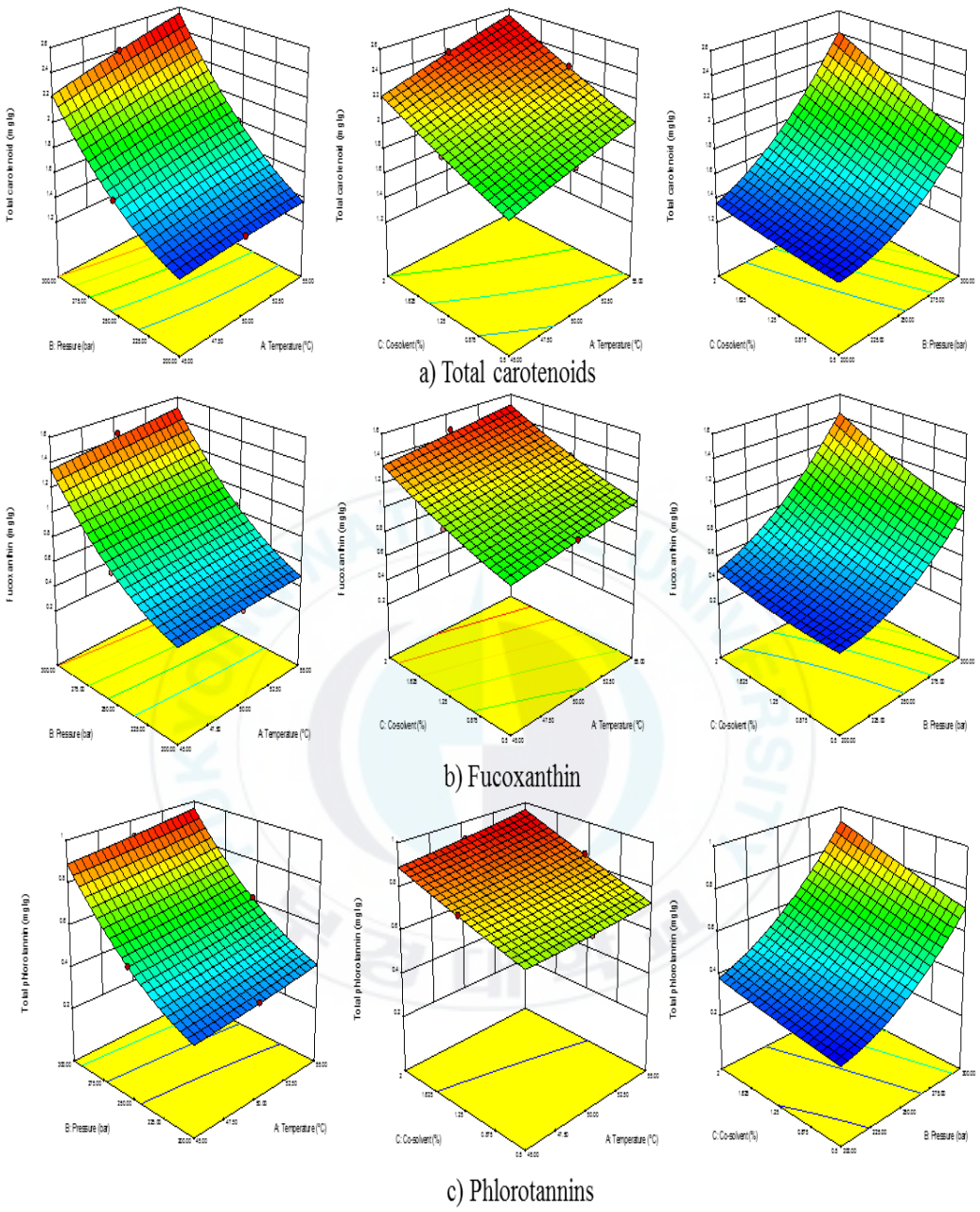

Fig. 2.3. Response surface plots showing combined effects of temperature, pressure and co-solvent flow rate on the yield of a) total carotenoids, b) fucoxanthin and c) phlorotannin.

that by using SFO as co-solvent, the yield of FX can be increased nearly six times.

2.3.3. Measurement of FA composition

The FA compositions for the optimized condition (SC-CO_2 with SFO), control (SC-CO_2 only for the optimized condition), and SFO were determined by gas chromatography, the results of which are shown in Table 3. The percentages of the total saturated FAs were high in the optimized condition extracts, while those of MUFAs were higher in the SFO extract. Oleic acid (C18:1n9c) was found in a high amount (5523.27 ± 60.41 µg/mL) in the optimized conditions, and at lower and higher levels in the control (2142.56 ± 25.64 µg/mL) and SFO (7625.85 ± 10.48 µg/mL) extracts, respectively. Levels of important PUFAs such as eicosapentaenoic acid (EPA; C20:5n3) were slightly increased when using the co-solvent. Under the optimized conditions, EPA was found at a concentration of 321.76 ± 17.05 µg/mL, while in the control, it was slightly lower (299.17 ± 12.33 µg/mL). Dawczynski et al. (2007) reported levels of EPA of 16.2 ± 8.90% in *Laminaria* sp. The obtained value of EPA from our sample is less, which may be due to seasonal and location variations between the seaweed samples.

2.3.4. Antioxidant activities

Antioxidant activity of optimized condition, control, and SFO oil reflects their ability to inhibit the production of free radicals. Antioxidant

Table 2.3. Fatty acid composition of various oils

Fatty acid compositions		(µg/mL)		
		SFO	**SC-CO_2**	**SC-CO_2 with SF**
Saturated fatty acid (SFA)	(C8:0) Caprylic acid	N.D	N.D	0.9±0.01
	(C10:0) Capric acid	N.D	N.D	0.77±0.02
	(C11:0) Undecanoic acid	N.D	N.D	N.D
	(C12:0) Lauric acid	0.43±0.10	1.89±0.01	3.03±0.05
	(C13:0) Tridecanoic acid	N.D	0.6±0.10	1.12±0.01
	(C14:0) Myristic acid	6.71±0.01	820.91±9.33	866.45±16.92
	(C15:0) Pentadecanoic acid	2.43±0.02	33.68±1.59	41.99±1.88
	(C16:0) Palmitic acid	664.27±4.24	1488.73±15.19	1941.24±30.44
	(C17:0) Heptadecanoic acid	424.32±5.98	298.12±1.82	481.21±8.43
	(C18:0) Stearic acid	540.16±3.12	193.42±2.56	393.96±9.27
	(C20:0) Arachidic acid	40.22±1.24	73.63±3.17	86.47±1.28
	(C21:0) Heneicosanoic acid	0.71±0.01	N.D	N.D
	(C22:0) Behenic acid	70.87±0.05	2.82±0.03	11.7±0.10
	(C23:0) Tricosanoic acid	N.D	745.21±10.47	679.7±0.61
	(C24:0) Lignoceric acid	8.35±0.06	0.91±0.01	1.27±0.00
	Total SFA	**1758.47 ± 14.83**	**3659.92 ± 44.28**	**4509.81± 69.02**
Monounsaturated fatty acid (MUFA)	(C15:1) cis-10-Pentadecanoic acid	N.D	N.D	N.D
	(C14:1) Mystoleic acid	N.D	5.23±0.01	6.39±0.02
	(C16:1) Palmitoleic acid	18.14±0.14	204.07±1.28	268.43±1.94
	(C17:1) cis-10-Heptadecenoic acid	7.68±0.05	11.23±0.02	3.56±0.76
	(C20:1) Gondoic acid	40.91±1.24	N.D	12.82±0.99
	(C18:1n9c) Oleic acid	7625.85±10.48	2142.56±25.64	5523.27±60.41
	(C18:1n9t) Elaidic acid	N.D	N.D	N.D
	(C22:1n9) Euric acid	N.D	N.D	N.D
	Total MUFA	**7692.58 ± 11.91**	**2363.09 ± 26.95**	**5814.47 ± 64.12**
Polyunsaturated fatty acid (PUFA)	(C18:2n6c) Linoleic acid	1248.87±15.19	527.62±10.20	1270.24±30.53
	(C18:2n6t) Linolelaidic acid methyl ester	N.D	N.D	N.D
	(C18:3n6) r-Linolenic acid	N.D	147.6±1.66	147.4±7.80
	(C20:2) cis-11,14-eicosadienoic acid	N.D	N.D	N.D
	(C18:3n3) Linolenic acid	10.98±0.02	195.87±5.14	177.46±10.22
	(C20:3n6) cis-8,11,14-Eicosatrienoic acid	N.D	31.85±1.21	27.98±0.20
	(C20:4n6) Arachidonic acid methyl ester	1.53±0.01	N.D	N.D
	(C22:2) cis-13,16-Docosadienoic acid	N.D	N.D	N.D
	(C20:5n3) EPA [Eicosapentaenoic acid]	N.D	299.17±12.33	321.76±17.05
	(C22:6nc) DHA [Docosahexaenoic acid]	N.D	N.D	N.D
	Total PUFA	**1261.38 ± 15.22**	**1202.11 ± 30.54**	**1944.84 ± 65.80**

Values are mean ± SD of three determinations. N.D means not detected.

levels using DPPH and $ABTS^+$ radical cation assays to evaluate free radical scavenging properties (Fig. 2.4). The optimized conditions showed lower IC_{50} values than the control and SFO. This indicates that the antioxidant activity was higher than the other two samples. It seems that when using SFO as co-solvent, the antioxidant activity of the seaweed extract was increased. Gallic acid showed an IC_{50} value of 109.5 µg/ml for DPPH assay, and trolox showed an IC_{50} value of 140.38 µg/ml for $ABTS^+$. Previously, studies on SFO showed that DPPH and $ABTS^+$ had similar high IC_{50} values (Janu et al., 2014). In a similar study, Lee et al. (2013) showed that SC-CO_2 extract of brown seaweed mixed with wheat germ oil showed high antioxidant activity.

2.3.5. Oil stability: Acid value, peroxide value, and Rancimat

Acid value (AV) and peroxide value (POV) were used to measure the quality of the oil during the storage period of 35 days. Fig. 2.5a and b shows the AV and POV of various oils stored at room temperature (20 ± 2°C). The AV of control and optimized condition oil showed an increase due to oxidation processes during the storage period of 35 days. On the other hand, the AV of SFO was slightly increased as the days extended. The POV of SFO rapidly increased during the storage period, but in the control and optimized condition, it was increased very slowly. The results of POV indicated that oils extracted with SC-CO_2 were effective in delaying the initial oxidation process. The AV and POV are dependent on the nature of processing and factors such as the surrounding moisture, air, and temperature (Jung et al., 2012).

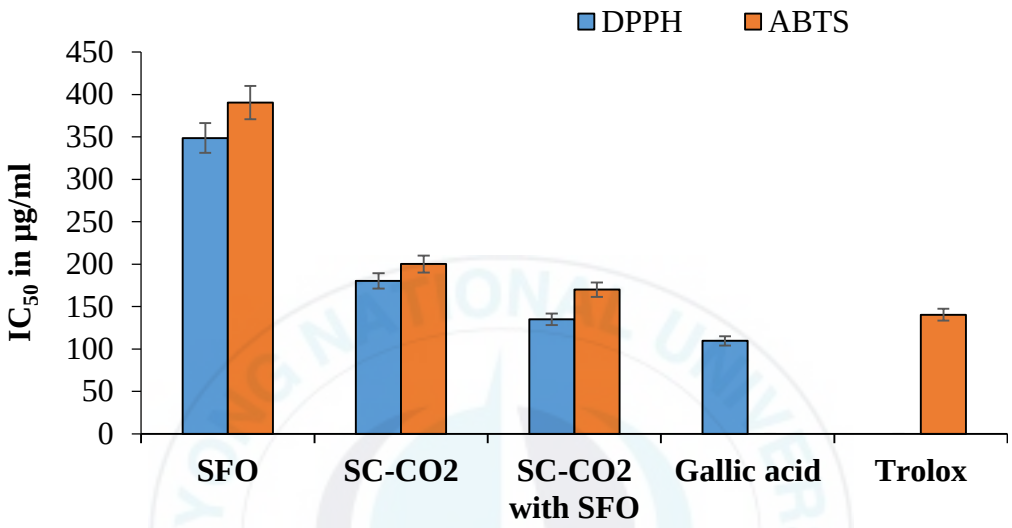

Fig. 2.4. Antioxidant activity of various oils.

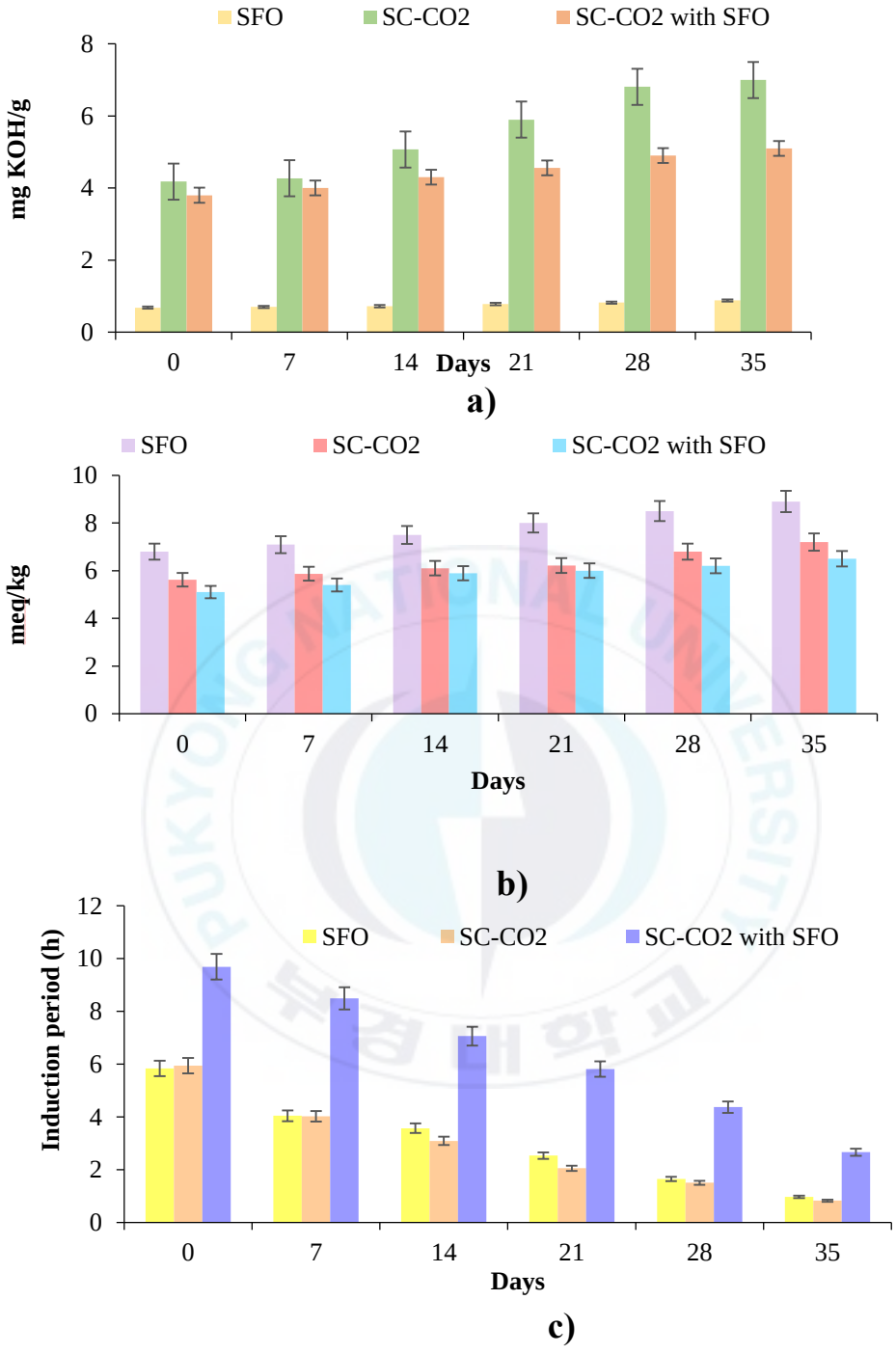

Fig. 2.5. Oil stability measurement of various oils at different storage days. a) Acid value b) Peroxide value and c) Rancimat.

The Rancimat method is commonly used to evaluate the induction period of various oils, and it was measured based on the increase in electrical conductivity due to the formation of volatile dicarboxylic acids as a result of lipid oxidation. Figure 2.5c shows the induction period of all the oils compared during the 35 days of storage. SFO showed an induction period of 5.84 to 0.97 h during the storage period, while the control and optimized conditions showed an induction period of 5.94 to 0.82 h and 9.69 to 2.66 h, respectively. It was found that SC-CO_2 with SFO extracted oil had greater efficiency to delay the oxidation in unfavorable environments, and it showed the longest induction period of 2.66 h at the end of 35 days. This may be due to the presence of some polyphenols that enhance the antioxidants. This finding agreed with our previous results (Lee & Chun, 2013).

2.3.6. Physical properties

The pH and viscosity of the various oils were measured; the values are shown in Table 2.4. The pH values for SFO (6.10 ± 0.05), control (6.68 ± 0.10), and optimized conditions (6.50 ± 0.02) were recorded. The pH of the obtained oils does not show much variation. Given the pH range 6–7, it appears that there is no change between SFO and SC-CO_2 with SFO after the extract.

The viscosity values varied for SFO (39.73 ± 2.23 cP), control (18.18 ± 1.85 cP), and SC-CO_2 with SFO (25.46 ± 3.16 cP) (Table 4). The viscosity values for the control were lower than those for SC-CO_2 with SFO; this is due to SFO, which was used as co-solvent.

Color is one of the most important quality parameters in foods. With regard to color coordinates (Table 2.5), lightness (*L**) values for SFO, control, and SC-CO_2 with SFO were 22.61 ± 2.81, 4.48 ± 1.21, and 6.69 ± 1.27, respectively. Lightness is influenced by the presence of pigments and hygroscopic substances, and an increase in lightness was noted when SFO was used as co-solvent. The lowest lightness values were observed in the control sample. The coordinate redness (*a**, red–green) showed values ranging between 0.56 and 1.85, while the coordinate yellowness (*b**, yellow–blue) showed values ranging between 0.65 and 4.12. The redness and yellowness values of the SFO were higher than those of the seaweed oils. The lower hue values (H) of the *S. japonica* oils could be related to pigment content because lower hue values indicate that higher redness is present in the sample. The chroma value (C) was in the range 4.52 to 0.86. The lightness is influenced by (i) the pigment present and (ii) the presence of hygroscopic substances which when treated thermally increasing their volume and increased light reflected and therefore lightness. The L∗ axis measures lightness (vertical axis), where L∗ =0 for black and L∗ =100 for white. The a∗ axis measures the amount of red and green: a positive a∗ value represents a tendency toward red, while a negative a∗ value indicates a tendency toward green. The b∗ axis measures the amount of yellow and blue: a positive b∗ value represents a tendency toward yellow and a negative b∗ value a tendency toward blue.

Table 2.4. Viscosity and pH measurement of various oils

Oils	Viscosity (Cp)	pH
SFO	39.73 ± 2.23	6.10 ± 0.05
SC-CO_2	18.18 ± 1.85	6.68 ± 0.10
SC-CO_2 with SFO	25.46 ± 3.16	6.50 ± 0.02

Table 2.5. Color properties of various oils

Oils	L*	a*	b*	C	H
SFO	22.61 ± 2.81	1.85 ± 0.49	4.12 ± 0.18	4.52 ± 0.50	65.81 ± 3.92
SC-CO_2	4.48 ± 1.21	0.56 ± 0.26	0.65 ± 0.04	0.86 ± 0.03	49.25 ± 4.18
SC-CO_2 with SFO	6.69 ± 1.27	0.82 ± 0.15	0.68 ± 0.08	1.07 ± 0.20	39.66 ± 1.98

2.3.7. Extraction kinetics for the optimized conditions

Overall extraction curves were determined for the optimized conditions, and the experimental data were used to estimate the kinetic parameters. The extraction curves show that yields increased with the extension of extraction time under all conditions (total extraction time was 4 h, and the samples were collected every 30 min). Figure 2.6 shows that the rate of extraction, or the slope of the curve, was still quite linear. This demonstrated that some of the TC, FX, and PT was still not completely extracted. So to obtain the final yield (E_∞) and k value, a nonlinear fitting method was performed. As is shown in Fig. 2.6 and Table 2.6, the experimental data fits well to the kinetic model. The correlation coefficients obtained were higher or equal to 0.99, indicating that the simplified extraction model used could be applied satisfactorily to estimate the extraction rate and maximum yields of TC, FX, and PT. The values of E_∞ and k are shown in Table 6 for each extraction yield. Further, the kinetic models of SC-CO_2 of TC, FX, and PT from *S. japonica* were set as $E = 2.7716 \times (1 - e^{-0.0082t})$, $E = 2.1602 \times (1 - e^{-0.0052\,t})$, and $E = 1.5167 \times (1 - e^{-0.0036t})$, respectively. Similar results were reported for the optimal extraction of lutein from *Chlorella pyrenoidosa* by subcritical CO_2 (Fan, Hou, Huang, Qiu, & Jiang, 2015).

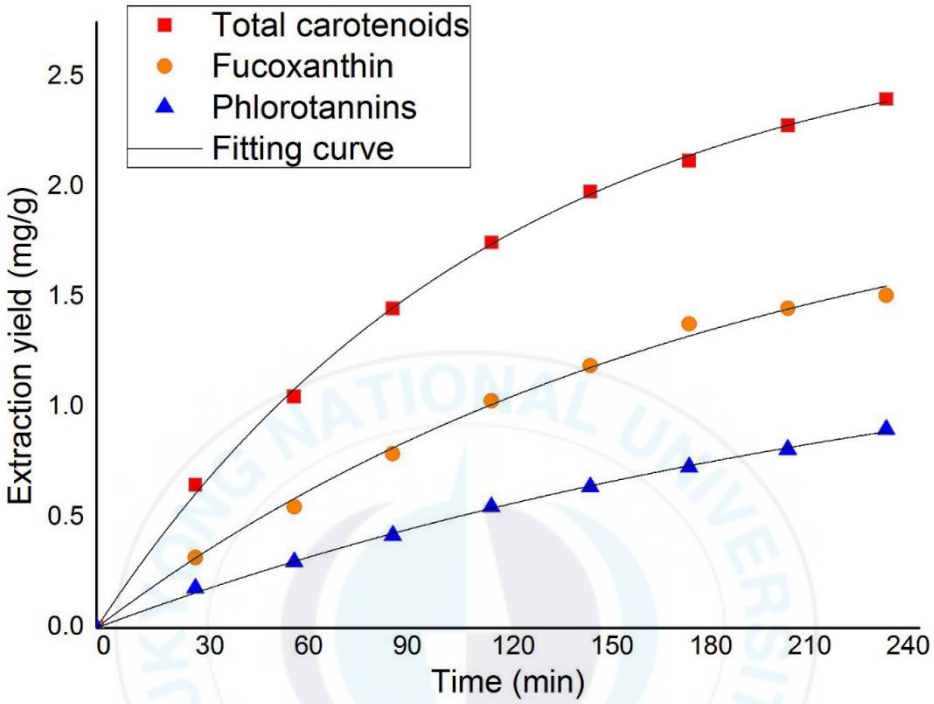

Fig. 2.6. Model fitting curve of extraction yield for optimized condition.

Table 2.6. E_∞ and k values from the fitting of Eq. (7) to the extraction curves of Fig. 2.6

Extraction yield	E_∞	k	R^2
Total carotenoids	2.7716±0.0444	0.0082±0.0002	0.9919
Fucoxanthin	2.1602±0.1327	0.0052±0.0005	0.9964
Phlorotannin	1.5167±0.0853	0.0036±0.0002	0.9987

2.4. Conclusion

The addition of vegetable oils and water as co-solvents significantly increased the efficiency of SC-CO_2 extraction of TC, FX, and PT from *S. japonica*. Of the five co-solvents tested, SFO was found to be the most effective in improving the extraction yield of TC and FX, while water as co-solvent improved the yield of PT. RSM was employed to optimize SC-CO_2 extraction conditions with SFO and water as co-solvents. The best conditions for the yield of TC and FX were 50.62°C, 300 bar, and 2.00% with SFO, while for PT, the best conditions were 48.98°C, 300 bar, and 2.00% with water. Experiments performed under the optimal conditions reached 2.391 ± 0.419 mg/g for TC, 1.421 ± 0.181 mg/g for FX, and 0.927 ± 0.026 mg/g for PT. Kinetic models were established for the optimized conditions, and the E_∞ and k values illustrate that the model can also be a significant guide to the study of the elucidation of the extraction process. The extracted oil from SC-CO_2 with SFO as co-solvent also showed a rich content of FAs, high antioxidant activity, and high oil stability. Therefore, the obtained oil, with its rich content of bioactive materials, can be used in foods, pharmaceuticals, and cosmetics.

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Chapter 3

Evaluation of the chemical composition of brown seaweed (*Saccharina japonica*) hydrolysate by pressurized hot water extraction

3.1. Introduction

Saccharina japonica (Dashi kombu) is an aquatic class of Phaeophyceae (brown algae), a kind of kelp or seaweed, which is widely cultured in China, Japan, and Korea. It is an industrially orientated species. *S. japonica*, also known as "Kombu" (in China "Haidai," in Korea "Dasima"), is a vital part of the diet for people in Japan. Bulky reaps are grown using rope farming, an easy way to cultivate macroalgae by hanging them to moving ropes along the sea. Recently, sulfated polysaccharides from the brown seaweed *S. japonica* were studied for their structures and functional materials (Vishchuk, Ermakova, & Zvyagintseva, 2011). A recent study showed that polysaccharides obtained from brown seaweeds were mainly composed of fucose, galactose, and sulfate groups, along with a trace amount of other monosaccharides (Synytsya et al., 2010). Sulfated polysaccharides from *S. japonica* are widely explored because they vary from common polysaccharides, which mostly contain sulfated fucose.

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These sulfated galactofucans have an extensive range of biofunctional properties (J. Wang, Zhang, Zhang, Zhang, & Niu, 2010).

Recent reports are focused on algae species because of their potential as a source of nutrition and nutritional characteristics, including their composition of fatty acids (Schmid, Guihéneuf, & Stengel, 2014), amino acids (Mišurcová et al., 2014), and dietary fiber (Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2010). The health benefits of the intake of seaweeds have also been reported (Sánchez-Muniz, 2012).

Water is a globally neutral solvent and nontoxic at room temperature, whereas H_2O molecule above its critical temperature and pressure, which is known as supercritical water, can behave as an organic solvent and acidic medium (Gallezot, 2012). Because of the alteration in this condition, some physical properties of water beyond their critical points (T_m > 374°C and T_p > 220 atm). It is an environmentally friendly processing agent and can also offer greater extraction yields from various samples (Özel & Göğüş, 2014; Vishchuk et al., 2011). Pressurized hot water extraction (PHWE) is performed by means of boiling water (from 100 to 374°C) with a pressure (usually from 10 to 60 bar) to uphold the water in liquid state. The key factor to study the various types of extraction procedures is the inconsistency of the dielectric constant with temperature. At room temperature, water is a polar solvent with a dielectric constant of 80. The dielectric constant significantly changes when the water is heated up to 250°C, where the value will be 27, but the liquid state can be maintained by retaining a suitable pressure (Carr, Mammucari, & Foster, 2011).

The equipment can be attached with a chilling machine for fast chilling of the product for immediately obtaining the pressurized hot water extract (Herrero, Cifuentes, & Ibanez, 2006). PHWE of biomass presents several advantages compared with traditional technologies (acid, alkali, and enzymatic hydrolysis). Its main advantage is that it does not use organic solvents, which is a factor of major importance in any process because organic solvents must be recycled, incinerated, or submitted to an appropriate unitary operation, resulting in a non-aggressive waste to the environment (Yu, Lou, & Wu, 2007). Moreover, it does not require biomass pretreatment; it is fast, presents lesser corrosion, lower residue generation, and lower sugar degradation than conventional hydrolysis methods (Zhao, Lu, Wu, Liu, & Wang, 2012).

On the other hand, it has been reported that during PHWE of macroalgae (Plaza, Amigo-Benavent, Del Castillo, Ibáñez, & Herrero, 2010), browning compounds, i.e., Maillard reaction products (MRPs), are formed due to the reaction of the carbonyl group of a reducing sugar with the free amino group of amino acids. It was identified to be useful as an antioxidant in herbal drugs and foods. In particular, it was reported that the antioxidant activity increased with the heating strength, in similar with color development and MRPs were responsible for the majority of the antioxidant activity, in hydrolysis macroalgae (Plaza et al., 2010). Therefore, it will be of good value to measure changes in MRP levels.

Thus, the aim of this study was to calculate the use of PHWE under different temperatures to identify the chemical composition of *S. japonica* like

total organic carbon (TOC), pH, MRPs, viscosity, color, amino acids, minerals, and monosaccharides obtained. Our findings could relate the nutritional composition of *S. japonica* obtained by PHWE, which can be considered important in human health and also in the fermentation industry.

3.2. Materials and methods

The brown seaweed *S. japonica*, J.E. Areschoug, 1851, Lane, Mayes, Druehl, and Saunders was collected from Guemil-eup, Wando-gun, Jeollanam-do, Republic of Korea. High-purity nitrogen gas (99.99%) was supplied by KOSEM (Yangsan, Republic of Korea). All reagents used were of analytical or high-performance liquid chromatography (HPLC) grade, and galactose, glucose, fructose, arabinose, mannose, mannitol, sorbitol, xylitol, and xylose standards (purity > 98%) were purchased from Sigma–Aldrich (St. Louis, MI, USA).

After washing fresh *S. japonica* samples with fresh water, unused materials, attached salts, and minerals were removed, and the samples were cut into small pieces. The pieces were dried at −80°C for 3 days in a freeze dryer (Eyela FDU-2100, Tokyo Rikakikai Co., LTD, Japan) equipped with a square-type drying chamber (Eyela DRC-1000, Tokyo Rikakikai Co., LTD, Japan). The dried samples were collected into sealed plastic bags. The samples were then finely ground using a mechanical blender (PN SMKA-4000 mixer, PN Co., Ltd., Ansan-si, Korea) and were sieved through a 710-μm stainless steel sieving mesh.

3.2.1. Pressurized hot water extraction

PHWE was performed in a 200-cm^3 batch reactor made of Hastelloy C276 (continuous-type supercritical water system, Phosentech, South Korea) with temperature control (Fig. 3.1). A total of 6 g of sample material was loaded into the reactor with 150 mL of distilled water. The vessel was then locked and heated using an electric heater to the required temperature (180°C–420°C). Pressures were determined on the basis of saturated steam to be between 13 bar and 520 bar for the temperature range studied. The temperature and pressure in the reactor were controlled using a temperature controller and pressure gauge, respectively. The sample was stirred using a four-blade stirrer at 150 rpm, and after reaching the desired temperature, 5 min of reaction time was maintained. Hydrolysate samples from the reactor were collected after reaching room temperature (within 1 or 2 h after stopping the reaction), filtered using Whatman nylon membrane filter (0.45 μm), and stored at 4°C. The residual samples recovered after PHWE were dried, and their weight was measured in grams (g).

3.2.2. Extraction yield

The extraction yield was calculated according to Plaza et al. (2013) with a slight modification.

Extraction yield (%) = dry extract weight /initial dry sample weight (1)

The *S. japonica* hydrolysate was kept in an oven at 120°C for 24 h to obtain the dry extract weight, and the initial dry sample weight of the freeze-dried *S. japonica* was calculated through the subtraction of water content with the total

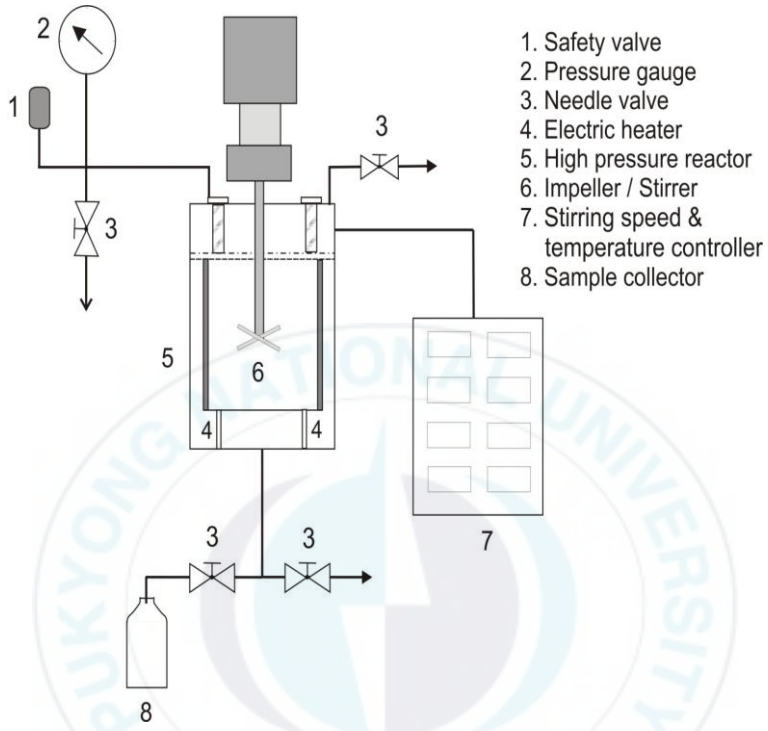

Fig. 3.1. Flow diagram of pressurized hot water extraction (PHWE).

weight. The freeze-dried *S. japonica* was taken to dryness in an oven at 120°C for 24 h. This process was conducted in triplicate. The percent of water content in the freeze-dried *S. japonica* was 10.69 ± 0.64%.

3.2.3. Analysis of total organic carbon and milliard reaction products

The carbon content of the *S. japonica* hydrolysate water was determined using TOC-Vcph, SSM (Shimadzu). The analysis was performed using 1000 ppm of hydrolysate water of each condition.

The browning intensities of *S. japonica* hydrolysate were determined using MRPs (melanoidins). These extracts were filtered, and the browning intensity was directly measured at 360 nm and 420 nm (Plaza et al., 2010). It was expressed as arbitrary absorbance units (A.U.).

3.2.4. Physical properties: Viscosity, color and pH

A Brookfield DVII + Pro viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) was used to measure the viscosity of *S. japonica* hydrolysate according to the method described by Ogawa et al. (2004) with slight modifications. In total, 8 mL of 0.1% (w/v) sample in 0.1 M acetic acid was incubated at 25°C for 10 min and then placed in a vessel. Spindle SC4-18 with agitation at 150 rpm was then used to measure viscosity, which was expressed as centipoise (cP).

The color of the *S. japonica* hydrolysate was studied in the CIELab color space using Minolta CM- 2600d (Minolta Camera Co., Osaka, Japan) with illuminant D65, 10° observer, SCI mode, 11-mm aperture of the instrument for

illumination and 8 mm for measurement. A low-reflectance glass (Minolta CR-A51/1829-752) was placed between the samples and equipment. The following color coordinates were determined: lightness (L*), redness (a*, ±red–green), and yellowness (b*, ±yellow–blue). From these coordinates, hue (H*) and chroma (C*) were calculated as follows:

$$\text{Hue} = \frac{\tan^{-1} b^*}{a^*} \qquad \text{Chroma} = (a^{*2} + b^{*2})^{1/2} \qquad (2)$$

3.2.5. Chemical composition

The freeze-dried algae samples (400 mg) were hydrolyzed with 6 N hydrochloric acid in an ampoule containing 0.1% phenol (for the protection of tyrosine) for 24 h at 110°C. After acid hydrolysis, 30 ml of citrate buffer (pH 2.2) was added, and the pH was adjusted between 0.5 and 1 with 7.5 N NaOH and to pH 2.2 with 1 N NaOH. The sample obtained was diluted to 100 ml with citrate buffer after adding 1 ml of 50 μM norleucine solution (as an internal standard), while the *S. japonica* hydrolysate obtained by PHWE were filtered (0.22-μm cellulose acetate filter) and loaded onto a S430 (SYKAM) amino acid autoanalyzer for free amino acid analysis. A cation separation column LCA K07/Li (4.6 × 150 mm) with a column temperature of 37°C–74°C and buffer pH range of 2.90–7.95 was used for free amino acid analysis. The mobile phase was 5 mM of p-toluenesulfonic acid solution at a flow rate of 0.45 mL/min. A mixture of 5 mM p-toluenesulfonic acid, 20 mM of bis-tris, and 100 mM of EDTA was used as the post-column reagent at a flow rate of 0.25 mL/min. Excitation and emission wavelengths were kept at 440 and 570 nm, respectively.

The individual amino acids stock solutions were prepared in 0.1 M HCl; tryptophan was prepared in water (Asaduzzaman & Chun, 2014).

The *S. japonica* hydrolysate and freeze-dried samples were prepared as described by Rocha et al. (2009) with minor modifications. The hydrolysate was filtered by 0.45-μm syringe filters (Sartorius), immediately acidified with HNO_3 to a pH of <2 and stored in a precleaned (rinsed with 10% HNO_3 followed by rinsing with MilliQ water) high-density polyethylene vial. Filtration was done just before measurement to eliminate tiny materials present in the sample, which are undesirable for PerkinElmer Elan 6100 ICPMS (PerkinElmer, Waltham, MA, USA) measurement.

The levels of galactose, glucose, fructose, arabinose, mannose, mannitol, sorbitol, xylitol, and xylose were measured using HPLC with an evaporative light scattering detector. HPLC analysis was performed using a Jasco HPLC (Easton, USA) model 400 equipped with a ChromNav analysis software. High-purity nitrogen (99.99%) from KOSEM Co. was used as the carrier gas. A Shodex (Japan) sugar column (SP0810) of 300 mm, thermostated to 80°C, was used to analyze monosaccharides. The water used for elution was filtered using a cellulose acetate filter (0.22 μm) and then sonicated. The flow rate of the eluent was maintained at 0.6 mL/min. Monosaccharides of freeze-dried algae samples (50 mg) were hydrolyzed with 1 M H_2SO_4 (3 ml) at 100°C for 3 h. The reaction medium was then neutralized with 1 mL N_4OH (15 M), and 1 mL of 2-deoxy-D-glucose (1 mg/mL) was added as the internal standard, while the hydrolysate samples were diluted four fold using filtered and sonicated water (HPLC grade).

The sample was filtered through a 0.2-μm nylon filter before they were analyzed by HPLC (Meillisa, Woo, & Chun, 2015).

3.2.6. Statistical analysis

All mean values were analyzed by one-way analysis of variance (ANOVA). Values are expressed as mean ± standard deviation. (SD; n = 3 replicates) (SPSS software; version 20 for windows, IBM, Chicago, IL, USA). Statistical analysis was performed using Tukey test, and $P < 0.05$ was considered to be significant.

3.3. Results and discussion

3.3.1. Extraction yield

PHWE experiment temperatures ranged from 180°C to 420°C, and the time taken to reach the desired temperature varied from 30 min to 105 min. The pressure was monitored using the pressure gauge, and it ranged from 13 bar to 520 bar. Usually, the product acquired was a solid–liquid mixture. After waiting (until it reaches room temperature) for precipitation to occur, two layers of the hydrolysate were formed. The upper part was an aqueous solution, which was clearer and less viscous, whereas the lower layer was a wet seaweed residue. Degraded products obtained from the hydrolysis of *S. japonica* appeared dark brownish and contained a blend of some liquid portion and little solid substances. The hydrolysate had a toasty aroma at low temperature, but it became more pungent with an increase in temperature. The samples were filtered using the Whatman nylon membrane filter (0.45 μm) and stored at 4°C. The residual

samples that were recovered after PHWE were dried, and their weight was measured in grams (Table 1). The residue content varied from 1.56 (180°C/13 bar) to 0.59 (420°C/520 bar). This clearly showed that a rise in temperature degrades the solid sample. Similar results were reported by Park et al. (2012).

The extraction yield of the *S. japonica* hydrolysate at different temperatures and pressures are shown in Table 1. They varied from 72.21 to 98.91 weight percent (dry weight). As can be seen in Table 1, temperature and pressure directly influenced the extraction yield. The effect of increasing yield with increasing temperature has been extensively observed in PHWE (Plaza et al., 2013), and it is explained by the increasing mass transfer, lower surface tension, and higher solubility of numerous compounds. Watchararuji et al. (2008) reported that PHWE of rice bran and soybean meal has a high hydrolysis yield because of strong accumulation through hydrophobic interactions; the resultant protein matter has low solubility in water at ambient temperature (Prado et al., 2014).

Corrosion in the PHWE environment is of concern. In particular, oxidizing and acidic environments can result in quick corrosion, and it will be more severe at subcritical conditions than at supercritical conditions owing to the relatively dense and polar character of subcritical water. The reactor frequently used for PHWE was made of nickel alloys such as Inconel 625 and Hastelloy C276; titanium alloys also have good resistance (Toor, Rosendahl, & Rudolf, 2011). In our experiment, we used Hastelloy C276 as it has a good resistance to corrosion when used at high temperature conditions. The

hydrolysate obtained from various PHWE conditions was examined for TOC, pH, MRPs, viscosity, and color, and amino acid, mineral, and monosaccharide contents.

3.3.2. Total organic carbon, milliard reaction products and pH

The amounts of TOC in the *S. japonica* hydrolysate obtained in each temperature condition are shown in Table 3.1. The 180°C/13 bar condition contained 18.64 ± 0.13 mg/L of carbon in the *S. japonica* hydrolysate, which essentially comprised sugars and organic acids. TOC of hydrolysate products obtained in each temperature condition varied. The amount of TOC (75.20 ± 1.69 mg/L) dramatically increased for the 420°C/520 bar condition. Thus, the result showed that TOC increases with an increase in temperature. Similar results were reported by Garcia et al. (2013) and Sereewatthanawut et al. (2008) reported that the TOC content constantly increased due to the raise in temperature and that these type results are found in some food items when we cook. Further, there was a possible reduction of organic carbon into carboneous gas products because of oxidation. Although vaporous content was not investigated in this study, earlier studies described that some products released from the oxidation of biomass contain volatile carbon and water (Sato, Quitain, Kang, Daimon, & Fujie, 2004).

The pH of the obtained *S. japonica* hydrolysate was measured; the values are shown in Table 3.1. The pH values varied from 4.91 ± 0.00 (180°C/13 bar) to 7.95 ± 0.02 (420°C/520 bar) in the *S. japonica* hydrolysate, and the

values were found to increase with an increase in temperature. The pH was increased at higher temperatures due to the formation of salts and degradation of all organic matter. The low pH was because of the degradation of sugars into organic acids, and these organic acids undergo a chain reaction, providing the acidity for increasing the speed of subsequent reactions as an autocatalytic process (Sasaki et al., 1998). Gao et al. (2014) reported that when sucrose was treated in subcritical water or water ethanol mixtures, the pH decreases due to the degradation of glucose and fructose products into acidic compounds. In subcritical water, the pH decreased further after the sucrose was completely hydrolyzed. Other than protein hydrolysis products, the products of hydrolysis of carbohydrate content of the rice bran were also resulted by non-selective hydrolysis reaction in subcritical water. Investigation on other possible uses of the hydrolysis product for human consumption such as uses as additives in bakery foods or drinks or as dietary supplements is currently underway. When carbohydrate reacts with hydronium and hydroxide ions, reducing sugars are produced. Lower yields obtained with alkali hydrolysis .could be a result of high pH condition which could lead to severe molecular cross-linking and rearrangements, resulting not only in the decrease in nutritive value, but also possible formation of toxic compounds such as lysinoalanine (Sasaki et al., 1998).

Table 3.1. Experimental summary of residue obtained after PHWE, yield, TOC, pH, Maillard reaction products (MRPs) and viscosity at different PHWE conditions

Conditions (°C/ bar)	Residue obtain after PHWE (g)	Yield (%)	TOC (mg/L)	pH	MRPs (A.U)		Viscosity (c.p)
					Absorbance at 360 nm	Absorbance at 420 nm	
180/13	1.56	72.21^{a}	18.64±0.13^{j}	4.91±0.00	2.51±0.00^{g}	1.90±0.02^{e}	6.80±0.20^{l}
200/17	1.35	75.01^{a}	19.05±0.00^{j}	4.94±0.00	3.00±0.00^{f}	1.95±0.02^{d}	6.50±0.15^{k}
220/25	1.23	78.10^{b}	20.01±0.31^{j}	5.05±0.00	3.21±0.05^{e}	1.96±0.01^{c}	6.10±0.10^{j}
240/34	1.18	79.82^{b}	22.20±0.13^{i}	6.03±0.01	3.30±0.01^{d}	1.97±0.00^{b}	5.80±0.05^{i}
260/49	1.03	81.37^{c}	25.53±0.10^{h}	6.57±0.00	3.40±0.01^{c}	1.97±0.02^{b}	5.50±0.00^{h}
280/72	0.95	85.64^{c}	30.14±0.06^{g}	6.75±0.00	3.61±0.01^{b}	1.97±0.02^{b}	4.80±0.45^{g}
300/100	0.87	90.20^{c}	39.45±0.43^{f}	6.84±0.03	3.61±0.01^{b}	2.00±0.01^{a}	4.50±0.18^{f}
320/120	0.79	92.45^{c}	47.16±0.22^{e}	6.96±0.02	3.99±0.11^{a}	2.00±0.00^{a}	4.10±0.90^{e}
350/150	0.75	94.04^{c}	56.50±0.80^{d}	7.28±0.01	4.00±0.00^{a}	2.00±0.01^{a}	3.90±0.05^{d}
375/260	0.71	94.15^{d}	58.41±0.21^{c}	7.45±0.00	4.00±0.00^{a}	2.01±0.01^{a}	3.50±0.00^{c}
400/400	0.64	95.67^{d}	61.19±0.49^{b}	7.64±0.01	4.00±0.00^{a}	2.01±0.00^{a}	3.10±0.01^{b}
420/520	0.59	98.91^{d}	75.20±1.69^{a}	7.95±0.02	4.00±0.01^{a}	2.01±0.00^{a}	2.90±0.05^{a}

Values are expressed as mean ± SD. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

It was broadly recognized that the different intensities of brown color can be efficiently used to observe non-enzymatic browning reactions, including the Maillard reaction. Visual observation was one of the simplest ways to detect the presence of MRPs. For this purpose, the obtained MRP value has been frequently engaged as an indicator of a wide range of Maillard reactions as an indicator of the occurrence of caramelization, also in foods. The increase in the browning effect is directly related to the advanced phases of the reaction. The absorbance at 360 nm and 420 nm is usually engaged to check the development of browning reaction of MRP (Plaza et al., 2013). The *S. japonica* hydrolysate had a significant content of MRPs based on the browning measurements at 360 nm and 420 nm (Table 3.1). The MRP content was high at the 420°C/520 bar (4.00 ± 0.01 A.U. at 360 nm, 2.01 ± 0.00 A.U. at 420 nm) and very low at the 180°C/13 bar (2.51 ± 0.00 A.U. at 360 nm, 1.90 ± 0.02 A.U. at 420 nm) conditions. These absorbance values were employed as indicators of caramelization and formation of brown advanced MRPs in thermally processed foods. Our data showed an increase in the formation of MRPs as the extraction temperature increased in PHWE of *S. japonica*. The formation of MRPs during PHWE of several samples has also been reported in other studies (He et al., 2012). MRPs include a wide range of compounds of significant importance for the nutritional value of food and beverages. Some of these compounds have strong antioxidant activities, but others such as hydroxymethylfurfural can be toxic and mutagenic (Vergara-Salinas, Vergara, Altamirano, Gonzalez, & Pérez-Correa, 2015).

3.3.3. Physical properties of seaweed hydrolysate

The viscosity of the obtained *S. japonica* hydrolysate was measured, and the values are shown in Table 3.1. The viscosity values varied from 6.8 ± 0.20 cP (180°C/15 bar) to 2.90 ± 0.05 cP (420°C/520 bar) in the *S. japonica* hydrolysate. The viscosities showed decreasing trends with an increase in temperature. Schrieber et al. (2007) reported that the molecular weight of hydrolysates can influence the viscosity of solutions and that hydrolysates with low molecular weight have a tendency to produce a solution of low viscosity that was easier to process even at a relatively high concentration. Hawthorne et al. (2002) described that a decrease in viscosity and surface tension with an increase in temperature improves the mass transfer rates of compounds from plant materials. Therefore, viscosity is an important measurement for the industry to monitor and control hydrolysis.

Color is one of the most important quality parameters in foods. With regard to color coordinates (Table 3.2), lightness (L^*) values ranged between 24.06 ± 3.12 and 37.10 ± 0.80, values obtained at different conditions of the hydrolysate. Lightness is influenced by the presence of pigments and hygroscopic substances, which when thermally treated increase their volume and reflected light. The lower lightness values observed in the hydrolysate at higher temperature could be associated with higher browning reactions that could take place in these samples. The coordinate redness (a^*, red–green) showed values ranging between 3.20 and 4.00, while the coordinate yellowness (b^*, yellow–blue) showed values ranging between 1.36 and 2.34. The redness

and yellowness values of the hydrolysate obtained at the 420°C/ 520 bar condition were higher than those at other conditions. The lower hue values (H) of the *S. japonica* hydrolysate could be related to browning reactions because lower hue values indicate that higher redness is present in the sample. The hue values increased with an increase in temperature. The chroma value (C) was in the range of 4.33 ± 0.14 to 6.41 ± 0.23.

3.3.4. Chemical composition of seaweed hydrolysate from various conditions

Amino acids play a key function in the human body to construct a mass of proteins and as intermediaries in metabolism. Various amino acids taste sweet and bitter and also give flavor to food (Wiboonsirikul, Hata, Tsuno, Kimura, & Adachi, 2007). The non-essential amino acid (NEAA) and essential amino acid (EAA) yields of the *S. japonica* hydrolysate generated by treatment at various temperatures are shown in Table 3.3. The NEAA yield was higher than the EAA yield in the extract. The highest yield of total amino acids of the *S. japonica* hydrolysate was found at 180°C (761.95 ± 14.54 mg/g). The amino acid yield was reduced at temperatures above 240°C. All EAAs, except phenylalanine and tryptophan, were detected in the *S. japonica* extract. Among EAAs, threonine, valine, isoleucine, and leucine were abundant. The highest yields for individual EAAs were for threonine (6.15 ± 0.37 mg/g), valine (12.63 ± 0.38 mg/g), isoleucine (4.03 ± 0.48 mg/100 g), and leucine (12.24 ± 0.65 mg/g) at 180°C.

All NEAAs, except proline, were detected in the *S. japonica* extract. The highest yields for NEAA were found at 180°C for serine (6.70 ± 0.36 mg/g), aspartic acid (280.13 ± 2.06 mg/g), glutamic acid (105.82 ± 1.54 mg/g), glycine (201.16 ± 7.14 mg/g), alanine (71.08 ± 0.86 mg/g), and arginine (61.66 ± 0.67 mg/g). As the temperature increased, the amino acid and total protein contents also decreased. At the 240°C/34 bar condition, the total protein content was (274.8 ± 12.48 mg/g). EAAs such as threonine (3.11 ± 0.30 mg/g), valine (6.26 ± 0.25 mg/g), isoleucine (1.96 ± 0.30 mg/100 g), and leucine (3.90± 0.26 mg/g) and NEAAs such as serine (5.20 ± 0.01 mg/g), aspartic acid (51.33 ± 2.68 mg/g), glutamic acid (63.63 ± 2.41 mg/g), glycine (51.17 ± 3.02 mg/g), alanine (34.88 ± 1.44 mg/g), and arginine (53.27 ± 1.80 mg/g) were found at the 240°C/34 bar condition. Asaduzzaman et al. (2014) reported that amino acids are converted into organic acids or volatile resources during hydrolysis, resulting in the substantial reduction of amino acid content using hydrolyzed processing of marine resources. Therefore, it can be assumed that high temperatures lead to the reduction of amino acids into organic acids or other byproducts. Cheng et al. (2008) et al. reported that the yields of most amino acids were high at reaction temperatures of 180°C–220°C.

Table 3.2. Color properties of *S. japonica* at different PHWE conditions

Conditions (°C/ bar)	Color				
	L*	a*	b*	C	H
180/13	37.10±0.80^{a}	4.89±1.09^{a}	1.36±0.14^{g}	5.08±0.05^{b}	15.54±1.89^{g}
200/17	34.56±5.35^{b}	4.78±0.40^{b}	2.1±0.32^{f}	5.22±0.02^{b}	23.71±2.07^{f}
220/25	32.68±6.32^{c}	4.00±2.14^{c}	2.34±0.86^{f}	4.63±0.17^{c}	30.32±1.03^{e}
240/34	31.38±7.85^{d}	3.82±3.23^{d}	2.46±0.53^{f}	4.54±0.21^{c}	32.78±5.00^{e}
260/49	30.97±3.45^{e}	3.20±0.10^{d}	2.91±0.90^{e}	4.33±0.14^{c}	42.28±2.78^{d}
280/72	30.08±6.00^{f}	2.89±0.43^{e}	3.01±0.25^{d}	4.17±0.11^{c}	46.16±3.94^{d}
300/100	29.10±7.21^{g}	2.71±0.71^{e}	3.6±0.10^{d}	4.51±0.05^{c}	53.02±1.65^{c}
320/120	28.49±5.10^{h}	2.56±1.23^{e}	4.29±0.77^{c}	5.00±0.02^{b}	59.17±0.77^{c}
350/150	27.99±6.90^{i}	2.21±0.13^{e}	4.56±0.35^{c}	5.07±0.02^{b}	64.14±0.19^{b}
375/260	27.45±4.50^{j}	2.01±0.54^{e}	5.10±0.02^{b}	5.48±0.06^{b}	68.48±1.80^{b}
400/400	25.12±8.25^{k}	1.97±0.24^{f}	5.48±0.20^{b}	5.82±0.04^{b}	70.22±2.31^{a}
420/520	24.06±3.12^{l}	1.89±0.65^{g}	6.12±0.15^{a}	6.41±0.23^{a}	72.83±2.07^{a}

L- lightness, a- redness, b- yellowness, C- chroma, and H-hue. Values are expressed as mean ± SD. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test

In the report by Mabeau and Fleurenc et al. (1993) the aspartic and glutamic acids levels were high and were major factors for the taste and flavor of seaweed. The total amino acid content in *S. japonica* was reported by Shin et al. (2011); when compared with this result, even after hydrolysis, almost all amino acid contents were maintained until 240°C, after which all amino acids were destroyed.

The dominant molecule in brown seaweed is a carbohydrate, with alginate identified as the major constituent. Alginate is found in most brown seaweeds as a physical constituent for the formation of cell wall, and it forms an insoluble salt, chiefly comprising calcium, with minor amounts of magnesium, sodium, and potassium (Meillisa et al., 2015). In the present study, various mineral components such as macrominerals (calcium, magnesium, sodium, phosphorus, and potassium), microminerals (copper, iron, iodine, manganese, zinc, and aluminum), and heavy metals (arsenic, cadmium, mercury, and lead) were determined in the *S. japonica* hydrolysate (Table 3.4). The *S. japonica* hydrolysate contained significant amounts of macro- and microminerals. Magnesium, calcium, potassium, and sodium contents were increased with an increase in temperature. The amount of each mineral between the 180°C/13 bar and 420°C/520 bar conditions was as follows: calcium [0.32 ± 0.01 μg/g in dry matter (DM) to 1.71 ± 0.04 μg/g in DM], magnesium (0.57 ± 0.02 μg/g in DM to 2.46 ± 0.02 μg/g in DM),

Table 3.3. Total amino acid yield from *S. japonica* at different PHWE conditions

	Conditions (°C/ bar)												
	Freeze dried *S. japonica*	**180/13**	**200/17**	**220/25**	**240/34**	**260/49**	**280/72**	**300/100**	**320/120**	**350/150**	**375/260**	**400/400**	**420/520**
	Essential amino acids (EAA)												
Threonine	432.17±10.02^{a,b}	6.15±0.37^{a}	5.69±0.12^{a}	5.22±0.20^{b}	3.11±0.30^{c}	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Valine	451.07±18.24^{a,b}	12.63±0.38^{a}	11.14±0.31^{b}	11.88±0.46^{b}	6.26±0.25^{f}	4.18±0.25^{c}	3.00±0.34^{d}	1.02±0.11^{e}	0.11±0.02^{g}	N.D	N.D	N.D	N.D
Methionine	201.15±14.17 ^{b,c}	0.03±0.01^{a}	0.02±0.01^{b}	0.01±0.01^{c}	0.01±0.01^{c}	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Isoleucine	301.78±20.41^{b}	4.03±0.48^{a}	3.27±0.40^{b}	3.26±0.30^{b}	1.96±0.30^{c}	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Leucine	419.19±4.74^{a,b}	12.24±0.65^{a}	9.74±0.37^{b}	8.25±0.30^{c}	3.90±0.26^{d}	2.25±0.25^{e}	1.02±0.01^{f}	N.D	N.D	N.D	N.D	N.D	N.D
Histidine	320.19±26.06 ^{a}	0.05±0.00^{c}	0.05±0.00^{c}	0.02±0.00^{d}	0.08±0.00^{a}	0.06±0.00^{b}	0.06±0.00^{b}	0.06±0.00^{b}	0.05±0.00^{c}	N.D	N.D	N.D	N.D
Lysine	408.06±19.37^{a,b}	0.09±0.00^{c}	0.05±0.00^{b}	0.02±0.00^{a}	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Total EAA	2533.61±113.01	35.22±1.89	29.96±1.21	28.66±1.27	15.32±1.12	6.49±0.50	4.08±0.35	1.08±0.11	0.16±0.02	N.D	N.D	N.D	N.D

	Non-essential amino acids (NEAA)												
Serine	400.10±39.10^{a,b}	6.70±0.36^{b}	5.78±0.14^{a}	5.51±0.26^{a}	5.20±0.01^{a}	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Aspartic acid	1040.22±12.50^{a}	280.13±2.06^{a}	226.82±1.16^{b}	87.88±1.44^{c}	51.33±2.68^{d}	41.84±0.08^{e}	30.33±1.35^{f}	9.49±0.27^{g}	N.D	N.D	N.D	N.D	N.D
Glutamic acid	987.41±17.86^{a}	105.82±1.54^{a}	83.24±2.25^{b}	77.34±1.02^{c}	63.63±2.41^{d}	22.29±1.32^{e}	10.05±0.23^{f}	N.D	N.D	N.D	N.D	N.D	N.D
Glycine	519.20±26.89^{a,b}	201.16±7.14^{a}	177.34±2.07^{b}	83.98±1.77^{c}	51.17±3.02^{d}	38.12±1.02^{e}	35.26±1.55^{e}	10.05±0.73^{f}	N.D	N.D	N.D	N.D	N.D
Alanine	508.47±30.35^{a,b}	71.08±0.86^{a}	61.82±0.62^{b}	50.03±0.60^{c}	34.88±1.44^{d}	20.67±0.54^{e}	5.00±0.23^{f}	N.D	N.D	N.D	N.D	N.D	N.D
Cysteine	98.76±11.09^{c}	0.10±0.01^{a}	0.05±0.01^{a}	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Tyrosine	143.25±13.88^{c}	0.08±0.01^{a}	0.05±0.01^{a}	0.01±0.01^{a}	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Arginine	410.31±18.29^{a,b}	61.66±0.67^{a}	59.82±0.24^{b}	58.38±0.12^{b}	53.27±1.80^{c}	50.47±0.64^{d}	49.82±0.76^{e}	30.42±0.72^{f}	10.51±0.15^{g}	N.D	N.D	N.D	N.D
Total NEAA	4107.72±169.96	726.73±12.65	614.92±6.50	363.13±5.22	259.48±11.36	173.39±3.60	130.46±4.12	49.96±1.72	10.51±0.15	N.D	N.D	N.D	N.D
Total amino acid	6641.33±282.97	761.95±14.54	644.88±7.71	391.79±6.23	274.8±12.48	179.88±4.1	136.37±4.47	51.04±1.83	10.67±0.17	N.D	N.D	N.D	N.D

Values are expressed as mean ± SD and in mg/g. Different letters indicate significant differences (P <0.05) according to Turkey's multiple range test. N.D, not detected. Phenylalanine, Tryptophan and Proline was not detected in all the conditions.

potassium (4.92 ± 0.22 µg/g in DM to 24.34 ± 0.18 µg/g in DM), and sodium (1.34 ± 0.01 µg/g in DM to 12.65 ± 0.08 µg/g in DM). The most abundant element in the *S. japonica* hydrolysate was potassium, and the sodium content strongly increased with an increase in temperature. In contrast, the aluminum content decreased with an increase in temperature from 180°C/13 bar (0.36 ± 0.05 µg/g in DM) to 420°C/520 bar (0.18 ± 0.01 µg/g in DM). Iron, iodine, manganese, and zinc were found in trace amounts from 0.01 ± 0.01 µg/g in DM to 0.03 ± 0.01 µg/g in DM in all hydrolysis conditions. The increase in sodium and potassium and decrease in aluminum contents clearly shows that the decomposition of the seaweed solid matter occurred very rapidly at high temperature. Heavy metals are considered as the major component in mineral composition. These heavy metals are considered as one of the important causes for polluting the environment due to their toxicity, tenacity, and ability to bioaccumulation (Dauvalter & Rognerud, 2001). The heavy metal composition of the *S. japonica* hydrolysate (arsenic, cadmium, mercury, and lead) is shown in Table 4. The heavy metal content of the *S. japonica* hydrolysate did not vary in any of the conditions. Arsenic was the most abundant heavy metal (3.25 ± 0.01 µg/g in DM); other heavy metals were found in very trace amounts in the *S. japonica* hydrolysate. The freeze-dried *S. japonica* showed 20.89 ± 0.10 µg/g in DM.

Taking these results into consideration, we can say that the contribution of aluminum from this seaweed hydrolysate is well below the specified provisional tolerable weekly intake (PTWI). The World Health Organization

(WHO) technical report series 959 has reported that worldwide, the total arsenic value ranges up to 236 ppm for 953 food samples (WHO, 2011). In addition, the European Commission (EC) has mentioned in food surveys conducted in Denmark and UK that more than 50% of arsenic in daily diet comes from seafood. The PTWI values specified by both the Joint Expert Committee on Food Additives (JECFA) and EC for Pb and Cd are 7 ppb and 25 ppb, respectively (EC, 2004; JECFA, 2003). The JECFA has recommended a PTWI value of 6 ppm for arsenic in foods (JECFA, 2010). Khan et al. (2015) reported that Al and As were found in high amounts in *S. japonica* (4.89 and 3.04 ppm, respectively), Hg content was very low (only 0.006 ppm), whereas the total arsenic content was below 4.49 ppm. Considering the various research findings and guideline values, the concentration of heavy metals was very low and their contribution to the total intake was very small. Thus, all *S. japonica* hydrolysates that were studied could be declared safe.

The PHWE processes can be used to break down the polysaccharide polymer in *S. japonica*. The extracts obtained were analyzed for their contents of sugars (Table 3.5). This table shows that various types of monosaccharides were identified such as galactose, glucose, gulose, fructose, arabinose, mannose, mannitol, sorbitol, xylitol, and xylose. Previously, in a study conducted at our laboratory, Meillisa et al. (2015) stated the presence of mannose and gulose at the subcritical level, which also has been included in Table 5. Therefore, we continue to conduct research at high temperature conditions to check the

degradation ability of brown seaweeds. The sugar content was changed in each condition.

Glucose, gulose, fructose, mannitol, and xylose were the most abundant monosaccharides found in the *S. japonica* hydrolysate. The amount of galactose (2.33 ± 0.25 g/L), glucose (6.70 ± 0.00 g/L), gulose (9.80 ± 0.40 g/L), fructose (8.40 ± 0.18 g/L), arabinose (1.50 ± 0.13 g/L), mannose (1.50 ± 0.20 g/L), mannitol (17.50 ± 0.07 g/L), sorbitol (1.30 ± 0.05 g/L), xylitol (3.48 ± 0.33 g/L), and xylose (5.30 ± 0.31g/L) were high at the 180°C/13 bar condition. The recovery of sugars was high at the 180°C/13 bar condition, but when the temperature and pressure were increased up to 420°C/520 bar, the concentration of sugars in the *S. japonica* hydrolysate gradually decreased: galactose (12.68 ± 1.81 g/L), glucose (8.15 ± 1.17 g/L), arabinose (12.15 ± 0.70 g/L), mannose (4.21 ± 0.52 g/L), mannitol (89.47± 12.04 g/L), and xylose (6.90 ± 0.10 g/L). Therefore, we can say that monosaccharides are not stable at higher temperature and pressure. The freeze-dried sample showed galactose (2.33 ± 0.25 g/L), glucose (6.70 ± 0.00 g/L), gulose (9.80 ± 0.40 g/L), fructose (8.40 ± 0.18 g/L), arabinose (1.50 ± 0.13 g/L), mannose (1.50 ± 0.20 g/L), mannitol (17.50 ± 0.07 g/L), sorbitol (1.30 ± 0.05 g/L), xylitol (3.48 ± 0.33 g/L), and xylose (5.30 ± 0.31g/L). From our result, it seems the monosaccharide content decreased as a function of temperature. Probably caramelization and MRPs are being promoting by the increase in temperature.

Table 3.4. Macro minerals, micro minerals and heavy metals contents obtained from *S. japonica* during different PHWE conditions

	Conditions (°C/ bar)												
	Freeze dried *S. japonica*	**180/13**	**200/17**	**220/25**	**240/34**	**260/49**	**280/72**	**300/100**	**320/120**	**350/150**	**375/260**	**400/400**	**420/520**
Macrominerals													
Calcium	4570.41±40.80^{c}	0.32±0.01^{g}	0.60±0.03^{e,f}	0.55±0.00^{f}	0.70±0.05^{e}	0.68±0.01^{e}	1.31±0.05^{d}	1.48±0.02^{c}	1.59±0.04^{b,c}	1.59±0.05^{b}	1.60±0.02^{b}	1.60±0.02^{a}	1.71±0.04^{b}
Magnesium	4190.38±30.06^{c}	0.57±0.02^{g}	0.60±0.02^{g}	0.56±0.00^{f,g}	0.89±0.05^{f}	0.30±0.02^{e}	1.47±0.03^{d}	2.01±0.02^{c}	2.23±0.12^{c}	2.20±0.05^{b}	2.30±0.02^{b}	2.37±0.03^{b}	2.46±0.02^{a}
Phosphorus	1500.26±10.47^{d}	0.52±0.02^{a}	0.35±0.00^{b}	0.30±0.01^{b}	0.23±0.01^{c}	0.22±0.01^{c}	0.08±0.00^{d}	0.09±0.04^{d}	0.09±0.00^{d}	0.09±0.00^{d}	0.09±0.00^{d}	0.01±0.00^{e}	0.01±0.00^{e}
Potassium	35870.47±19.21^{a}	4.92±0.22^{k}	6.59±0.06^{j}	6.55±0.05^{j}	9.18±0.09^{i}	9.89±0.26^{h}	10.35±0.10^{g}	13.19±0.09^{f}	16.35±0.07^{e}	16.48±0.07^{d}	17.70±0.28^{c}	20.48±0.10^{b}	24.34±0.18^{a}
Sodium	9980.24±37.89^{b}	1.34±0.01^{l}	1.71±0.01^{k}	2.41±0.11^{j}	3.14±0.08^{i}	4.25±0.13^{h}	5.00±0.14^{g}	6.23±0.12^{f}	7.00±0.14^{e}	8.23±0.12^{d}	10.10±0.05^{c}	10.19±0.09^{b}	12.65±0.08^{a}
Microminerals													
Iron	2.14±0.10^{i}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	N.D	N.D	N.D	N.D	N.D	N.D
Iodine	30.60±0.48^{e}	0.03±0.01^{b}	0.02±0.01^{a}	0.02±0.01^{b,c}	0.02±0.01^{b,c}	0.01±0.01^{c}	0.09±0.01^{a}	0.07±0.01^{a}	0.08±0.01^{a}	0.07±0.01^{a}	0.07±0.01^{a}	0.09±0.01^{a}	0.08±0.01^{a}
Manganese	1.98±0.50^{i}	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}
Zinc	10.80±0.21^{g}	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Aluminum	31.46±0.17^{e}	0.36±0.05^{a}	0.20±0.07^{b}	0.20±0.01^{b}	0.20±0.01^{b}	0.21±0.01^{b}	0.19±0.01^{b}	0.18±0.01^{b}	0.18±0.01^{b}	0.18±0.01^{b}	0.18±0.01^{b}	0.18±0.01^{b}	0.18±0.01^{b}
Heavy metals													
Arsenic	20.89±0.10^{f}	3.25±0.01^{a}	3.25±0.01^{a}	3.25±0.01^{a}	3.25±0.01^{a}	3.25±0.01^{a}	3.25±0.01^{a}	3.25±0.01^{a}	3.25±0.01^{a}	3.25±0.01^{a}	3.25±0.01^{a}	3.25±0.01^{a}	3.25±0.01^{a}
Cadmium	0.50±0.01^{j}	0.03±0.00^{a}	0.03±0.00^{a}	0.03±0.00^{a}	0.03±0.00^{a}	0.03±0.00^{a}	0.03±0.00^{a}	0.03±0.00^{a}	0.03±0.00^{a}	0.03±0.00^{a}	0.03±0.00^{a}	0.03±0.00^{a}	0.03±0.00^{a}
Mercury	5.06±0.01^{h}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}
Lead	0.35±0.01^{k}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}

Values are expressed as mean ± SD and in ug/g in DM (dry matter). Different letters indicate significant differences ($P < 0.05$) according to Turkey's multiple range test. N.D, not detected. Copper was not detected in all the conditions.

Table 3.5. The monosaccharides content from *S. japonica* at different PHWE conditions

Conditions (°C/ bar)	Galactose	Gulose	Glucose	Fructose	Arabinose	Mannose	Mannitol	Sorbitol	Xylitol	Xylose
Freeze dried *S. japonica*	12.68±1.81^{b}	N.D	8.15±1.17^{c}	N.D	12.15±0.70^{b}	4.21±0.52^{a}	89.47±12.04^{a}	N.D	N.D	6.90±0.10^{d}
180/13	2.33±0.25^{a}	9.80±0.40^{a}	6.70±0.00^{a}	8.40±0.18^{a}	1.50±0.13^{a}	1.50±0.20^{a}	17.50±0.07^{a}	1.30±0.05^{a}	3.48±0.33^{a}	5.00±0.31^{a}
200/17	2.17±0.02^{b}	8.30±0.13^{a}	6.50±0.12^{a,b}	8.00±0.05^{a,b}	1.25±0.1^{b}	1.20±0.15^{a}	15.90±0.20^{b}	1.25±0.02^{a}	3.10±0.31^{b}	5.30±0.05^{a}
220/25	1.87±0.03^{c}	9.00±0.36^{a,b}	6.30±0.00^{b}	7.90±0.15^{b,c}	1.05±0.05^{b,c}	1.10±0.20^{b,c}	13.85±0.42^{c}	1.19±0.04^{a,b}	2.85±0.02^{b}	5.30±0.17^{a}
240/34	1.74±0.03^{c}	8.50±0.07^{b}	6.30±0.00^{b}	7.50±0.09^{c}	0.99±0.06^{b,c}	1.20±0.02^{a,b,c}	11.15±0.10^{d}	1.19±0.05^{a}	2.54±0.10^{b,c}	5.30±0.10^{a}
260/49	1.56±0.07^{d}	9.10±0.12^{b}	6.25±0.02^{b}	7.00±0.36^{c}	0.97±0.01^{b,c}	1.00±0.12^{b,c,d}	8.75±0.37^{e}	1.14±0.16^{a}	2.16±0.05^{d,e}	4.80±0.11^{a,b}
280/72	1.43±0.03^{d}	6.00±0.25^{c}	5.85±0.16^{c}	6.40±0.04^{d}	0.92±0.02^{c}	0.95±0.02^{c,d,e}	6.20±0.10^{f}	1.10±0.26^{a}	2.10±0.11^{b,c}	4.50±0.05^{b,c}
300/100	1.20±0.05^{e}	5.26±0.13^{d}	5.50±0.15^{c}	6.35±0.01^{d,e}	0.87±0.01^{c}	0.85±0.00^{d,e,f}	4.75±0.32^{g}	0.98±0.01^{a,b,c}	2.00±0.02^{d,e}	4.20±0.30^{c}
320/120	1.14±0.05^{e}	4.16±0.09^{e}	5.00±0.10^{c}	6.30±0.13^{d,e}	0.68±0.04^{d}	0.81±0.01^{d,e,f,g}	3.16±0.47^{h}	0.95±0.02^{a,b,c}	1.99±0.05^{d,e}	3.85±0.23^{d}
350/150	1.01±0.02^{f}	3.83±0.21^{e}	4.80±0.10^{c}	5.80±0.10^{d}	0.44±0.01^{e}	0.76±0.08^{d,e,f,g}	2.41±0.20^{i}	0.89±0.06^{a,b,c}	1.74±0.02^{e}	3.62±0.28^{d,e}
375/260	0.85±0.04^{g}	2.52±0.18d^{f}	4.50±0.25^{d}	5.40±0.10^{e}	0.35±0.02^{e}	0.69±0.02^{e,f,g}	1.85±0.13^{i,,j}	0.87±0.05^{b,c}	1.20±0.09^{f}	3.36±0.18^{d,e}
400/400	0.77±0.02^{g}	1.82±0.04^{f}	4.00±0.01^{d}	4.30±0.12^{f}	0.34±0.03^{e}	0.60±0.03^{f,g}	1.45±0.02^{j}	0.85±0.02^{c}	0.88±0.05^{f,g}	3.04±0.02^{f}
420/520	0.31±0.03^{h}	0.51±0.01^{g}	3.80±0.15^{e}	3.00±0.18^{g}	0.13±0.04^{f}	0.50±0.01^{g}	1.04±0.03^{j}	0.79±0.05^{c}	0.64±0.03^{g}	2.57±0.05^{f}

Values are expressed as mean ± SD and in g/L. Different letters indicate significant differences ($P < 0.05$) according to Turkey's multiple range test. N.D, not detected.

Our *S. japonica* hydrolysate showed very high content of mannitol (17.50 ± 0.07 g/L) at the 180°C/13 bar condition. In *S. japonica*, the mannitol content is approximately 5% of its dry weight, but it increases in summer up to >30% of its dry weight when the growth of kelp is maximum (Honya, Kinoshita, Ishikawa, Mori, & Nisizawa, 1993). Ito et al. (1989) reported that 24.3% of dry weight is because of mannitol in *S. japonica*.

Mannose is a type of sugar that is most commonly used as a source for bioethanol production (Balat, 2011). Mannose is reduced to mannitol, which has various industrial uses and is mostly used to make tablets or medicine (Y. Wang, San, & Bennett, 2013). Mannitol is classified as a sugar alcohol. Further, byproducts of sugar alcohols consist of xylitol and sorbitol. These two are isomeric sugars; the lone variance is the positioning of the hydroxyl group on carbon (Song & Vieille, 2009). The WHO has listed mannitol as one of the important drugs; it was also listed as a very vital medicine for the well-being of humans. The global market for sugar alcohols in 2000 was $1.3 billion. The largest sugar alcohol in terms of volume and dollar sales was sorbitol. The bulk prices for liquid and crystalline sorbitol are approximately $0.55–0.65 per kg and $1.61–2.26 per kg, respectively. The annual mannitol market was estimated at approximately 30,000 tons. However, according to a recent issue of the Chemical Market Reporter, the bulk price of mannitol (powdered) is $7.32 per kg. For most sugar alcohols, the market is mature, and volume growths are expected to follow the trends of large-scale consumer products in which sugar alcohols are used (Weymarn, 2002).

3.4. Conclusion

This study showed that PHWE of *S. japonica* affects the yield, TOC, pH, MRPs, and viscosity, and amino acid, mineral, and monosaccharide contents during different temperature conditions. High temperature causes TOC, pH, and the MRP content to increase. EAAs were recovered well at 180°C than at other conditions. The contents of minerals such as calcium, magnesium, potassium, and sodium were found to be increased with an increase in temperature, whereas cadmium, mercury, arsenic, and lead were found in trace amounts under all conditions. The monosaccharide profile showed that the increase in temperature decomposes sugars. The mannitol content (17.5 g/L) was very high at 180°C compared with other sugars. It can be concluded that 180°C is the optimum condition to have a good nutritional composition of amino acids, minerals, and sugars. At high temperature conditions, all chemical compositions were degraded in the brown seaweed. Therefore, the pressurized hot water extract of *S. japonica* can be used as a good source of bioenergy, raw material source in the fermentation industry, and human food. Further research is ongoing to isolate and detect the structure of monosaccharides such as gulose and mannitol from *S. japonica* extracts.

3.5. References

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Chapter 4

Structural, antioxidant, and biological activities of fucoidan from *Saccharina japonica* using pressurized liquid extraction

4.1. Introduction

Brown seaweeds are a group of marine algae that contains nearly 2000 species (Rodriguez-Jasso, Mussatto, Pastrana, Aguilar, & Teixeira, 2011). These seaweeds have numerous biofunctional materials with important economical applications, such as organic products, micro- and macro-elements, polysaccharides, fatty acids, and vitamins (Craigie, 2011). Fucoidan is a key compound in brown seaweed; studies have shown various biological activities, including anti-inflammatory, anticoagulant, antitumoral, antithrombotic, antioxidant, and antiviral activities (Jhamandas, Wie, Harris, MacTavish, & Kar, 2005). Generally, fucoidan comprises α-(1→3)- and (1→4)-linked L-fucose residues that may be organized in stretches of (1→3)-α-fucan or of alternating α-(1→3)- and α-(1→4)-bonded L-fucose residues. These residues may be replaced with sulfate (SO_3) on the C-2 or C-4 (rarely on C-3) (Ale, Mikkelsen, & Meyer, 2011). In addition to fucose, fucoidan also includes other monosaccharaides, such as glucose, galactose, rhamnose, xylose, mannose, and uronic acids (Foley, Szegezdi, Mulloy, Samali, & Tuohy, 2011).

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Saccharina japonica is a well-known Chinese herbal medicine and it is consumed for its treatment of iodine deficiency and diuretic swelling; additionally, it is rich in fucoidan. *S. japonica* has three types of polysaccharides: fucoidan, alginate, and laminarin. Alginate is composed of guluronic acid and mannuronic acid, whereas laminarin comprises glucose. Alginate and fucoidan are usually found in intercellular spaces and cell walls, whereas laminarin is present in cytoplasm (Wang, Wang, Yun, Zhang, & Zhang, 2012).

Sulfated polysaccharides (SP) can be isolated and purified from brown algae via several methods, such as by using hot water, dilute alkali, dilute acid, pretreatments with enzymes, and various purification and fractionation processes using a large volume of solvents needing long extraction time. The above-mentioned processes have several drawbacks, such as being difficult to extract and time and energy consuming, using a large amount of reactants, and having high labor costs, besides being hazardous to the environment (C.-Y. Huang, Wu, Yang, Kuan, & Chen, 2016).

Water is a globally neutral solvent and nontoxic at room temperature, whereas the H_2O molecule above its critical temperature and pressure, which is known as supercritical water, can behave as an organic solvent and acidic medium (Gallezot, 2012). Pressurized liquid extraction (PLE) of biomass presents several advantages compared with traditional technologies (acid, alkali, and enzymatic hydrolysis). Its main advantage is that it does not use solvents, which is a factor of major importance in any process because organic solvents

must be incinerated, recycled, or submitted to an appropriate unitary operation, resulting in a nonaggressive waste to the environment (Yu, Lou, & Wu, 2008). Various literatures showed that PLE was a promising method for extracting active ingredients from a variety of natural resources, including seaweeds (Dominguez, 2013; Herrero, Castro-Puyana, Mendiola, & Ibañez, 2013). However, no information is available regarding fucoidan extraction using PLE; moreover, little information is available on hydrothermal processing of SP (Balboa, Rivas, Moure, Domínguez, & Parajó, 2013; Rodriguez-Jasso, Mussatto, Pastrana, Aguilar, & Teixeira, 2014).

In the current study, CF was extracted from *S. japonica* by PLE. The influence of temperature, pressure, and solvents on CF yield was examined. The chemical composition, molecular weight, and antioxidant activity of the obtained CF were also explored. Furthermore, CF was checked for its antimitotic and anticancer activity.

4.2. Materials and methods

In June 2015, *S. japonica* (Areschoug, Lane, Mayes, Druehl, and Saunders, 1851) was collected from Guemil-eup, Wando-gun, and Jeollanam-do, Republic of Korea. The obtained samples were prepared as reported in our previous study (Saravana, Choi, Park, Woo, & Chun, 2016), and the samples were de-oiled using a supercritical CO_2 process for seaweed oil extraction (Sivagnanam et al., 2015).

4.2.1. Extraction of fucoidan

PLE was operated in a 200 cm^3 batch system built with Hastelloy C276 (continuous-type supercritical water system, Phosentech, South Korea) as reported in our previous work (Saravana et al., 2016). Here, 9.65 g of seaweed was loaded into the reactor with 160 mL of solvents, such as water (distilled water), 0.1% sodium hydroxide, 0.1% formic acid, 70% ethanol, 50% ethanol, and 25% ethanol. The reactor was closed, purged with nitrogen gas through a valve, and maintained at its required temperature (80–200 °C), pressure (5–100 bar), agitation speed (200 rpm), and reaction time (5 min). The temperature and pressure in the reactor were controlled by a temperature controller and pressure gauge, respectively. The hydrolyzed samples from the reactor were immediately collected after the reaction using a cooling chamber, filtered using membrane filters (0.45 μm pore size, Fisher Scientific), and stored at 4 °C. The residual samples recovered after PLE were dried at 35 °C and weighed to calculate the residue amount attained. Then, an equal volume of 1% (w/v) $CaCl_2$ solution was mixed to the hydrolysate and kept at 4 °C (overnight) for removing alginate. Then, alginate was separated by filtration and dried at 35 °C. To the resultant filtrate, a double volume of absolute ethanol was added and stored at 4 °C (overnight). SP was obtained by centrifugation (8500 rpm, 20 min, 4 °C), and it was dried at 35 °C, powdered, and kept for further analysis. Crude fucoidan yield (% CF) and alga degradation (% AD) were calculated as follows:

$$\% \text{ CF} = \frac{\text{WM}_{\text{OH}}}{\text{WA}} \times 100 \quad (1)$$

$$\% \text{ AD} = \frac{\text{WA} - \text{WA}_{\text{PLE}}}{\text{WA}} \times 100 \quad (2)$$

where WM_{OH} is the dry mass weight obtained after ethanol precipitation; WA is the algal weight used in each experiment; WA_{PLE} is the dry algal weight recovered after PLE.

De-oiled *S. japonica* was used to extract CF using a conventional method (García-Ríos, Ríos-Leal, Robledo, & Freile-Pelegrin, 2012), with slight modifications to have comparative data with the PLE. Here, 5 g of seaweed was suspended in 100 mL of 0.05 M HCl and agitated for 2 h at 25 °C using a separation process, which is the same as that used in Section 2.2.1

4.2.2. Chemical composition

Here, 10–15 mg of CF was hydrolyzed with 4 N HCl (2 mL) at 121 °C for 2 h. The obtained hydrolysis was used to measure the total sugar content (glucose used as the standard) (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), and the sulfate content was measured by the barium chloride–gelatin method (Dodgson & Price, 1962). The method of Bitter et al. (1962) was used to estimate the uronic acid content. The protein content was determined by the Lowry method (bovine serum albumin used as the standard) (Lowry, Rosebrough, Farr, & Randall, 1951), and polyphenols were determined using the Folin–Ciocalteau reagent with phloroglucinol as the standard (Kuda &

Ikemori, 2009). The colorimetric assay was achieved on a microplate using the Synergy™ HTX multi-mode microplate reader (BioTek Instruments, Winooski, VT, USA) and processed with Gen-5 software (BioTek, Colmar, France).

4.2.3. Screening of fucoidan content

Here, 200 μL of sulfuric acid (18 M) and 50 μL of the sample (0.3 mg/mL) were added to a 96-well plate. It was mixed well and heated (80 °C/30 min). After cooling, 3% (w/v) L-cysteine hydrochloride (8 μL) was added and incubated at 4 °C for 1 h. Then, absorbance was measured at 405 nm.

$$OD_{fucoidan} = [OD_{405\ (sample)} - OD_{405\ (blank)}] - [OD_{450\ (sample)} - OD_{450\ (blank)}] \quad (3)$$

Commercial fucoidan (25–500 μg/mL) was used to determine the fucoidan content. Distilled water was used as the blank, and a triplicate was performed (Sabourał et al., 2014).

4.2.4. Determination of monosaccharide composition

The CF sample (6 mg) was hydrolyzed for 4 h in 2 M trifluoroacetic acid (0.3 mL) at 105 °C. The hydrolyzed CF sample was dissolved in 1 mL of distilled water and filtered with a 0.22-μm membrane filter (Millipore). A 20 μL sample was injected into an HPLC at a flow rate of 1 mL/min through a Prevail carbohydrate ES column (5 μm, 250 mm length × 4.6 mm i.d., Alltech, USA) with acetonitrile (A) and water–acetonitrile (95:5, v/v) (B) as solvents. The ratio of solvent flow was 80 (A)/20 (B). An evaporative light-scattering detector was

used [ELSD, Jasco HPLC (Easton, USA) model 400 equipped with a ChromNav analysis software]. The results were compared with standard solutions of fucose, arabinose, mannose, glucose, galactose, xylose, rhamnose, galacturonic acid, and glucuronic acid**.**

Hydrolyzed CF (5 μg/spot) and standards (0.5 μg/spot) were applied on silica gel 60 F254 plates (Merck, Germany). The plates were eluted with acetonitrile:water (80:20), dried, sprayed with a mixture 0.2% w/v naphtoresorcinol in ethanol:H_2SO_4 (96: 4, v:v), and heated at 105 °C for 15 min to visualize the monosaccharide bands.

4.2.5. Determination of average molecular weight

The CF molecular weight (M_w) was determined by HPSEC-ELSD (high-performance size exclusion chromatography–evaporative light-scattering detector). The column used was a TSKgel $GMPW_{XL}$ (7.8 mm × 300 mm), and the guard column was TSK PW_{XL} (6.0 mm × 4.0 mm). The mobile phase comprised an HPLC-grade water solution filtrated on 0.22-μm filters. The flow rate was 1 mL/min, and analyses were performed at 30 °C. A 5-mg sample was suspended in 1 mL of HPLC-grade water and then filtered on 0.22-μm filters. Pullulan samples (Shodex Standard P-82, Phenomenex) were used as standards.

4.2.6. Characterization of crude fucoidan

The ultraviolet (UV) absorbance spectrum from 200 to 400 nm for the sample solution (1 mg/mL in distilled water) was measured by a microplate reader**.** CF was mixed with potassium bromide and measured using spectrum GX Fourier transform infrared spectroscopy (FTIR**)** (Perkin Elmer, Inc., USA) equipped with a DTGS detector. IR spectra were recorded within the range of 400–4000 cm^{-1}**.** X-ray diffraction (XRD) patterns of the CF powders were measured by a Brüker D8-Advance diffractometer (Brüker, Germany. Data were collected in the 2θ range of 5°–80° with a step size of 0.05° and a counting time of 5 s/step. Thermal gravimetric analysis (TGA) was conducted using a TGA-50 (Shimadzu Corporation, Kyoto, Japan) instrument in a nitrogen atmosphere. Here, 10–13 mg of CF was heated from room temperature to 700 °C at a rate of 10 °C min^{-1}. Carbon, hydrogen, nitrogen, and sulfur contents were analyzed using a CHNS Analyzer Series II, Model # 2400 (Shimadzu, Japan).

4.2.7. Biological activities: Antioxidant, antimitotic and anticancer activity

The AA was evaluated using DPPH and $ABTS^{+}$, as described by Khanam et al. (2012). CF (150 µL) with a concentration of 1 mg/ ml and 150 µL of freshly prepared DPPH solution (0.05 mg/mL methanol) were mixed together and incubated in the dark for 30 min at room temperature. The absorbance was measured at 517 nm using a microplate reader.

CF samples (150 µL) of various concentrations were mixed to react with 2.85 mL of $ABTS^+$ solution [solutions were prepared as described by Khanam et al. (2012)] for 2 h in the dark. The absorbance was taken against methanol at 734 nm. The percentages of inhibition of DPPH and $ABTS^+$ from CF were measured as follows:

$$\text{Inhibition}\% = (1 - A_{\text{test sample}}/A_{\text{blank}}) \times 100, \quad (4)$$

where A_{blank} is the absorbance of the water blank, and $A_{\text{test sample}}$ is the absorbance of the CF. Trolox was used as the reference standard.

An equal weight of green gram seeds were allowed to sprout in 500 µL of solution containing samples (1-10 mg/mL). Next day the seeds were dried out and its mass was calculated. Distilled water was used as control and doxorubicin was used as standard (Jose, Radhakrishnan, & Kurup, 2015). Length of the radical was visualized for its morphological studies. Percentage inhibition of water imbibition was measured as follows:

$$\%\ \text{of inhibition} = \frac{(Ww - Dw)_{control} - (Ww - Dw)_{test}}{(Ww - Dw)_{control}} \quad (5)$$

Where Ww - Wet weight of seeds, Dw - Dry weight of seeds.

Allium cepa (onion) roots were allowed to sprout in water for 48 h in room temperature for growing onion bulbs. Uniformly developed roots were immersed in sample (1 mg/mL) for 3 h. Doxorubicin was used as a standard (1 mg/mL) and distilled water was used as a control. Then the roots were wash

using a fixing solution (Ethanol: acetic acid ratio of 3:1 V/V) for 10-12 h (Saboo, Thorat, Tapadiya, & Khadabadi, 2012). Then acetocarmine was used to stain the roots, observed and images were obtained using (Motic, BA310, China). MI and inhibition of mitosis was determined by the formula:

$$\text{MI} = \frac{Number\ of\ dividing\ cells}{Number\ of\ total\ cells} \times 100 \quad (6)$$

$$\%\ \text{of miotic inhibition} = \frac{MI_{control} - MI_{test}}{MI_{control}} \quad (7)$$

Human cervical cancer cell line HeLa cells, neuroblastoma cells SK-N-SH and SH-SY-5Y cells, hepatocellular carcinoma Hep3B, (Korean Cell Line Bank, Seoul, Korea) were cultured in MEM medium (Corning, Manassas, VA, USA) supplemented with 10 % fetal bovine serum (FBS) (Corning) and 1% antibiotics solutions (100 U/mL penicillin and 100 mg/ml streptomycin) (Corning). Human gastric cancer cell line MKN-28 cells were cultured in RPMI 1640 (Corning, Manassas, VA, USA) supplemented with 10% FBS and 1% antibiotics solutions. Cells were maintained in a humidified incubator with 5% CO_2 at 37 ℃.

HeLa, SK-N-SH, MKN-28, SH-SY-5Y or Hep3B cells ($1.0x10^4$) cells/well were added on 96-well cell culture plates and incubated for 24 h. After incubation, cells were treated with a different kind of samples for 24 h. In order to treat with samples for 72 h, cells were plated on 24-well cell culture plates at 2.0x10^4 cells in each well and incubated with 24 h. Cells were exposed to derivates for 72 h. Medium was exchanged every 24 h. WST-1 solution (Daeil

Lab service, Seoul, Korea) was added to the medium for 3 h and an absorbance was measured at 460 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The results are presented as means ± SD from three independent experiments. Inhibition graphs were plotted using mean values obtained from each concentration relative to control values.

$$\text{Cell viability (\%)} = \left(1 - \frac{(Abs\ of\ control - Abs\ of\ blank) - (Abs\ of\ sample - Abs\ of\ blank)}{(Abs\ of\ control - Abs\ of\ blank)}\right) \times 100 \qquad (8)$$

4.2.8. Statistical analyses

Experimental values were shown as mean ± standard deviation (SD). Differences concerning parameters were analysed by the one-way ANOVA test to compare different treatments ($p < 0.05$). All calculations were done via statistical software (SPSS 16.0).

4.3. Results and discussion

4.3.1. Effect of temperature, pressure and solvents on the yields

The yields of AD, crude alginate, and CF after various extractions are shown in Fig. 4.1. The AD was increased as the temperature increased (Fig. 4.1a), and the crude alginate decreased as the temperature increased. The highest CF yield was 8.23%, obtained from 140 °C and 50 bar (0.1% NaOH). The temperature played a key role on the fucoidan yields. After 140 °C, the yield decreased as the temperature increased. Considering the solvent systems, 0.1% NaOH gave a high yield whereas formic acid and water as solvents also

increased the yield. However, using ethanol with various concentrations, the yield was very poor. The conventional method gave a yield of 1.82%, which was lower than the PLE method, while AD was (37.58%) and crude alginate was 8%. From these results, it appears that PLE can significantly improve the yield of CF.

The justification for the high yield with NaOH may have been caused by cellulose swelling, which disrupted the hydrogen bonds between hemicellulose and cellulose in the cell wall, resulting in hemicellulose solubilization. Therefore, it could effectively release insoluble polysaccharides and convert them into soluble polysaccharides. However, minerals and protein firmly connected with SP were also extracted (Peasura, Laohakunjit, Kerdchoechuen, & Wanlapa, 2015). The yield of CF from formic acid is slightly lower than that from NaOH, perhaps because of acid hydrolysis. Formic acid may have hydrolyzed the polysaccharide structure, including hemicellulose, cellulose, and lignin, which was released into SP. However, SP could also be hydrolyzed by acid; as they were mainly short-chain polysaccharides, this would reduce the final recovery (S.-Q. Huang et al., 2010). Water as a solvent gave less yield of CF because it can dissolve only long-chain soluble polysaccharides. CF extracted from *S. japonica* using chitosan as an extractant was reported having a yield of 1.68% (Xing et al., 2013); another report on CF yield from *S. japonica* using ultrasonication showed 5.75% (Wan et al., 2015).

4.3.2. Chemical composition of crude fuciodan

The fucoidan content (Fig. 4.2a) was high in 140 °C, 50 bar NaoH (4.15%). Zhang et al. (2003) reported that *S. japonica* has a fucoidan content of approximately 1%–5%, while Xing et al. (2013) demonstrated a high content of fucoidan (5.51%) extracted from *S. japonica* using chitosan as an extractant. The fucoidan content in our study is varied due to several reasons, such as different extraction techniques and samples collected in different seasons and locations. A previous report from Wijesinghe and Jeon (2012) also suggested that the fucoidan content alters with the location, season, and species.

The sulfate content of CF extracted with NaOH, formic acid, and water initially increased with an increase in temperature but did not further increase after 170 °C and 75 bar. The lowest sulfate content was found while using 70% ethanol (Fig. 4.2b). CF extracted with NaOH had the highest sulfate content because of ester linkages. These chemical bonds between the polysaccharide chain and sulfate group were not easily cleaved by NaOH (Buranov & Mazza, 2009). The sulfate content in the water extract was higher than that from formic acid; perhaps, water extraction at high temperature breaks down the ester bonds in the SP chain (Peasura et al., 2015).

The uronic acid content of CF extracted with formic acid was higher when compared to other solvents (Fig. 4.2c); the highest amount of uronic acid was 13.45% at 110 °C and 25 bar. However, it reduced as the temperature increased. Similarly, Vriesmann et al. (2011) found that the uronic acid content increases with decreasing extraction pH; furthermore, uronic acid decreases

when there is an increase in temperture. These results were found during the extraction of pectin from cacao pod husks.

CF extracted at 140 °C and 50 bar with formic acid and NaOH had the highest total sugar (30.70% and 29.48%, respectively), followed by those extracted using water and ethanol (Fig. 4.2d). CF extracted with acid had the highest total sugar content because the glycosidic bond in the polysaccharide chain could be hydrolysed by acid more readily than by alkali and distilled water (Peasura et al., 2015).

Proteins are the main impurities of cell wall polysaccharides, majorly because they form part of the structure of cell walls and are closely associated with polysaccharides. All CF extracts showed a low content of protein because of the ester sulfate moieties, which can form strong anions and attract positively charged proteins. It was possible that PLE could decrease protein contamination in CF. The protein content of CF with water (1.13%–2.40%) was lower than that with formic acid (0.80%–2.39%) and NaOH (1.54%–3.64%) extraction (Fig. 4.2e).

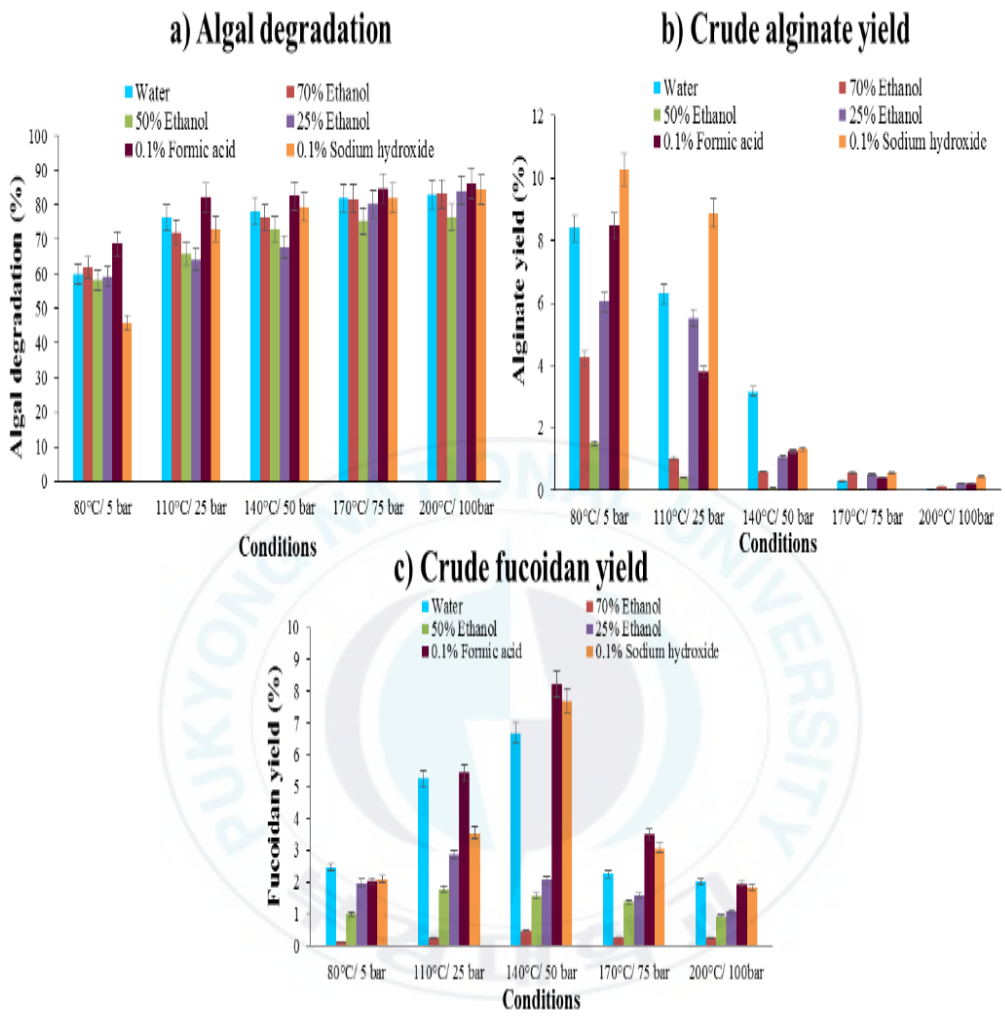


Fig. 4.1. Effect of PLE on the yield of: a) Algal degradation, b) Crude alginate and c) Crude fucoidan

Studies suggest that polyphenols are prone to form a complex with fucoidan and alginic acid. Therefore, CF may contain several impurities, such as phenolic compounds (Imbs, Skriptsova, & Zvyagintseva, 2015). The polyphenol contents for CF are presented in Fig. 4.2f. The impurity of CF by polyphenols was detected in high amounts in NaOH (1.54%–3.64%) followed by formic acid, water, and ethanol. However, for 70% ethanol, the polyphenol content is extremely low due to the extraction of all polyphenols during the PLE process, but considering the yield of CF, it was too low. These results indicate that the PLE process cannot remove the impurities such as alginate, polyphenols, and protein, but it may possibly reduce the quantity of impurities in CF.

4.3.3. Monosaccharides and molecular weight

CF obtained from 140 °C and 50 bar conditions were characterized for their monosaccharide composition (Table 4. 1). L-Fucose, galactose, glucose, mannose, and glucuronic acid were found in all the samples. Fucose is the major component of all CF, while mannose and glucuronic acid contents were the highest monomers present in the samples. Generally, CF extracted from *S. japonica* was mainly composed of fucose, glucose, mannose, galactose, xylose, rhamnose, and D-glucuronic acid (Jin, Zhang, Wang, & Zhang, 2013). The variance in the monosaccharide content was possibly due to the differences in extraction processes. TLC analysis showed that all extracted CF contained L-fucose, galactose, glucose, mannose, and glucuronic acid by comparing the Rf values and spot color between monosaccharide standards and samples (Fig. 4.3).

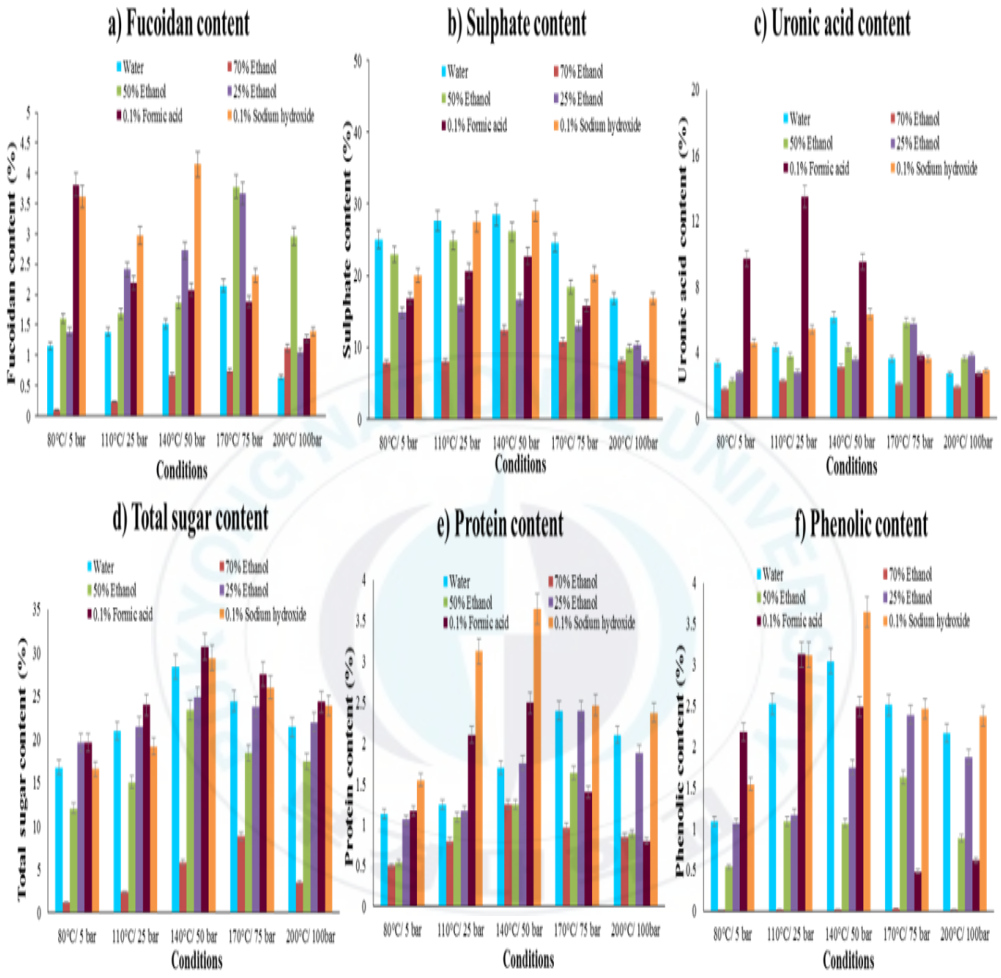


Figure 4.2. Effect of PLE on the composition of the CF: a) Fucoidan, b) Sulphate, c) Uronic acid, d) Total sugar, e) Protein and f) Phenolic content.

Table 4.1 shows the average M_w of CF extracted by PLE. On the basis of the calibration with standard pullan, the molecular weights of CF extracted at 140 °C and 50 bar were between (83.39–183.32 kDa). For the conventional method, it was 216.90 kDa, and the polydispersity was between 1.06 and 1.37. The CF M_w from *S. japonica* was reported to have around 87 KDa (Wang, Zhang, Zhang, Zhang, & Niu, 2010). The difference in M_w from the same species can be due to the different fucoidan extraction processes, and M_w distribution of the SP was related with polysaccharides with sulfate (Zvyagintseva et al., 2003). The M_w of CF extracted with formic acid was apparently lower than that of CF extracted with other solvents, showing that SP chains may have been disturbed and decomposed during acid extractions.

4.3.4. Characterization of crude fuoidan

All the CF extracted using various solvent systems (140 °C, 50 bar) showed significant UV absorbance near 260 nm, corresponding to the absorbance of aromatic compounds covalently bound to polysaccharide (Fig. 4.4a). Similar kinds of results were reported for *Fucus evanescens* (Imbs et al., 2015) CF obtained under 140 °C and 50 bar conditions, as well as standard fucoidan and CF obtained from the conventional method, were examined by FTIR to confirm the exact absorption bands present in CF (Fig. 4.4b). The FTIR spectra clearly show that all the evaluated samples exhibited absorption bands typical of fucoidans. The absorption band at 1240–1255 cm^{-1} (S=O stretching) confirmed the presence of sulfate in CF. The sharp band at 840 cm^{-1} and the

shoulder at 820 cm^{-1} (C–S–O) propose a complex pattern of substitution, primarily at the C-4 position (axial C-4 substitution of α-linked l-fucopyranose) with other substitution at C-2 or/and C-3 (equatorial positions) in lower amounts (Rodriguez-Jasso et al., 2011). XRD is a potent method used to illustrate SP structures and to confirm the crystalline structure of the sample. XRD patterns recorded for CF were between 5° and 80° (Fig. 4.4c). All CF showed low overall crystallinity including the standard. Figure 4c suggests that CF was a semicrystalline polymer; the crystalline region was observed at the angle (2θ) 23.61°. This result was similar to the one obtained by Jeddou et al. (2016) who reported on polysaccharides from water-soluble polysaccharides from potato peels.

TGA curves of CF extracted using various solvents at 140 °C and 50 bar are shown in Fig. 4.4d. TGA results can be explained in three stages. First, the curve showed the mass% decrease to the weight loss due to dehydration, which occurred between 25 and 130 °C. Then, pyrolysis reactions of the samples started at 120 °C. The second stage started at 210 °C and involved in the devolatilization of the sample, with evolution of the volatile matter mainly occurring between 220 and 490 °C. Last, the third stage started near 500 °C and was upheld up to 700 °C. The leftover samples at the final stage of the process resembles the ash content in the sample. The remaining mass perhaps consists of phosphates, sulfates, and carbonates, which are minerals generally found in SP structures like fucoidan (Rodriguez-Jasso et al., 2011).

Table 4.1. Monosaccharide composition in molar ratio of the CF obtained from various extracts

Samples	Monosaccharide composition (mol %)[a]					Mw[a] (kDa)	M_w/M_n[a]
	Fucose	Galactose	Glucose	Mannose	Glucuronic acid		
Conventional method	4.89	0.98	0.46	2.04	2.04	216.90	1.16
Water	5.78	1.20	0.56	2.14	2.34	183.32	1.37
0.1% Sodium hydroxide	6.39	1.46	0.69	2.38	2.18	175.96	1.07
0.1% Formic acid	6.24	1.81	0.60	2.30	2.51	83.39	1.20
70% Ethanol	3.17	0.64	0.34	0.61	0.81	129.01	1.02
50% Ethanol	4.65	0.79	0.42	0.99	1.08	120.47	1.08
25% Ethanol	5.67	1.07	0.56	1.52	1.65	117.76	1.18

[a] Values are means from three independent tests. Arabinose, Rhamnose, Galacturonic acid and Xylose are not detected.

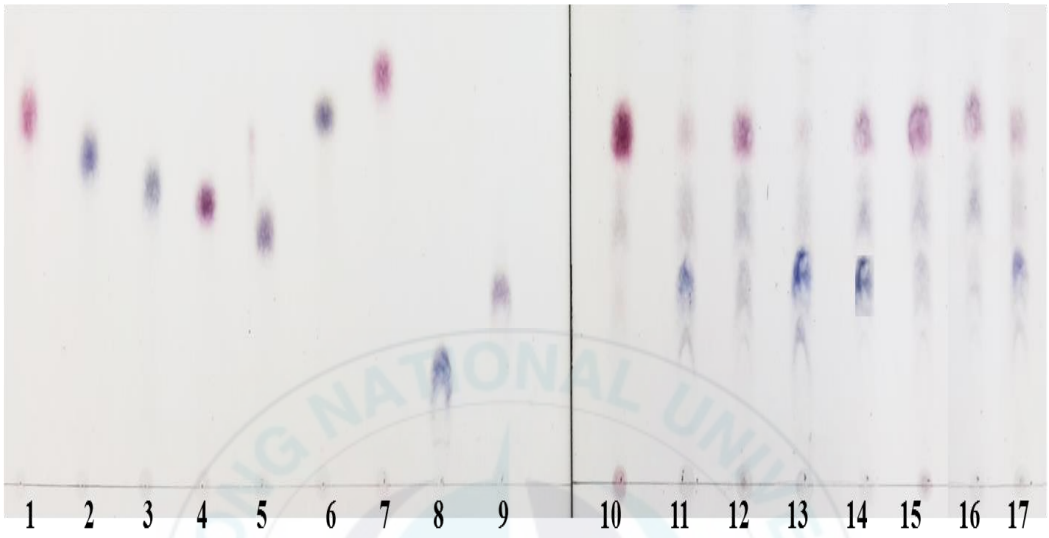


Fig. 4.3. Thin layer chromatography of monosaccharides constitutive of the crude fucoidan. Lane: 1, Fucose; 2, Arabinose; 3, Mannose; 4, Glucose; 5, Galactose; 6, Xylose; 7, Rhamnose; 8, Galacturonic acid; 9, Glucuronic acid; 10, Hydrolysed fucoidan standard; 11, Conventional method; 12, Water; 13, NaoH; 14, Formic acid; 15, 70% Etoh; 16, 50% Etoh; 17, 25% Etoh.

As shown in Table 4.2, the sulfur content (S%) was high in NaOH-derived CF. The S% was also the highest in comparison with fucoidan (95% pure). The presence of traces of nitrogen content (N%) found in all CF suggests the presence of some amino-containing compounds (e.g., protein or amino sugars). In contrast, the standard fucoidan does not have N% (Ale & Meyer, 2013).

4.3.6. Biological activities of crude fucoidan

The study indicated the DPPH scavenging activity for CF obtained from various solvents at 140 °C and 50 bar (Fig. 4.5). The inhibition of CF was high in 0.1% NaOH (41.88%); however, standard fucoidan (48.28%) and trolox (71.26%) showed high activity. These results showed that AA is not only due to sulfate content but also due to other factors, such as impurities of protein, polyphenols, and monosaccharide content that can subsidize to AA. The scavenging capacity of SP extracted with NaOH and water may be attributed to the presence of hydrogen atoms from the specific monosaccharide compositions and their side-chain linkages. In the present study, the $ABTS^{+}$ radical scavenging activity showed high activity for 0.1% formic acid than the other samples (Fig. 4.5). These results suggest that the chemical structure of SP plays a role in the H abstraction reaction by the $ABTS^{+}$ cation radical (Peasura et al., 2015). The obtained results were similar to the earlier study that witnessed a reduction in the activity towards the radical cation with an increase in sulfate

content for commercial κ-, ι-, and λ-carrageenans (Barahona, Chandía, Encinas, Matsuhiro, & Zúñiga, 2011).

The CF and standard fucoidan showed inhibition of water imbibition (Table 4.3). The CF showed an IC_{50} value of 395.57 to 549.48 mg/mL, while standard fucoidan showed 329.00 ± 5.17 mg/ml. The standard doxorubicin, a known anticancer drug was used as a positive control and showed an IC_{50} value of 192.31 ± 2.69 (Table.4.3). The physical investigation showed that CF has a dose-dependent inhibition of seed germination (Figure 4.6). The biochemical connection between animals and plants can be exploited to use plants as an substitute system for animal experiments for the development of new drugs (Nayak & Lakshmi, 2014). Similar trend on seed germination using CF from *Padina tetrastromatica* was shown to have antimitotic activity (Jose et al., 2015).

The MI of the onion root tips treated with various CF and standard fucoidan is given in Table 4.3. Here, also the CF showed moderate activity when compared with standard fucoidan (Figure 4.7). The MI were ranged from 4.19 to 27.19% and 34.13% for fucoidan standard, and doxorubicin showed 75.81%, respectively. The inhibition of mitosis in onion root tip is considered as an easy and sensitive method for the examination of cytotoxicity of drugs (Thenmozhi, Nagalakshmi, & RAO, 2012). Thus, we suggest that the cytotoxic action of fucoidans can involve disruption of mitotic processes in the fast-dividing cancer cells which will be favourable for cancer treatment.

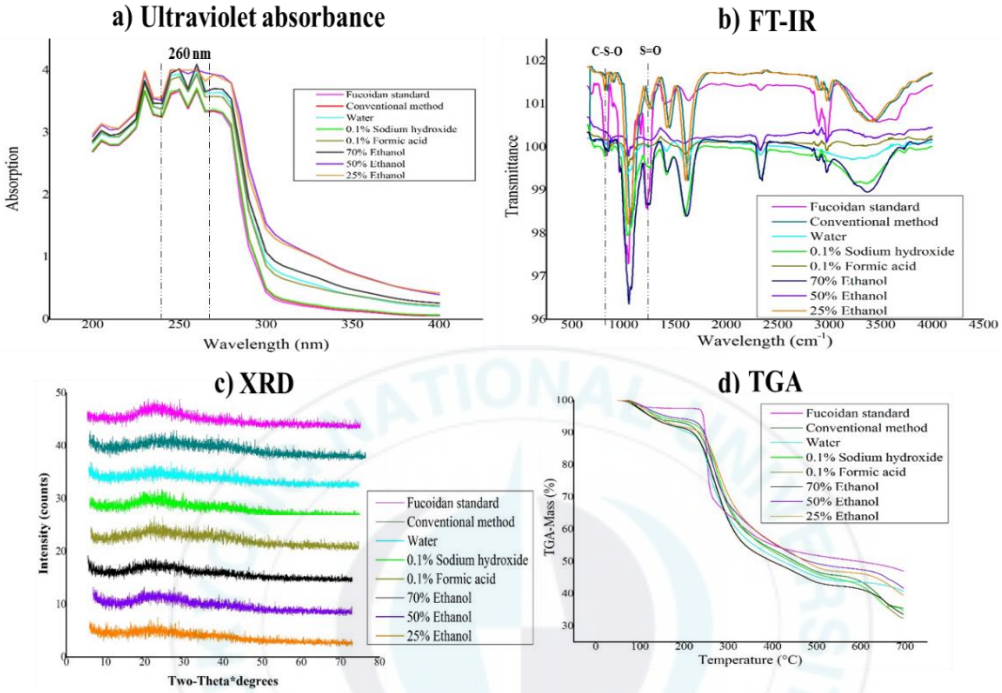


Fig.4.4. Characteristics of the crude fucoidan obtained from various extracts: a) UV-vis, b) FT-IR, c) XRD and d) TGA.

Table 4.2. Elemental analysis of the crude fucoidan obtained from various extracts

Sample	C%	H%	N%	S%
Fucoidan standard	24.11	3.47	0.00	8.49
Conventional method	25.35	3.45	0.24	3.57
Water	24.67	3.51	0.32	2.21
0.1% Sodium hydroxide	26.19	3.70	0.70	2.99
0.1% Formic acid	26.20	3.65	0.15	2.17
70% Ethanol	23.18	3.18	1.07	2.16
50% Ethanol	24.48	3.56	0.99	2.53
25% Ethanol	25.59	3.42	0.64	2.75

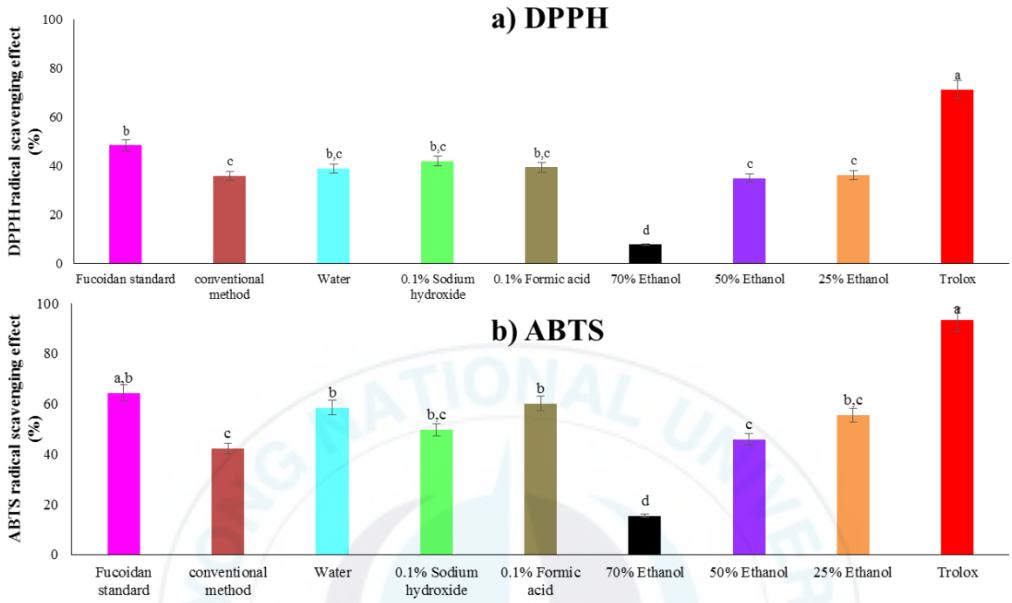


Fig. 4.5. Antioxidant activities of the crude fucoidan obtained from various extracts: a) DPPH, and b) ABTS. [a-d] Different letters indicate a statistically difference ($p < 0.05$).

Table 4.3. IC_{50} of water imbibition in green gram seeds and Mitotic index showing inhibition in onion root tips by crude and standard fucoidan

Sample	IC_{50} of water imbibition (mg/ml)	Mitotic index in %
Doxorubicin	192.31	75.81
Fucoidan standard	329.55	34.13
Water	445.77	20.72
NaOH	489.87	23.22
Formic acid	395.87	27.19
70%EtOH	549.48	N.D
50%EtOH	536.91	4.19
25%EtOH	466.25	12.96

Figure 4.6. Antimitotic activity of crude and standard fucoidan by inhibiting sprouting in green gram seeds.

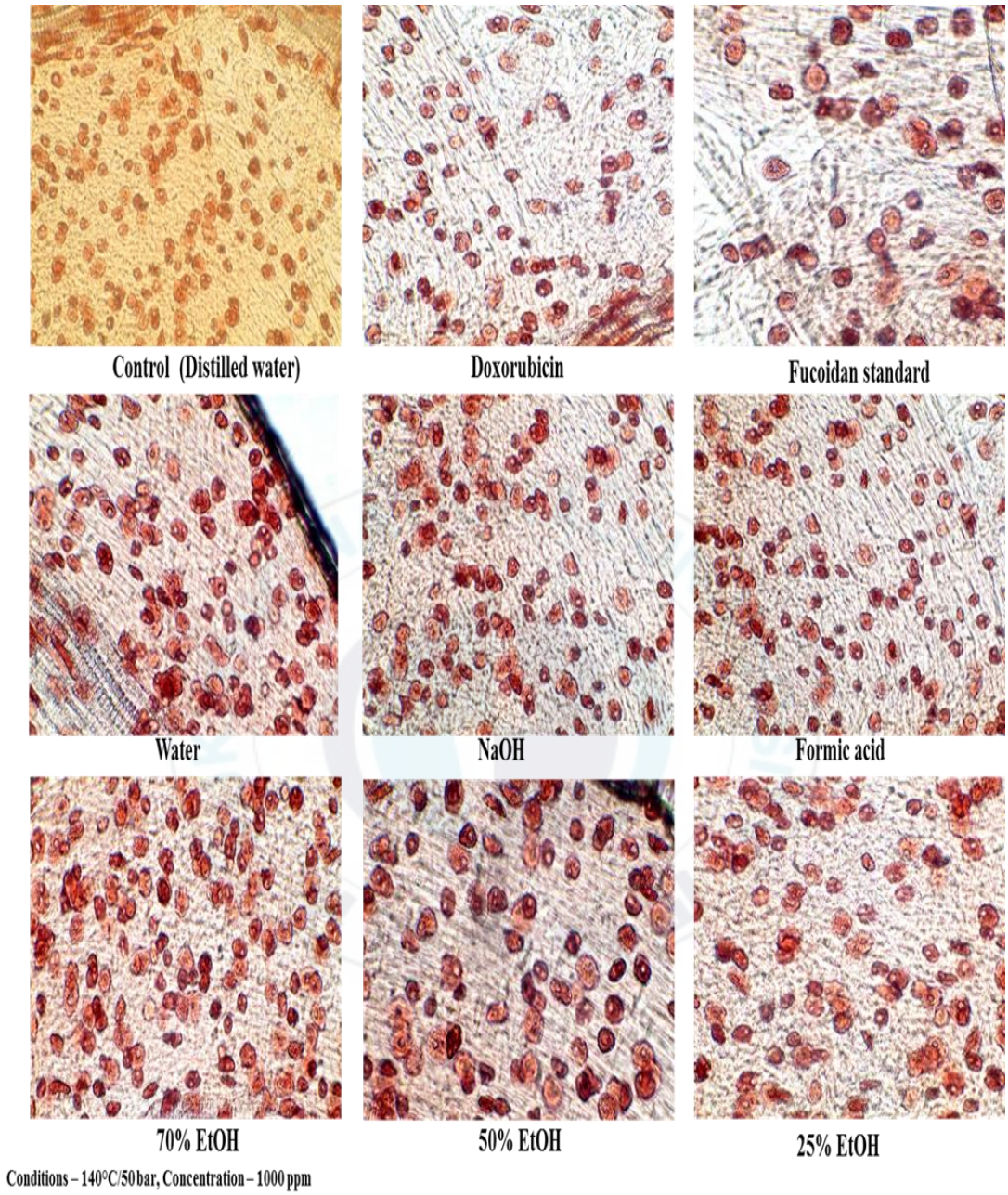
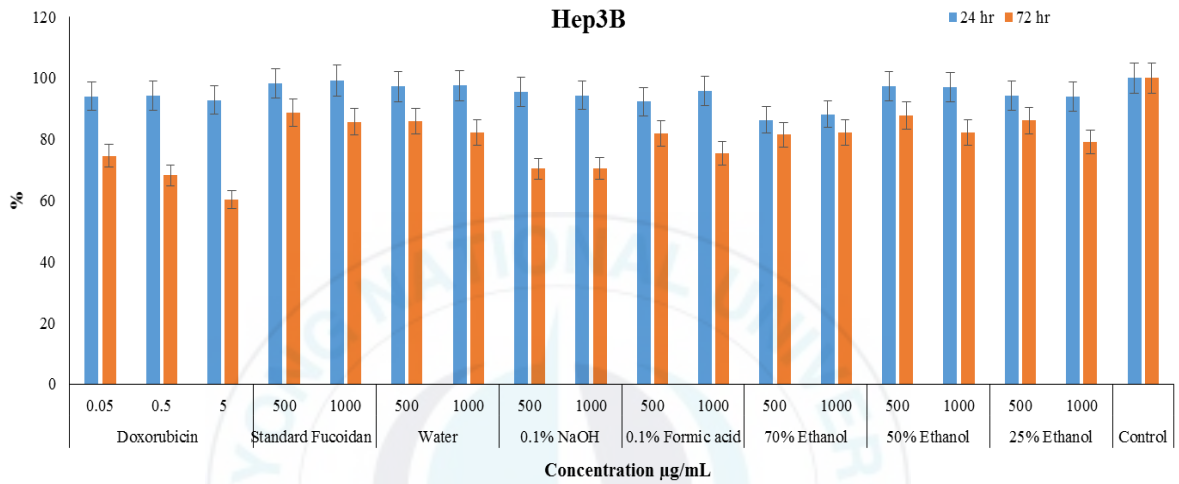


Fig.4.7. Antimitotic activity of crude and standard fucoidan by inhibiting cell division in onion root tip.

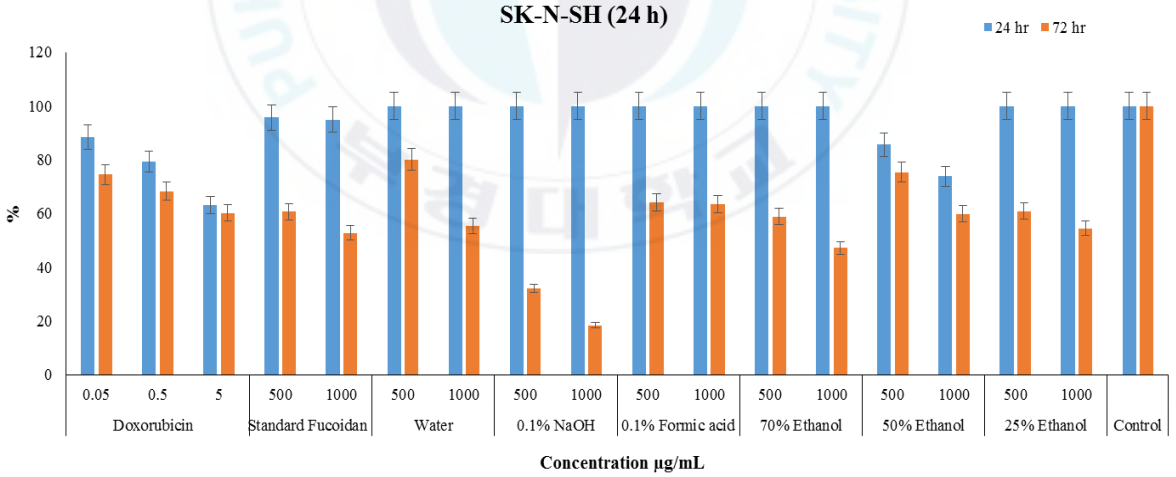
The crude fucoidan was studied for *in vitro* anti-proliferative activity against HeLa, SK-N-SH, MKN-28, SH-SY-5Y or Hep3B cells with two different concentrations of 500 and 1000 μg/mL. Doxorubicin was used as a positive control with a concentration of 0.05, 0.5 and 5 μg/mL, while standard fucoidan (500 and 1000 μg/mL concentration) from sigma was also used to compare with our CF. The results of anti-proliferative activity are shown in Fig. 4.8. Evaluation of *in vitro* anti-proliferative activity was carried out in triplicate for all the samples. CF fucoidan showed potent anti-proliferative activity against all the cells except HeLa cell lines. The lowest anti-proliferative activity with 40% was found against MKN-28 after 72 hr. Most of the cell lines showed moderate activity for the CF after 72 hr. A moderate activity was found in CF at a concentration of 1000 μg/mL against Hep3B, SK-N-SH and slightly higher activity was found in MKN-28 and SH-SY-5Y. Doxorubicin showed a clear growth inhibition in all the cell lines, while standard fucoidan has similar activity when compared with CF.

A previous study showed similar results as our report, it says that a strong inhibition of CF from *Utricularia aurea* was obtained when compared with the purified fractions against KB nasopharynx cancer cells (Choosawad, Leggat, Dechsukhum, Phongdara, & Chotigeat, 2005). Another report says, that fucoidan with low molecular weight and high amount of sulfate content will also have an impact to reduce the cell viability (Yang et al., 2008). The low/moderate anti-proliferative activity against the cell lines in this work might be attributed due to a high molecular weight of the fucoidan (152.45 KDa) and

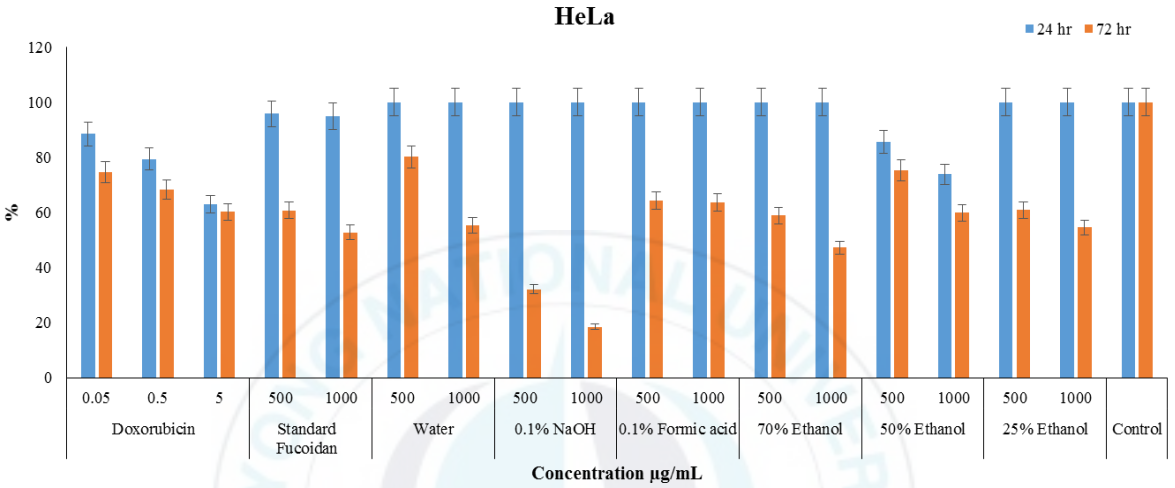
a)


Hep3B
24 hr
72 hr
%
120
100
80
60
40
20
0
0.05
0.5
5
500
1000
Doxorubicin
Standard Fucoidan
Water
0.1% NaOH
0.1% Formic acid
70% Ethanol
50% Ethanol
25% Ethanol
Control
Concentration µg/mL

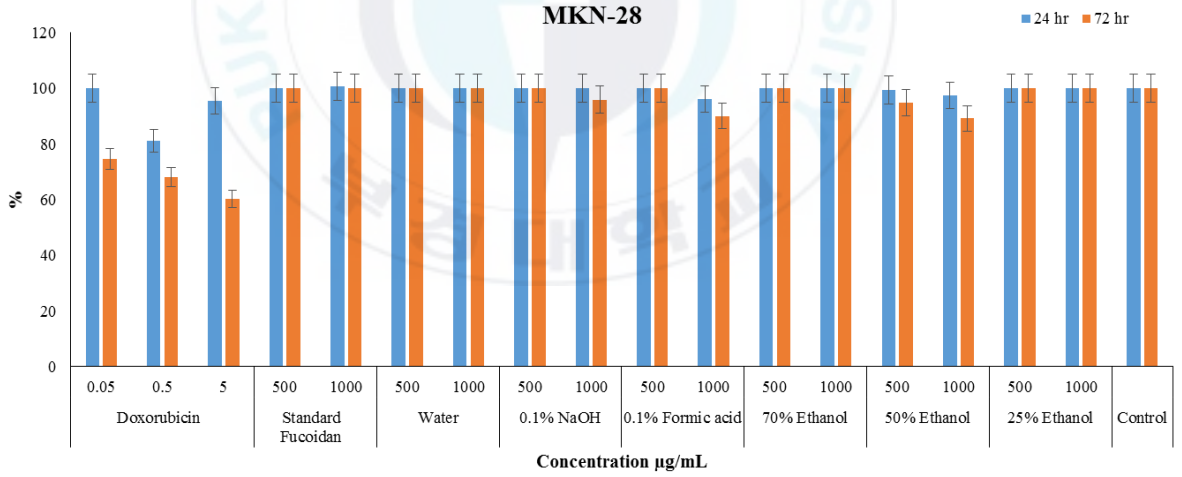
b)


SK-N-SH (24 h)
24 hr
72 hr
%
120
100
80
60
40
20
0
0.05
0.5
5
500
1000
Doxorubicin
Standard Fucoidan
Water
0.1% NaOH
0.1% Formic acid
70% Ethanol
50% Ethanol
25% Ethanol
Control
Concentration µg/mL

c)


HeLa
24 hr
72 hr
%
120
100
80
60
40
20
0
0.05
0.5
5
500
1000
Doxorubicin
Standard Fucoidan
Water
0.1% NaOH
0.1% Formic acid
70% Ethanol
50% Ethanol
25% Ethanol
Control
Concentration µg/mL

d)


MKN-28
24 hr
72 hr
%
120
100
80
60
40
20
0
0.05
0.5
5
500
1000
Doxorubicin
Standard Fucoidan
Water
0.1% NaOH
0.1% Formic acid
70% Ethanol
50% Ethanol
25% Ethanol
Control
Concentration µg/mL

e)

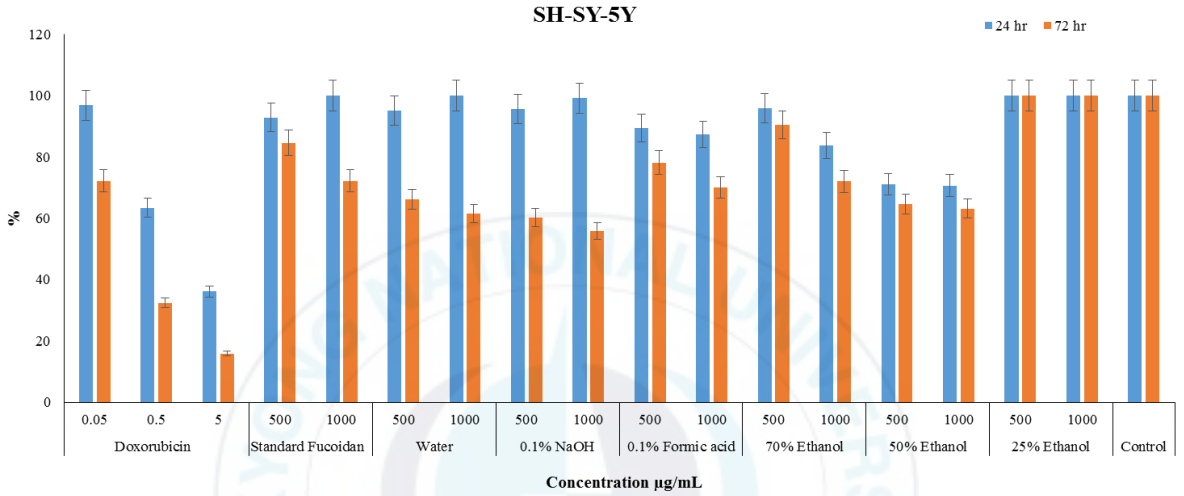


Fig.4.8. The anti-proliferation effects of fucoidan on various human cancer cell lines. (A) Hep3B cells, (B) SK-N-SH, (C) HeLa, (D) MKN-28 and (E) SH-SY-5Y cells. Each bar represents the mean ± SD, n = 3.

low sulfate contet (28.64%). Other factors that may have cell reducing capacity effects which include the high amount of xylose present in CF [40]. Xylose was used a component to develop anticancer drugs, so this might be a reason to trust that xylose content in CF may play a key role in the cell reducing capacity of fucoidans (Mak et al., 2014).

4.4. Conclusions

The results obtained in the present study demonstrate that fucoidans could be successfully extracted from *S. japonica* by PLE technology. In addition, the yield of CF can be improved when compared with the conventional method. The characterization of CF shows that fucoidan recovered from 140 °C and 50 bar with various solvents has a similar composition to the commercially available standard fucoidan. AA shows that fucoidan recovered from 140 °C and 50 bar has similar DPPH and $ABTS^{+}$ scavenging effects compared to standard fucoidan. Moreover, CF demonstrated a modest antimitotic and anticancer activities. To conclude, PLE can be considered a more environmentally friend technique than the traditional extraction processes. These results support the utility of PLE technology for an efficient method to extract fucoidan.

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Summary

From overall studies we conclude that

- The addition of vegetable oils and water as co-solvents significantly increased the efficiency of SC-CO_2 extraction of TC, FX, and PT from *S. japonica*. SFO was found to be the most effective in improving the extraction yield of TC and FX, while water as co-solvent improved the yield of PT.
- PHWE of *S. japonica* affects the yield, TOC, pH, MRPs, viscosity, and amino acid, mineral, and monosaccharide contents during different temperature conditions. High temperature causes TOC, pH, and the MRP content to increase, while monosaccharide profile showed that the increase in temperature decomposes sugars. It can be concluded that 180 °C is the optimum condition to have a good nutritional composition of amino acids, minerals, and sugars.
- Fucoidans could be successfully extracted from *S. japonica* by PLE technology. The characterization of CF recovered from 140 °C/ 50 bar with various solvents has a similar composition to the standard fucoidan. The high yield of CF was 8.23%, obtained from 140 °C and 50 bar (1% sodium hydroxide as solvent).

Abstract (In Korean)

고압분리공정을 이용한 다시마로부터 유용물질의 추출공정 최적화 및 생리활성

P. S. Saravana

부경대학교 대학원 식품공학과

요약

가장 대중적으로 이용되고 있는 다시마는 가래제거 혹은 감소, 체중감소를 위한 일반적인 치료제로 천 년에 걸쳐 사용되어져 온 해양생물이다. 다시마는 생물학적 기능소재가 풍부하며, 이로 인해 많은 연구들이 푸코잔틴과 푸코이단이 항염증, 항바이러스, 항응고제, 항암, 항산화와 항 동맥경화와 같은 광범위한 약리학적 특성을 나타내는 것이라고 검증하였다. 본 연구에서는 이러한 가치 있는 소재를 추출하기 위하여 녹색기술공정(Green technology)이 사용되었다.

첫 번째 연구에서 다시마로부터 총 카로티노이드, 푸코잔틴, 플로로탄닌을 추출하기 위해 초임계 이산화탄소(supercritical carbon dioxide, SC-CO_2)와 보조용매로 해바라기씨유, 대두유, 카놀라유, 에탄올과 물을 사용하였다. 최적의 추출조건을 확인하기 위해 반응표면방법론(Response surface methodology)을 이용하였으며, 이를

통해 얻은 조건 (온도 [45–55°C]와 압력 [200–300 bar] 그리고 보조용매의 유속[0.50–2.00 (% of CO_2, w/w)])을 이용한 최급상승법 (steepest ascent method)은 추출 수율을 향상시키기 위한 최상의 보조용매량을 결정하기 위해 사용되었다. 총 카로티노이드와 푸코잔틴의 추출 최적 조건은 50.62℃, 300 bar, 보조용매(해바라기씨유) 2.00%으로 확인되었으며, 플로로탄닌의 최적조건은 48.98℃, 300 bar, 보조용매(물) 2.00%으로 확인되었다. 추출 곡선은 최적화된 조건에 대해 추출곡선을 확인하였으며, 실험데이터는 유동적 변수를 평가하는데 사용되었다. 해바라기씨유를 보조용매로 사용한 경우, SC-CO_2 만 사용하였을 때 보다 높은 지방산 함량 및 항산화능 그리고 오일 안정성을 보였다.

두 번째 연구에서는 다시마를 180-420℃의 온도와 13-520 bar 의 압력 범위에서 가압열수추출(Pressurized hot water extraction, PHWE)로 처리하고, 수득한 가수분해물에 대해 수율, 총 유기탄소(TOC), pH, Maillard 반응 생성물, 점도, 색도, 아미노산, 미네랄 그리고 단당류 함량을 조사하였다. 추출 수율은 온도와 비례하게 증가했으며, 72.21%에서 98.91%까지 다양하게 나타났다. 온도의 증가와 함께 총 유기탄소량과 pH, 칼륨 및 나트륨함량은 증가하였으나, 점도는 감소하였다. 발린과 라이신과 같은 필수아미노산과 아스팜산, 글루탐산, 글리신과 시로신과 같은 비 필수아미노산은 낮은 온도에서 더욱 많이

회수되었고, 비소, 카드뮴, 수은, 납과 같은 중금속의 함량은 매우 적은 함량을 보였다. 전체 아미노산은 180℃/13bar 의 조건에서 최대치(761.95 ± 14.54 mg/g)를 보였으며, 글루코즈(6.70 g/L), 프락토즈(8.40 g/L)와 만니톨(17.50 g/L)과 같은 주요 단당류의 함량 또한 동일한 조건에서 매우 높게 발견되었다. 이 결과는 다시마의 가압열수추출은 발효산업에서의 사용될 수 있는 긍정적인 가능성과 식량으로써 사용될 수 있음을 보여주었다.

세 번째 연구에서는 가압액체추출(Pressurized liquid extraction, PLE)을 이용하여 다시마로부터 푸코이단(fucoidan)을 추출하였다. 다양한 온도(80-200℃), 압력(5-100 bar), 및 용매(물, 0.1%수산화나트륨, 0.1% 포름산, 70% 에탄올, 50% 에탄올, 그리고 25% 에탄올)의 조건에서 수득한 푸코이단의 특성을 가늠하였다. 푸코이단의 최고 수율은 8.23%로, 0.1% 수산화나트륨을 용매로 사용하였을 때 140℃, 50bar 의 조건에서 나타났다. 조성분석, FT-IR, 분자량, 단당류, TGA, UV-Vis, XRD 그리고 원소분석을 통해 추출된 다당류가 푸코이단의 특징을 나타내는 것을 확인하였다. 푸코즈(Fucose)는 다양한 용매에서 얻어진 푸코이단에 있는 주요 단당류이다. 다양한 용매로부터 수득한 푸코이단은 항산화, 세포분열저지, 항암활성을 보였다. 이 연구는 가압액체추출이 다시마의 다당류의 추출 수율을 향상시키기 위한 효과적인 방법이며, 그것은 천연 항산화제와 항암제로써 이용될 가능성이 있음을 입증한다.

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