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ORIGINAL PAPER

# Chemical composition and antioxidant activity of sulphated polysaccharides extracted from *Fucus vesiculosus* using different hydrothermal processes

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Sulphated polysaccharides (SP) were extracted from *Fucus vesiculosus* seaweed by using two different hydrothermal processes: microwave-assisted extraction (MAE) and autohydrolysis (AH). The extraction yields, chemical composition, and antioxidant activity of the polysaccharides extracted were determined and compared. Although both processes afforded SP with similar yields (18.2 mass % and 16.5 mass %, for MAE and AH, respectively) and L-fucose as the main monosaccharide, the heterogeneous structure of the polysaccharide recovered was significantly affected by the AH process. The SP obtained by MAE contained 53.8 mole % of fucose, 35.3 mole % of xylose, and 10.8 mole % of galactose; while the SP obtained by AH was composed of 76.8 mole % of fucose and 23.2 mole % of galactose. Both samples presented comparable values of antioxidant activity by the di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (2,2-diphenyl-1-picrylhydrazyl, DPPH), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), and lipid oxidation inhibition methods, but the polysaccharide obtained by AH exhibited a higher antioxidant potential by the differential pulse voltammetry technique. This study demonstrates that the chemical composition and antioxidant activity of SP obtained from *F. vesiculosus* vary according to the process used for their extraction. However, the SP obtained by MAE or AH both have the potential for use as natural antioxidants in industrial applications.


**Keywords:** antioxidant activity, autohydrolysis, fucan, *Fucus vesiculosus*, microwave-assisted extraction, sulphated polysaccharides

## Introduction

Oxidative stress induced by oxygen radicals is regarded as a primary factor in various degenerative and chronic diseases including alcoholic liver disease, ageing, and diabetes. Reactive oxygen species (ROS), such as the superoxide anion-radical ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), and other non-radicals oxygen derivatives such as hydrogen peroxide ($H_2O_2$) are constantly generated by normal metabolic processes as part of controlled inflammatory reactions, and as a result of exposure to environmental factors (Halliwell, 2012). The excessive production of ROS may result in oxidative damage to many large biomolecules, such as

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lipids, DNA, and proteins, leading to destruction of the cellular membrane or tissue damage (Wang et al., 2010; Sokolova et al., 2011). In addition, lipid peroxidation is a major cause of pathological effects such as cardiovascular disease, cancer, and brain dysfunctions; it also leads to the development of food rancidity and off-flavours (Lim et al., 2002).

A great deal of attention has recently, been paid to those natural substances that can delay or prevent the oxidation of cellular substrates, as it is highly desirable to avoid the use of synthetic compounds related to toxic and carcinogenic effects on health. As the ocean is viewed as a source of potential drugs, it has been searched for novel bioactive metabolites for prospective pharmaceuticals and healthy foods. Approximately 16000 natural products have already been isolated from marine organisms, and several of these exhibit the potential for applications in the medical, pharmaceutical, cosmetics, and food industries (Bhakuni & Rawat, 2005). Among marine species, seaweeds are known for their ability to generate bioactive compounds (with various biological and physiological activities) that protect them from external factors such as UV radiation, stress, and herbivores (Wijesekara et al., 2011). The sulphated polysaccharides of seaweeds, for example, are chemically different from those of land plants and may have important biological properties (Haroun-Bouhedja et al., 2000; Mao et al., 2006; Wijesinghe & Jeon, 2012).

Brown seaweed sulphated polysaccharides (fucans) are molecules mainly composed of fucose and sulphate groups, but which may also contain minor amounts of other sugars (including galactose, xylose, glucose, among others) in their composition. Such molecules represent significant economic importance, and several studies have demonstrated their potential as free-radical scavengers and antioxidants for the prevention of oxidative damage in living organisms (Rupérez et al., 2002; Bhakuni & Rawat, 2005; Costa et al., 2010). Moreover, fucans as antioxidants have been classified as non-toxic and water-soluble macromolecules, playing an important role in assisting their water insoluble counterparts in the removal of reactive oxygen species from the body (Rupérez et al., 2002; Sokolova et al., 2011). However, fucans' structure and biological properties, including antioxidant capacity, vary according to algal species, environmental conditions, life-stage of the seaweed and extraction procedure (Wijesinghe & Jeon, 2012).

Hydrothermal processes, which use only water as a reactant under high temperature and pressure conditions, have been seen as an environmentally friendly and cost-effective technology for biomass fractionation into its main constituents (Garrote et al., 1999). In our previous studies, two hydrothermal processes, microwave-assisted extraction and autohydrolysis, were used to extract fucoidan from the brown seaweed *Fucus vesiculosus*, and the conditions for maximising the extraction yields were established (Rodriguez-Jasso et al., 2011, 2013). Due to the differences in the conditions used for extraction, it is possible that the polysaccharides obtained by the microwave and autohydrolysis processes present different chemical compositions and biological properties. Therefore, the aim of the present study was to investigate and compare the chemical composition and antioxidant activity of sulphated fucans extracted from *F. vesiculosus* by these two hydrothermal processes. The antioxidant activity was estimated by different methods including di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (2,2-diphenyl-1-picrylhydrazyl, DPPH), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), lipid oxidation inhibition (LOI), and differential pulse voltammetry (DPV).

## Experimental

### *General*

DPPH, ABTS, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®), and linoleic acid (95 %) were obtained from Sigma–Aldrich (USA). All other reagents used were of analytical grade and obtained from either Sigma–Aldrich or Merck (Germany).

### *Seaweed and extraction of sulphated polysaccharides*

*Fucus vesiculosus* seaweed was collected from Praia Norte (Portugal) in May 2010. To be used in the extraction experiments, the seaweed was washed thoroughly under fresh tap water in order to remove sand and epiphytes, dried at 35 °C, milled in a domestic blender, and stored in plastic bags at ambient temperature in a dry place. Particles smaller than 1 mm were not used in the experiments. The conditions used to obtain the maximum recovery of sulphated polysaccharides from *F. vesiculosus* were established in previous studies (Rodriguez-Jasso et al., 2011, 2013).

Microwave-assisted extraction was performed in a digestion oven MDS-2000 (CEM Corporation, USA). For the extraction reaction, the milled seaweed was suspended in distilled water to obtain an alga/water mass/volume ratio of 1 : 25. The suspension was placed in the extraction vessel and irradiated at 172 °C for 1 min prior to cooling in an ice bath. Subsequently, the hydrolysate thus produced was separated from the residual algal material by filtration through a nylon fibre filter (Rodriguez-Jasso et al., 2011).

For the autohydrolysis process, the milled seaweed was suspended in distilled water to obtain an alga/water mass/volume ratio of 1 : 25 and the mixture was placed in a stainless steel cylinder reactor that was submerged in a silicon oil bath (Julabo

Labortechnik, Germany). The autohydrolysis process was carried out at 180 °C for 20 min. At the end of the reaction, the reactors were immediately cooled in an ice bath and the hydrolysate thus produced was separated from the residual algal material by vacuum filtration (Rodriguez-Jasso et al., 2013).

In order to separate the sulphated polysaccharides from the hydrolysates produced by the microwave and autohydrolysis processes, a 1 mass % solution of $CaCl_2$ was added to the hydrolysate ($\varphi_r = 1 : 1$) for alginate removal (4 °C, overnight storage). The free alginate liquor was recovered by filtration using a qualitative filter paper, then double the volume of absolute ethanol was added to the resultant filtrate and the mixture was stored at 4 °C for 8 h. The precipitated polysaccharide was recovered by centrifugation (8500 $min^{-1}$, 4 °C, 15 min) dried at 35 °C, milled, and stored for analyses.

### *Analytical determinations*

The concentration of monosaccharides was determined by hydrolysis of the fucoidan samples (10–15 mg) with 2 M trifluoroacetic acid (0.5 mL) at 121 °C for 2 h, in glass tubes filled with nitrogen. After the reaction, the tubes were cooled in an ice-water bath, centrifuged at 5000 $min^{-1}$ for 5 min and the supernatant was neutralised to pH 7 with 2 M NaOH. The resulting samples were analysed by HPLC using a Jasco chromatograph (Japan) equipped with a refraction-index detector and a MetaCarb 87P (300 mm × 7.8 mm) column at 80 °C. Deionised water was used as the mobile phase at 0.4 mL $min^{-1}$.

Total content of phenolic compounds was estimated by the Folin–Ciocalteau assay, and the content of the sulphate groups was quantified through the barium chloride–gelatine method (Dodgson, 1961). The extraction yield was calculated from the ratio between the dry mass of sulphated polysaccharide and the algal mass used for extraction.

### *Antioxidant activity assays*

In order to determine the antioxidant activity of the sulphated fucans, solutions were prepared by solubilising these polysaccharides in distilled water to acquire 0.5 mg $mL^{-1}$ and 1.0 mg $mL^{-1}$. Then, the total antioxidant activity of these polysaccharides solutions was estimated by the methods described below.

#### *Radical scavenging activity by DPPH method*

The DPPH scavenging activity was determined by measuring the ability of the samples to scavenge the free radical DPPH. For the reactions, 50 μL of sample were added to 2950 μL of a 60 μM methanolic DPPH solution, and the mixture was shaken and incubated in darkness at ambient temperature for 30 min. Then, the absorbance at 517 nm was determined. The control solution consisted of distilled water used instead of the sample. The DPPH radical scavenging activity was expressed as the inhibition percentage using Eq. (1):

$$\% \text{ inhibition of DPPH} = \left(1 - \frac{\text{AS}}{\text{AC}}\right) \times 100 \qquad (1)$$

where AC and AS are the absorbance of the control and the absorbance of the sample solution, respectively.

#### *Radical scavenging activity by ABTS method*

For radical ABTS formation, a stock solution was prepared by mixing 12.5 mL of 2.45 mM potassium persulphate with 25 mL of 7 mM ABTS solution; once prepared, the mixture was kept in the dark at ambient temperature for 12–16 h prior to use. Then, the absorbance was measured at 734 nm, and the ABTS solution was diluted with ethanol to an absorbance of 0.70 ± 0.01. For the assays, 950 μL of fresh ABTS solution was mixed with 50 μL of the sample and the absorbance was measured after 1 min of reaction. The control solution contained distilled water instead of the sample. The ABTS radical scavenging activity was expressed as the inhibition percentage using Eq. (2):

$$\% \text{ inhibition of ABTS} = \left(1 - \frac{\text{AS}}{\text{AC}}\right) \times 100 \qquad (2)$$

### *LOI assay*

The LOI assay was performed using linoleic acid as the lipid source. The substrate solution was prepared by diluting 0.56 g of linoleic acid and 1.5 g of Tween® 20 in 8 mL of 96 vol. % ethanol. For the assays, 50 μL of sulphated polysaccharide solution was mixed with 100 μL of linoleic acid solution and 1.5 mL of 0.02 M acetate buffer with pH 4.0. The mixtures were homogenised in a vortex mixer and the emulsions incubated at 37 °C for 1 min. Then, 750 μL of 50 M $FeCl_2$ solution (0.0994 g $FeCl_2$ and 0.168 g EDTA diluted to 1 L with distilled water) was added in order to induce the oxidation of linoleic acid. After 1 h of reaction, 1 mL of 0.1 M NaOH in 10 vol. % ethanol was added to 250 μL of the mixture to stop the oxidation process. Subsequently, 2.5 mL of 10 vol. % ethanol was added and the absorbance was measured at 232 nm against 10 vol. % ethanol as blank. A control solution was prepared using distilled water instead of the polysaccharide sample. The per-cent of antioxidant activity was calculated according to Eq. (3):

$$\% \text{ inhibition of lipid oxidation} = 100 - (100B - A) \qquad (3)$$

where $A$ is the difference between the absorbance of the control sample (with distilled water) after 24 h

and at the start time of incubation (0 h) and $B$ is the difference between the absorbance of the sample after 24 h and at the start time (0 h) of incubation.

### *DPV method*

The principle of this method involves recording the voltammograms of the cathodic reduction of oxygen by means of any voltammetric analyser using differential voltammetry. The DPV measurements were carried out using Epsilon Potentiostat/Galvanostat equipment (BASi, USA) with a standard three-electrode electrochemical cell. A platinum foil electrode was used as the working electrode and a platinum spiral electrode as the counter. All potentials were referred to an Ag/AgCl 3 M KCl reference electrode. The height of the pulses was adjusted to 80 mV with a potential range from 0 mV to 1700 mV. Pulse amplitude and width were 200 ms and 50 ms, respectively. The electrochemical evaluation of the sulphated fucans antioxidant potential was compared to a standard solution of Trolox® (1 mg $mL^{-1}$).

### *Statistical analysis*

All analytical determinations were performed in triplicate and the results are presented as average values ± standard deviations. Data were analysed using one-way analysis of variance (ANOVA) and the Tukey multiple comparison test was used to determine significant differences ($p < 0.05$) between mean values. OriginPro 8 (OriginLab Corporation, USA) was the software used for data analyses.

## Results and discussion

### *Extraction yield and chemical composition*

Sulphated polysaccharides from brown seaweeds like *F. vesiculosus* usually contain a main structure composed of fucose and sulphate groups, with minor proportions of other monosaccharides (which may include mannose, galactose, glucose, and xylose, among others) linked to the fucose backbone (Li et al., 2008; Jiao et al., 2012; Wijesinghe & Jeon, 2012). A typical structure of sulphated polysaccharides (fucoidan) from *F. vesiculosus* is shown in Fig. 1. However, the composition of these polysaccharides varies according to the extraction procedure (Wijesinghe & Jeon, 2012).

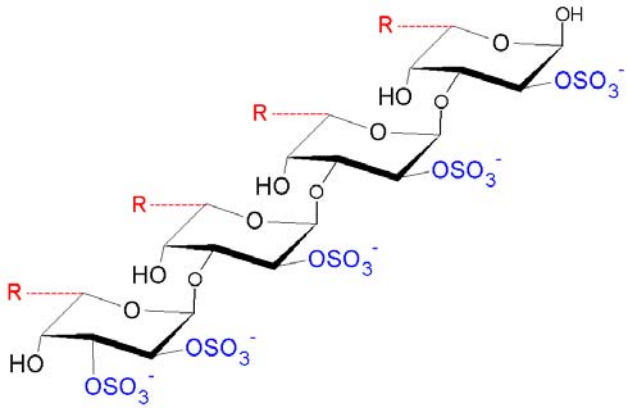


**Fig. 1.** Structure of sulphated polysaccharides from *Fucus vesiculosus* brown seaweed. R is a monosaccharide sugar that may include mannose, galactose, glucose, or xylose, among others (This Fig. was created by the author, Mussatto S. I., and is part of an unpublished study).

The chemical composition of the sulphated polysaccharides obtained in the present study by the microwave-assisted extraction (MSP) and by autohydrolysis process (ASP) of *F. vesiculosus* is shown in Table 1. Both polysaccharides were obtained under conditions previously selected to promote their maximum extraction from *F. vesiculosus* seaweed (Rodriguez-Jasso et al., 2011, 2013). Table 1 reveals that MSP and ASP were obtained with similar extraction yields ((18.2 ± 1.4) mass % and (16.5 ± 1.2) mass % respectively), and L-fucose was the main monosaccharide in both samples. However, the heterogeneous and branched structure of the fucans was highly affected by the autohydrolysis, which yielded a polysaccharide containing 76.8 % fucose and 23.2 % galactose. On the other hand, MSP afforded a sulphate polysaccharide composed of fucose, xylose, and galactose sugars. The significant difference between the structures of these fucans can be related to the longer extraction time used for autohydrolysis (20 min) when compared to the time used for the microwave-assisted

**Table 1.** Chemical characterisation of sulphated polysaccharides obtained from *Fucus vesiculosus* seaweed by microwave-assisted extraction (MSP) or by autohydrolysis process (ASP)

| Sulphated fucan sample | Extraction yield per 100 g of seaweed/g | Monosaccharides composition/mole % | | | $SO_3$/% | Total content of phenolics per 100 mg of sulphated fucan/mg |
|---|---|---|---|---|---|---|
| | | Fucose | Xylose | Galactose | | |
| MSP | 18.2 ± 1.4 | 53.8 ± 1.2 | 35.3 ± 0.8 | 10.8 ± 0.2 | 21.1 ± 1.7 | 5.0 ± 0.6 |
| ASP | 16.5 ± 1.2 | 76.8 ± 2.3 | 0.0 | 23.2 ± 0.7 | 21.2 ± 0.8 | 5.6 ± 0.8 |

extraction (1 min). This longer time could have affected the sugars present in the side-chains of the fucose backbone.

The sulphate content was similar (≈ 21 mass %) in both fucoidan samples (Table 1). This value was close to that reported for fucoidan samples recovered by solvent extraction of *F. vesiculosus* (Rioux et al., 2007) and was higher than the contents observed in sulphated polysaccharides recovered by cold or hot (80 °C) water extraction of *F. vesiculosus* (9.65 mass % and 14.51 mass %, respectively) (Jiao et al., 2012). It should be noted that 21 mass % is an elevated sulphate content and this is an important characteristic of the recovered polysaccharides, since fucans with a high content of sulphate groups have been reported to present important biological functions (Schaeffer & Krylov, 2000; Wijesinghe & Jeon, 2012). Finally, a low polyphenols content (≈ 5 mass %) was found in both MSP and ASP samples. These low values can be associated with the use of water as the extraction solvent, since phenolics are generally more soluble in polar organic solvents than in water (Kim & Lee, 2002; Martins et al., 2012).

The results obtained in this part of the study reveal that the chemical composition of the sulphated polysaccharides recovered from *F. vesiculosus* is affected by the method used for their extraction. This finding accords with the findings of Jiao et al. (2012), who observed that the extraction of polysaccharides from *F. vesiculosus* with hot (80 °C) or cold water generated polysaccharides containing different amounts of fucose as main sugar. In addition, both the hot and cold water-extracted polysaccharides presented different contents of minor sugars, which included galactose, xylose, and glucose.

### *Antioxidant activity*

According to the current literature, many different methods have been used to evaluate antioxidant activity in food and biological systems, hence it is difficult to compare the results presented in different studies. Moreover, since each method is based on a different reaction, it is advisable to determine the antioxidant potential of a sample by different methods in order to be better able to interpret the results. In the present study, the antioxidant activity of the sulphated fucans extracted from *F. vesiculosus* was evaluated by four different methods (DPPH, ABTS, LOI, and DPV). To determine the antioxidant activity of the sulphated polysaccharides, samples of the fucans were dissolved to provide two different concentrations (0.5 mg $mL^{-1}$ and 1.0 mg $mL^{-1}$), and, the higher the concentration of fucoidan in the sample, the higher the antioxidant activity observed by all the methods evaluated.

DPPH and ABTS assays were carried out in order to verify the radical scavenging capacity of the sulphated polysaccharides extracted from *F. vesiculosus*. The DPPH free radical-scavenging model is widely accepted as a tool for estimating the free radical-scavenging activities of antioxidants. In this method, the absorbance decreases as a result of a colour change as the radical is scavenged by antioxidants through the donation of hydrogen to form the stable DPPH–H molecule (Hu et al., 2004). In the present study, the highest DPPH radical-scavenging effects were observed for the fucoidan samples obtained by autohydrolysis (ASP), with an inhibition value of (23.0 ± 3.5) % for a polysaccharide concentration of 1 mg $mL^{-1}$ (Fig. 2).

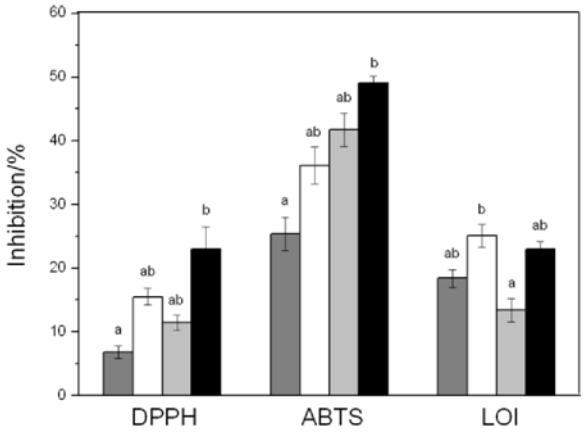


**Fig. 2.** Antioxidant activity (DPPH, ABTS, and LOI assays) of sulphated fucans extracted from *Fucus vesiculosus* by microwave-assisted extraction (MSP) or autohydrolysis process (ASP). Polysaccharide solution prepared at: ■ – 0.5 mg $mL^{-1}$ (MSP), □ – 1.0 mg $mL^{-1}$ (MSP), ■ – 0.5 mg $mL^{-1}$ (ASPF), and ■ – 1.0 mg $mL^{-1}$ (ASP). For each method, columns with the same letter denote no statistically significant differences between samples at $p < 0.05$.

The results obtained for ABTS inhibition ranged from 25 % to 50 % according to the polysaccharide sample analysed, which represents a higher variation than that observed for DPPH inhibition (from 6 % to 23 %) (Fig. 2). When comparing the three chemical methods used to determine the antioxidant activity (DPPH, ABTS, and LOI), it may be noted that the sulphated polysaccharides evaluated in the present study were more reactive towards the ABTS radical inhibition.

As with the DPPH assay, the highest ABTS inhibition values were also observed for the samples obtained by autohydrolysis (ASP), with the maximum inhibition value of (49.1 ± 1.1) % for a polysaccharide concentration of 1 mg $mL^{-1}$. According to Barahona et al. (2011), the chemical structure of fucans plays some role in the H abstraction reaction by the ABTS cation radical, and the scavenging effect of fucoidan could be due to the sulphate group position at O-2, which is close to the glycosidic bond. In contrast with the DPPH and ABTS results, the LOI method gave very similar results for MSP and ASP fucoidan sam-

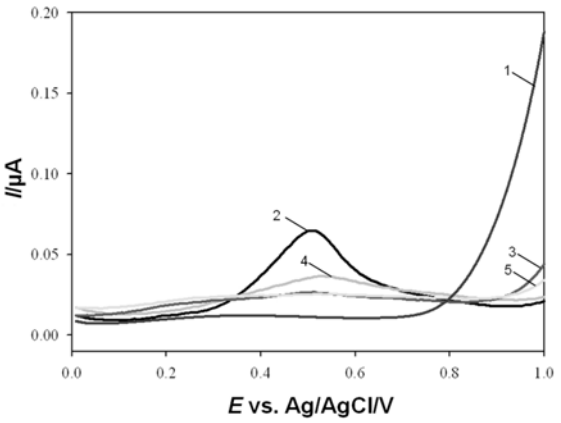


**Fig. 3.** Differential pulse voltammetry of the sulphated fucans extracted from *Fucus vesiculosus* seaweed by microwave-assisted extraction (MSP) or autohydrolysis process (ASP). Polysaccharide solution prepared at concentrations of 0.5 mg $mL^{-1}$ and 1.0 mg $mL^{-1}$. A Trolox® solution was used as control for comparative purposes; 1 – Trolox®, 2 – 1.0 mg $mL^{-1}$ (ASP), 3 – 1.0 mg $mL^{-1}$ (MSP), 4 – 0.5 mg $mL^{-1}$ (ASP), 5 – 0.5 mg $mL^{-1}$ (MSP). Potentials were measured against standard Ag/AgCl electrode.

ples at 1 mg $mL^{-1}$, with a percentage of inhibition higher than 22 % (Fig. 2).

It should be noted that, although the DPPH and ABTS methods revealed higher radical scavenging effects for the ASP sample at 1 mg $mL^{-1}$, an analysis of the variance of the results obtained for each method revealed no significant differences at $p < 0.05$ for MSP and ASP samples at 1 mg $mL^{-1}$ or at 0.5 mg $mL^{-1}$. Fig. 2 shows that, for each method (DPPH, ABTS, and LOI), columns with the same letter denote no statistically significant differences between samples at $p < 0.05$. These results reveal that MSP and ASP presented comparable antioxidant potentials by DPPH, ABTS, and LOI methods.

Recently, alternative methods for the determination of antioxidant capacity based on electrochemical principles have been proposed. Such methods establish an interesting relationship between the electrochemical behaviour of the antioxidant compounds and their resultant "antioxidant power" (Barros et al., 2008). DPV is a simple technique that involves recording the current of the electrochemical oxygen reduction at the electrode surface in order to generate the data necessary to calculate the antioxidant capacity (Korotkova et al., 2002). The voltamperograms generated after DPV with MSP and ASP samples are presented in Fig. 3, where the values of the intensities and potentials of the sulphated polysaccharides are recorded as peak signals.

Fig. 3 shows that both MSP and ASP were oxidisable when subjected to voltammetric experiments, and well-resolved voltammetric peaks were obtained. However, the peak potentials and voltammetric responses ($I$) were strongly influenced by the sample as well as by the concentration of polysaccharide in the sample. A remarkable difference was also observed for the sample of the Trolox® solution used as control. The maximum peaks obtained by DPV were recorded at 0.49 V, with intensity of 0.02 μA and 0.064 μA for MSP and ASP samples, respectively, at 1 mg $mL^{-1}$. These values reveal a 32-fold increase in the intensity value for the ASP sample compared with the MSP sample. From these results, ASP can be considered as having a stronger antioxidant activity than MSP because the intensity value obtained by DPV is directly related to the antioxidant activity (Korotkova et al., 2002).

According to some authors, the antioxidant capacity of the sulphated polysaccharides appears to be related to the molecular mass and sulphate content present in the structure (Qi et al., 2005; Yuan et al., 2005). Considering that, in the present study, both polysaccharides samples (MSP and ASP) presented similar sulphate contents (Table 1), it is possible that the difference in the antioxidant potential observed by using the DPV method could be related to differences in the structure of the fucans extracted by these two different methods.

## Conclusions

Sulphated polysaccharides can be extracted with similar yields from *Fucus vesiculosus* by the microwave-assisted extraction or autohydrolysis processes. Although L-fucose was the main monosaccharide in both samples, the heterogeneous structure of the recovered polysaccharide was highly affected by the AH process, probably due to the longer time used for the extraction, which may have affected the sugars present in the side-chains of the fucose backbone. In terms of antioxidant potential, both sulphated polysaccharides present comparable antioxidant activities by the DPPH, ABTS, and LOI methods, but the polysaccharide obtained by autohydrolysis presented higher antioxidant potential by the differential pulse voltammetry technique. This study reveals that the chemical composition and antioxidant capacity of sulphated polysaccharides obtained from *F. vesiculosus* vary according to the process used for their extraction. However, both fucans obtained by microwave-assisted extraction or by autohydrolysis process have the potential for use as natural antioxidants in industrial applications.

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