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THESIS

DEVELOPMENT OF METHODS FOR DETERMINING THE TOTAL CONCENTRATION AND SPECIES OF NON-METALS IN SEAWEED FROM THE ANTARCTIC

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Thesis presented, in accordance with the international doctoral cotutelle agreement, to the Chemistry Postgraduate Program from the Federal University of Pelotas (UFPel, Brazil) and to the Doctoral School of Exact Sciences and their Applications from Université de Pau et des Pays de l'Adour (UPPA, France), as a partial requisite to obtain the degree of Doctor of Science (Analytical Chemistry).

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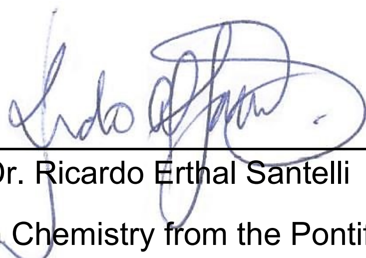

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“Don't worry about a thing
'Cause every little thing
Gonna be all right”
(Bob Marley, 1977)

Abstract

Antarctic is the main thermal regulator of the Earth, and the characteristics of the climate in the Southern Hemisphere are essentially generated and controlled by masses of cold air coming from this continent. Generally, it is in Antarctic that the first changes undergone by the planet are observed, and the damage caused by climate changes, consequently, is reflected to the rest of the planet. The evolution of different life forms in Antarctic took place under extreme conditions of cold, ice and limited exposure to sunlight. Due to these special conditions, several species present in Antarctic are endemic to the region. Given the uniqueness of Antarctic, chemical information on the composition of its fauna and flora becomes of great importance with regard to environmental issues. Chemical information about Antarctic seaweed reported in the literature is limited to a few organic species and metals. However, information about nonmetals, such as halogens and sulfur, is of great relevance since the determination of these elements can be used for purposes such as marine and atmospheric environmental monitoring. Halogens and sulfur when released into the atmosphere generate reactive species that cause the depletion of the ozone layer. On the other hand, these elements are part of the composition of marine seaweed, presenting important metabolic functions, as well as being essential in the synthesis of several compounds. Thus, in view of the scarcity of information and the importance of determining halogens and sulfur in Antarctic marine species, in this work, methods were developed and optimized for the determination of halogens and sulfur and their species in Antarctic seaweed. By using a multi-technique approach involving different sample preparation methods and high sensitivity determination techniques, it was obtained comprehensive information about total halogens and sulfur concentration, distribution of bromine, iodine, and sulfur in different seaweed fractions in ten seaweed species.

Keywords: Algae, Sample preparation, Liquid chromatography, Mass spectrometry, Speciation

Resumo

A Antártica é o principal regulador térmico da Terra, e as características do clima do Hemisfério Sul são essencialmente geradas e controladas por massas de ar frio provenientes deste continente. Geralmente, é na Antártida que se observam as primeiras mudanças sofridas pelo planeta, e os danos causados pelas mudanças climáticas, consequentemente, se refletem no resto do planeta. A evolução de diferentes formas de vida na Antártica ocorreu sob condições extremas de frio, gelo e exposição limitada à luz solar. Devido a essas condições especiais, várias espécies presentes na Antártica são endêmicas da região. Dada a singularidade da Antártica, as informações químicas sobre a composição de sua fauna e flora tornam-se de grande importância no que diz respeito a questões ambientais globais. As informações químicas sobre algas marinhas antárticas relatadas na literatura são limitadas a algumas espécies orgânicas e metais. No entanto, informações sobre não metais, como halogênios e enxofre, são de grande relevância, uma vez que a determinação desses elementos pode ser utilizada para fins como monitoramento ambiental marinho e atmosférico. Halogênios e enxofre quando liberados na atmosfera geram espécies reativas que causam a destruição da camada de ozônio. Por outro lado, esses elementos fazem parte da composição das algas marinhas, apresentando importantes funções metabólicas, além de serem essenciais na síntese de diversos compostos. Assim, tendo em vista a escassez de informações e a importância da determinação de halogênios e enxofre em espécies marinhas antárticas, neste trabalho foram desenvolvidos e otimizados métodos para a determinação de halogênios e enxofre e suas espécies em algas marinhas antárticas. Usando uma abordagem multitécnica envolvendo diferentes métodos de preparo de amostras e técnicas de determinação de alta seletividade e sensibilidade, foram obtidas informações abrangentes sobre a concentração total de halogênios e enxofre e a distribuição de espécies de bromo, iodo e enxofre em diferentes frações de algas marinhas em dez espécies de algas marinhas.

Palavras-chave: Algas, Preparo de amostra, Cromatografia líquida, Espectrometria de massas, Especiação

Résumé

L'Antarctique est le principal régulateur thermique de la Terre, et les caractéristiques du climat de l'hémisphère sud sont essentiellement générées et contrôlées par des masses d'air froid provenant de ce continent. Généralement, c'est en Antarctique que l'on observe les premiers changements subis par la planète, et les dommages causés par le changement climatique, par conséquent, se répercutent sur le reste de la planète. L'évolution des différentes formes de vie en Antarctique s'est déroulée dans des conditions extrêmes de froid, de glace et d'exposition limitée au soleil. En raison de ces conditions particulières, plusieurs espèces présentes en Antarctique sont endémiques à la région. Compte tenu du caractère unique de l'Antarctique, les informations chimiques sur la composition de sa faune et de sa flore deviennent d'une grande importance au regard des enjeux environnementaux mondiaux. Les informations chimiques sur les algues antarctiques rapportées dans la littérature se limitent à quelques espèces organiques et métaux. Cependant, les informations sur les non-métaux, tels que les halogènes et le soufre, sont d'une grande importance, car la détermination de ces éléments peut être utilisée à des fins telles que la surveillance de l'environnement marin et atmosphérique. Les halogènes et le soufre, lorsqu'ils sont libérés dans l'atmosphère, génèrent des espèces réactives qui provoquent la destruction de la couche d'ozone. D'autre part, ces éléments entrent dans la composition des algues marines, présentant des fonctions métaboliques importantes, en plus d'être indispensables à la synthèse de plusieurs composés. Ainsi, compte tenu de la rareté des informations et de l'importance de la détermination des halogènes et du soufre dans les espèces marines de l'Antarctique, dans ce travail, des méthodes ont été développées et optimisées pour la détermination des halogènes et du soufre et de leurs espèces dans les algues marines de l'Antarctique. En utilisant une approche multitechnique impliquant différentes méthodes de préparation d'échantillons et des techniques de détermination à haute sélectivité et sensibilité, obtenir des informations complètes sur la concentration totale d'halogènes et de soufre et la distribution des espèces de brome, d'iode et de soufre dans différentes fractions d'algues marines chez dix espèces d'algue.

Mots clés : Algues, Préparation d'échantillons, Chromatographie liquide, Spectrométrie de masse, Spéciation

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List of abbreviations

AC	alternating current
ASE	accelerated solvent extraction
API	atmospheric pressure ionization
CD	conductivity detection
CRM	certified reference material
DC	direct current
DIT	diiodotyrosine
DMS	dimethyl sulfide
ESI	electrospray ionization
GC	gas chromatography
HPLC	high-ppressure liquid chromatography
IC	ion chromatography
IC-CD-MS	ion chromatography with conductimetry detector coupled to mass spectrometry
ICP	Inductively coupled plasma
ICP-MS	inductively coupled plasma mass spectrometry
ISE	ion selective electrode
LC	liquid chromatography
LIT	linear ion trap
LOD	Limit of detection
LOQ	Limit of quantification
MAE	microwave-assisted extraction
MIC	microwave-induced combustion
MIT	monoiodotyrosine
MS	mass spectrometry
m/z	mass-to-charge
NAA	neutron activation analysis
RP	reverse phase
SEC	size-exclusion chromatography
SFE	supercritical fluid extraction
UAE	ultrasound-assisted extraction
UV	ultraviolet
XAS	x-ray absorption spectrometry

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1 Introduction

In the last decade, several climate changes have been observed in Antarctic continent, such as an increase in atmospheric temperature and variations in the frequency of snow precipitation (BRACEGIRDLE, 2012; DAMERIS, 2010). As a result, glaciers and frozen ground on the Antarctic continent have been deteriorating over time. Thus, considering that Antarctic is the main thermal regulator of the planet, mainly in the Southern Hemisphere, any change in this region directly affects the climate in the rest of the world. In this sense, although anthropogenic activities are identified as the main cause of these changes, natural sources, such as seaweed, contribute to such variations, through some emissions into the atmosphere (ABBATT; THOMAS; ABRAHAMSSON; BOXE *et al.*, 2012).

Among the elements emitted by these organisms, through metabolic processes, we can mention the halogens (bromine, chlorine, fluorine, and iodine) and sulfur species, which may be able to change some climatic characteristics (ABBATT; THOMAS; ABRAHAMSSON; BOXE *et al.*, 2012). Halogenated and sulfur compounds, such as volatile halogenated organic compounds and dimethyl sulfide (DMS), when released into the atmosphere, tend to react with ozone molecules (O_3), in a continuous cycle, resulting in the depletion of this gas, which is responsible for absorbing part of the solar energy (SINGH; BHARGAWA, 2019; VON GLASOW; VON KUHLMANN; LAWRENCE; PLATT *et al.*, 2004). Additionally, the presence of sulfur compounds in the atmosphere favours an increase in the acidity of rainfall, which can cause several environmental problems (SMYTHE-WRIGHT; BOSWELL; LUCAS; NEW *et al.*, 2005).

Despite the importance in determining halogens and sulfur species in seaweed from the Antarctic in order to characterize them and monitoring the concentrations of these elements, most of the works reported in the literature involving elemental determination or speciation studies, perform the determination of metals (MAJER; PETTI; CORBISIER; RIBEIRO *et al.*, 2014; MESKO; PICOLOTO; FERREIRA; COSTA *et al.*, 2015; PICOLOTO; PEREIRA; COSTA; HARTWIG *et al.*, 2017). Whereas for nonmetals, as halogens and sulfur, the studies are limited to determination of volatile organic halogenated

compounds. This is probably linked to the difficulty in determining these elements in complex solid samples, such as seaweed. Thus, reliable and adequate methods for preparing this type of sample must be developed in order to adequately quantify these elements. In this way, microwave-induced combustion (MIC) is a suitable tool for the preparation of marine macroalgae, aiming at the subsequent determination of halogens and sulfur, since, among other advantages, this method allows the use of alkaline solutions for the absorption of elements, which are suitable for this purpose (NÓBREGA; SANTOS; DE SOUSA; CADORE *et al.*, 2006).

However, despite total concentration is an important information regarding a sample, only this data is not sufficient to obtain comprehensive understanding about the different species presents in the samples, as well as their distribution among the fractions of the samples. In this sense, fractionation and speciation analyses are extremely important for studies aimed at characterizing of a group of elements in a sample. In addition, the identification of new halogenated compounds is described in the literature for different species of seaweed since some compounds, such as halogenated polyphenols, present beneficial biological activities (CABRITA; VALE; RAUTER, 2010). In this sense, Antarctic seaweed are interesting organisms to evaluate the presence of this kind of compounds, since the extreme condition in which they have developed. For this, several extraction methods are reported in the literature for further speciation of different compounds and elements. However, to perform the determination of total content as well as species of halogens and sulfur is necessary the use of sensitive and selective analytical techniques, such as chromatographic and spectrometric, as well as their hyphenation.

Thus, the objective of the present study was to develop and optimize an analytical method, proposing a sample preparation method for Antarctic seaweed aiming at the subsequent determination of the total concentrations of halogens and sulfur in a single analysis by ion chromatography with conductimetric detection coupled to mass spectrometry (IC-CD-MS). For that, the ideal and safe conditions for sample preparation by MIC, such as absorbing solution and sample mass, were carefully evaluated and optimized. In addition, in order to obtain comprehensive information about the halogen and sulfur species present in Antarctic seaweed, this study also applied and optimized

different extraction methods for further identification and quantification of halogenated and sulfur species. For this purpose, it was used a multi-technique approach, employing the use of highly selective and sensitive chromatographic and spectrometric.

2 Literature review

2.1 Seaweed

2.1.1 Generalities

Algae make up a group of organisms, and have been known since ancient civilizations. These organisms are found in almost any environment that can provide them enough light and moisture to carry out the photosynthesis process, such as in seas, rivers, lakes, on the ground or on snow, and also in animals and plants, as partners symbionts (EL GAMAL, 2010). Regarding size, algae can be classified into two types, microalgae, which range in size from unicellular organisms to sizes of 1 mm, and macroalgae which can grow up to 50 cm per day, reaching up to 70 m in length (RAVEN; GIORDANO, 2014). Macroalgae are also called seaweed, term to which they will be addressed throughout the text.

Seaweed are divided according to their predominant pigment into three large groups: green algae (Chlorophyta), brown algae (Phaeophyta) and red algae (Rhodophyta) (EL GAMAL, 2010). These aquatic organisms represent a significant proportion of the world's biodiversity, being a large group that performs important ecological functions (WIJESINGHE; JEON, 2011). They are the base of the food chain in the aquatic environment as primary producers, being important for the maintenance and support of life in oceans and seas (ANASTASAKIS; ROSS; JONES, 2011).

Seaweed are autotrophic organisms, thus, through photosynthesis, seaweed combine carbon dioxide present in the atmosphere with the water and light within the chloroplasts to form glucose and, consequently, release oxygen to the environment (ANASTASAKIS; ROSS; JONES, 2011). In this sense, besides seaweed are responsible for the production of up to 80% of the oxygen produced on our planet (CARR; FRIEDRICHS; SCHMELTZ; NOGUCHI AITA *et al.*, 2006; HUANG; NICHOLSON; HUANG; CASSAR, 2021), they also perform the recycling of carbon dioxide present in the environment. In addition, seaweed absorb nutrients from the environment in which they live, such as chemical species, which can later be recycled in the ecosystem by decomposers or released into the environment through metabolic processes (ANASTASAKIS; ROSS; JONES, 2011).

2.1.2 Composition and bioaccumulation of seaweed

Since time immemorial, humans have used seaweed for different purposes, from decorative items to food (WIJESINGHE; JEON, 2011). Currently, seaweed have high economic value, due to the great diversity of compounds present in the most varied species of seaweed. Among the compounds mentioned, it can be highlighted vitamins, proteins, minerals, fatty acids, enzymes, among others (ECHAVE; FRAGA-CORRAL; GARCIA-PEREZ; POPOVIĆ-DJORDJEVIĆ *et al.*, 2021; EL GAMAL, 2010; LAURITANO; ANDERSEN; HANSEN; ALBRIGTSEN *et al.*, 2016). These compounds are associated to several functions, such as defence, nutritional, as well as in metabolic processes (WIJESINGHE; JEON, 2011). In this sense, some exclusive metabolites appear as promising ingredients for use in industry, such as in the food, cosmetic, pharmaceutical, agricultural, among other areas. In addition, seaweed can be used as a complement to feed, fertilizers, in water treatment, as a biofuel and also as a source of chemical compounds, such as agar, carrageenans and alginates (ALLEN; JASPARS, 2009; CARDOZO; GUARATINI; BARROS; FALCÃO *et al.*, 2007).

The synthesis of compounds by seaweed is directly related to the incorporation of elements in their structures from the environment in which they develop, due to the presence of different functional groups and proteins in their superficies (FARíAS; ARISNABARRETA; VODOPIVEZ; SMICHOWSKI, 2002). Thus, in addition to the aforementioned applications, seaweed have been widely used as biomarkers in aquatic environments (MAJER; PETTI; CORBISIER; RIBEIRO *et al.*, 2014; MESKO; PICOLOTO; FERREIRA; COSTA *et al.*, 2015; PICOLOTO; PEREIRA; COSTA; HARTWIG *et al.*, 2017). In this sense, it is reported that the family, genus, species, light exposure time and geographic origin of seaweed directly affect their ability to accumulate different elements from the environment where they inhabit (CABRITA; MAIA; OLIVEIRA; SOUSA-PINTO *et al.*, 2016; FARíAS; ARISNABARRETA; VODOPIVEZ; SMICHOWSKI, 2002; WANG; WANG; KE, 2014).

Despite the wide use of seaweed for environmental biomonitoring, in most of studies reported in the literature, the objective is to determine metals (FARíAS; ARISNABARRETA; VODOPIVEZ; SMICHOWSKI, 2002; MAJER; PETTI;

CORBISIER; RIBEIRO *et al.*, 2014; MESKO; PICOLOTO; FERREIRA; COSTA *et al.*, 2015). On the other hand, the determination of non-metals, such as halogens and sulfur, in seaweed is also discussed in the literature, with iodine receiving particular attention due to its importance for thyroid function, and also because some seaweed species can accumulate a high amount of this element (KÜPPER; CARPENTER; LEBLANC; TOYAMA *et al.*, 2013). However, the determination of other halogens and sulfur is very important not only in nutritional terms, but also in terms of monitoring environmental changes, identifying new compounds with possible biological activities, as well as characterizing marine species for which no data is available in the literature.

2.1.3 Halogens and sulfur in seaweed

Given seaweed can absorb chemical species present in environmental where they live, the presence of halogens and sulfur in their composition is reported in the literature (GIORDANO; NORICI; RATTI; RAVEN, 2008; MALINOWSKI; MACIEL; CHAVES; BARBOSA *et al.*, 2022). Halogens and sulfur are absorbed by seaweed mainly from seawater, which have high concentration of these elements in salt forms (540 mmol L^{-1} chloride, 800 µmol L^{-1} bromide, 74 µmol L^{-1} for fluoride, 0.5 µmol L^{-1} iodide, and 29 mmol L^{-1} sulfate), thus, the halogens are absorbed as halides from the seawater and sulfur is absorbed as sulfate by seaweed (AL-ADILAH; FEITERS; CARPENTER; KUMARI *et al.*, 2022; GIORDANO; NORICI; RATTI; RAVEN, 2008). These elements have important functions in seaweed metabolism.

Regarding halogens, chlorine is related to essentiality roles in seaweed, such as the active transport and cell expansion, in chloride form (RAVEN, 2016). Iodide is the most effective halide against most reactive oxygen species (ROS), and bromide complements iodide for detoxifying superoxides in seaweed (KÜPPER; CARPENTER; LEBLANC; TOYAMA *et al.*, 2013). On the other hand, no clear information is known about fluorine accumulation or functions in seaweed (AL-ADILAH; FEITERS; CARPENTER; KUMARI *et al.*, 2022).

Halogens need to undergo some oxidative activation to carry out reactions such as incorporation into organic compounds, given the lack of reactivity of the stable halide anions (LUDEWIG; MOLYNEUX; FERRINHO;

GUO *et al.*, 2020). In this sense, enzymes present in seaweed, such as haloperoxidases and halogenases are required for the halogen oxidation and further synthesis of halogenated compounds (TIMMINS; DE VISSER, 2015). For example, in the synthesis of iodinated tyrosines, generally found in seaweed (PENG; YU; XU; CAO, 2018; ROMARÍS-HORTAS; BERMEJO-BARRERA; MOREDA-PIÑEIRO, 2012; SHAH; WUILLOUD; KANNAMKUMARATH; CARUSO, 2005; WANG; XU; WANG; GAO *et al.*, 2019). The diverse group of halogenated compounds released from and contained in seaweed are thought to serve as a defence mechanism (AL-ADILAH; FEITERS; CARPENTER; KUMARI *et al.*, 2022). They assist in preventing grazing on seaweed, managing bacterial, fungal, and microalgal epiphytes, and reducing bacterial and fungal infection (PAUL; POHNERT, 2011; WEINBERGER; COQUEMPOT; FORNER; MORIN *et al.*, 2007). Furthermore, most of the halogenated secondary metabolites identified from seaweed are biologically active and exhibit anti-viral, anti-inflammatory, cytotoxic, insecticidal properties, among others (CABRITA; VALE; RAUTER, 2010; JESUS; CORREIA-DA-SILVA; AFONSO; PINTO *et al.*, 2019).

In turn, sulfur appears in many oxidation states, ranging from -2 to +6, which makes it present in a wide range of compounds, such as proteins, peptides, amino acids, polysaccharides, and others (BARAHONA; RODRÍGUEZ SÁNCHEZ; NOSEDA; MANSILLA *et al.*, 2021; SCHAUMLÖFFEL; GIUSTI; PREUD'HOMME; SZPUNAR *et al.*, 2007; WANG; FENG; LU; LI *et al.*, 2007). These substances seem to be involved in the transfer of inorganic to organic sulfur, since organisms can only accumulate the sulfate anion in environments with high concentrations of this ion. Sulfation is also an effective way of making certain substances water soluble, thus favouring their excretion (CARVALHO; ROQUE, 2000).

The cell wall of seaweed is mainly composed by sulfated polysaccharides, fucans in brown seaweed and agar and carrageenans in red seaweed (GÓMEZ-ORDÓÑEZ; ALONSO; RUPÉREZ, 2010; RUPÉREZ; SAURA-CALIXTO, 2001). In addition to important properties for the food industry, such as thickeners, numerous health advantages, including antioxidant, anticancer, antiinflammation, among others, have been attributed to the sulfated polysaccharides (PATEL, 2018), which makes this class of compounds of great

interest in different areas, such pharmaceutical, and cosmetical (CUNHA; GRENHA, 2016).

Ultimately, it is important to mention that seaweed, through metabolic processes, release halogenated and sulfur compounds into the atmosphere. In this sense, some halogenated and sulfur compounds, such as volatile halogenated organic compounds and DMS, contribute to the depletion of the ozone layer (SAIZ-LOPEZ; PLANE; BAKER; CARPENTER *et al.*, 2012; VON GLASOW; VON KUHLMANN; LAWRENCE; PLATT *et al.*, 2004), affecting mainly the Earth poles, as Artic and Antarctic. These reports associated with the lack of information present on halogens and sulfur in seaweed from the Southern Hemisphere (AL-ADILAH; FEITERS; CARPENTER; KUMARI *et al.*, 2022) further evidence the need for speciation studies of halogens and sulfur in seaweed from the Antarctica.

2.1.4 Antarctic seaweed

Due to the abundance of nutrients present in the Antarctic environment, the development of organisms such as seaweed is favoured. Thus, the generated seaweed biomass plays a very important role in the ecosystems of the region, contributing as a source of food, and serving as a habitat for a wide variety of species of marine fauna (GÓMEZ; HUOVINEN, 2020). Seaweed from the Antarctic are organisms that developed for thousands of years under extreme environmental conditions, such as low temperatures and short periods of exposure to the sun light, that certainly influenced their chemical composition and consequently their metabolism (BECKER; GRAEVE; BISCHOF, 2010; BECKER; QUARTINO; CAMPANA; BUCOLO *et al.*, 2011; TEIXEIRA; SANTOS; TURATTI; PAZIANI *et al.*, 2019). Thus, due to the strong geographic isolation of the Antarctic region, the evolutionary development of seaweed to adapt to these conditions had great effects on biodiversity (WIENCKE; AMSLER, 2012). In this sense, in 2017, the latest survey on the diversity of seaweed in Antarctica indicated that there are 151 species on the continent, of which 41 (about 27%) are endemic species (PELLIZZARI; SILVA; SILVA; MEDEIROS *et al.*, 2017).

Studies have been carried out in order to characterize the composition of Antarctic seaweed. Among these studies, it can be mentioned the identification

of mycosporins, amino acids, fatty acids, sterols, and polysaccharides (BARAHONA; RODRÍGUEZ SÁNCHEZ; NOSEDA; MANSILLA *et al.*, 2021; BERNEIRA; DE SANTI; DA SILVA; VENZKE *et al.*, 2021; PEREIRA; NUNES; ZAMBOTTI-VILLELA; STREIT *et al.*, 2017). Some of the reported compounds presented biological activities, such as antiviral, acting against the herpes and HIV viruses (SALEHI; SHARIFI-RAD; SECA; PINTO *et al.*, 2019; SANSONE; BRUNET; NOONAN; ALBINI, 2020; VO; NGO; TA; KIM, 2011), antileishmanial (CLEMENTINO; ODA; TEIXEIRA; TAVARES *et al.*, 2021), anticancer (FRASSINI; SILVA; MOURA; VILLELA *et al.*, 2019; MARTINS; NEDEL; GUIMARÃES; DA SILVA *et al.*, 2018), and antiparasitic effects (SANTOS; RANGEL; TEIXEIRA; GASPAR *et al.*, 2020). Furthermore, other compounds showed photoprotective effects against UV radiation (RANGEL; VILLELA; PEREIRA; COLEPICOLO *et al.*, 2020), demonstrating potential for use in cosmetics.

On the other hand, as for seaweed in general, the most of studies that perform elemental determination in Antarctic seaweed are focused on metal and selenium (FARíAS; ARISNABARRETA; VODOPIVEZ; SMICHOWSKI, 2002; GUILHERME; PACHECO; SOARES; PEREIRA *et al.*, 2020; MAJER; PETTI; CORBISIER; RIBEIRO *et al.*, 2014; MESKO; PICOLOTO; FERREIRA; COSTA *et al.*, 2015; PICOLOTO; PEREIRA; COSTA; HARTWIG *et al.*, 2017). Although there is a considerable number of publications about halogens and sulfur in seaweed, most of these studies are limited to determination of one or some elements or species of these elements (AL-ADILAH; PETERS; AL-BADER; RAAB *et al.*, 2020; MESKO; TORALLES; CRIZEL; COSTA *et al.*, 2014; ROMARÍS-HORTAS; BERMEJO-BARRERA; MOREDA-PIÑEIRO, 2012; SUN; WANG; CHENG; LIU *et al.*, 2015), whereas for Antarctic seaweed the works are limited to volatile organic compounds determination (LATURNUS, 2001; LATURNUS; ADAMS; GOMÉZ; MEHRTENS, 1997; LATURNUS; GIESE; WIENCKE; ADAMS, 2000; LATURNUS; WIENCKE; KLÖSER, 1996). Thus, in view of the lack of information, the possibility of discovering new compounds, as well as the possibility of using seaweed as biomonitors in aquatic and atmospheric environments, the characterization of the composition of Antarctic seaweed in terms of total concentrations of halogens and sulfur and their species becomes extremely relevant.

2.2 Analytical techniques for halogens and sulfur determination

For the selection of the determination technique to be used, some factors must be considered, such as the type and composition of the sample, the sample preparation method used, the analytes and their concentration present in the sample, as well as the availability of equipment (KNAPP; MAICHIN; FECHER; HASSE *et al.*, 1998; VARGA, 2007). In this sense, among the techniques used for the determination of halogens and sulfur in seaweed, it can be cited spectrometric techniques such as inductively coupled plasma mass spectrometry (ICP-MS) and X-ray absorption spectroscopy (XAS); chromatographic techniques, including gas chromatography (GC) and high-performance liquid chromatography (HPLC); electroanalytical techniques, such as ion selective electrode (ISE); radiochemical techniques, such as neutron activation analysis (NAA) and its variations, as well as the titrimetric and gravimetric methods (AL-ADILAH; PETERS; AL-BADER; RAAB *et al.*, 2020; EL ZOKM; ISMAIL; EL-SAID, 2021; HOU; YAN, 1998; MESKO; TORALLES; CRIZEL; COSTA *et al.*, 2014; ROMARÍS-HORTAS; BERMEJO-BARRERA; MOREDA-PIÑEIRO, 2012; 2013; SHAH; WUILLOUD; KANNAMKUMARATH; CARUSO, 2005; WHYTE; ENGLAR, 1976; YEH; HUNG; LIN, 2014).

Neutron activation analysis and its variations are analytical techniques for direct analysis of samples that have been used due to their high specificity and sensitivity for the analysis of elements with very low concentrations (FRONTASYEVA, 2011). However, in addition to exposing the analyst to risks due to radiation, other disadvantages are associated, such as the low throughput and the high cost of acquisition and maintenance in view of the use of a nuclear reactor (HAMIDATOU; SLAMENE; AKHAL; ZOURANEN, 2013; VANDECASTEELE; BLOCK, 1997). The XAS technique is used since it is able to map the distribution of chemical elements present in different regions of the sample. In addition to allowing the identification of the oxidation state of the elements present in the samples, this technique has the multi-element determination capability and, like NAA, XAS is a non-invasive technique, which makes it possible to eliminate or reduce the sample preparation step before analysis (FEITERS; KUPPER; MEYER-KLAUCKE, 2005; KÜPPER; MILLER; ANDREWS; HUGHES *et al.*, 2018).

However, biological samples, as seaweed, generally present a complex matrix containing high amounts of soluble solid substances and inorganic compounds (BULSKA; RUSZCZYŃSKA, 2017). In this sense, since direct analysis techniques present some limitations regarding the calibration step, problems related to the lack of homogeneity of the sample and matrix effects, their use is not suitable for complex matrices analysis. In view of this, another spectrometric technique well established in the literature and widely used for the determination of non-metals in seaweed is ICP-MS. This technique also has the multi-element determination capability, as well as high sensitivity and selectivity. However, the low mass-to-charge (*m/z*) ratio, the high ionization potential and the presence of polyatomic species in the argon plasma are the main challenges for the determination of halogens and sulfur by ICP-MS (MONTASER, 1998; NELMS; BEAUCHEMIN, 2007).

Thus, as an alternative to spectrometric methods, liquid chromatography, more precisely ion chromatography, has been widely used for the determination of all halogens and sulfur in their anionic forms. Among its advantages, it can highlight the multielement determination capability, the low cost of acquisition and, mainly, the low cost of operation when compared to spectrometric techniques (MELLO; BARIN; DUARTE; BIZZI *et al.*, 2013).

Determination of halogens and/or sulfur is reported in literature for diverse seaweed species(COELHO JUNIOR; PEREIRA; HARTWIG; TORALLES *et al.*, 2017; GÓMEZ-ORDÓÑEZ; ALONSO; RUPÉREZ, 2010; HOU; YAN, 1998; MALINOWSKI; MACIEL; CHAVES; BARBOSA *et al.*, 2022). Most of studies focus on determination of some analytes and/or their species, with the majority of studies focused on the determination of iodine and its species. For this, the most used techniques are ICP-MS, HPLC using different separation modes, as well as the combination between these techniques, mainly in order to perform speciation analysis. These techniques will be discussed in more detail in the next items, since these were the techniques used in the present study.

2.2.1 Liquid chromatographic techniques

Liquid chromatography (LC) is one of the most analytical techniques used for the identification of halogens and sulfur, as well as their species (MALINOWSKI; MACIEL; CHAVES; BARBOSA *et al.*, 2022; MELLO; NOVO; COELHO JUNIOR; SCAGLIONI *et al.*, 2020; MESKO; BALBINOT; SCAGLIONI; NASCIMENTO *et al.*, 2020; MESKO; TORALLES; COELHO JUNIOR; RONDAN *et al.*, 2020). This technique is based on the physical separation, in which the components present in a matrix are distributed between two phases, the stationary phase and the mobile phase. In LC, the stationary phase is composed by a solid material, while mobile phase is liquid, varying its composition according the chromatography method to be used. Among the LC techniques, high-performance liquid chromatography or high-pressure liquid chromatography is widely used for the effective separation and identification of solutes from complex matrices. During HPLC analysis, in order to ensure a constant flow rate, and hence a reproducible chromatography, a mobile phase under high pressure is delivered through the stationary phase, which is packed into a column capable of withstanding such high pressures.

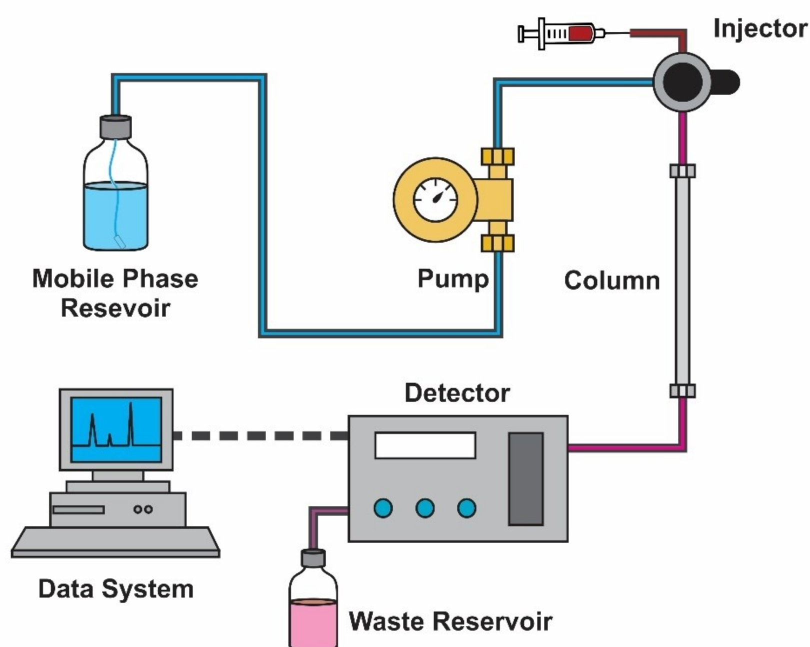

Figure 1. Basic components of a HPLC system.

Analytes can be eluted under isocratic circumstances, which results in similar elution conditions throughout the separation, or under gradient elution, which modulates the eluting force of mobile phase and provides superior resolution. The basic configuration of an HPLC system is mainly composed of a mobile phase reservoir, a pumping system, which allows the circulation of the eluent and the sample, an injector, a separation column, and a detector (SKOOG; HOLLER; CROUCH, 2007), which are illustrated in Figure 1. Furthermore, depending on analytes and type of separation, different systems of detection can be used, such as universal detectors, which respond to all or most of the analytes, and selective detectors, which respond only to certain analytes (HADDAD, 2010). In this sense, LC can be classified according to the form of separation, such as ion chromatography (IC), size-exclusion chromatography (SEC) and reverse-phase (RP), for example (XU, 2013). The reproducibility and selectivity of chromatographic procedures are also dependent on a number of factors, such as salt content, pH, and solvent molarity, which affect analyte interactions and partitioning between stationary and mobile phases (MALLIK; QIU; TAKAFUJI; IHARA, 2018; VALLS; MILLÁN; MARTÍ; BORRÀS *et al.*, 2009).

2.2.1.1 Ion Chromatography

Ion chromatography is based on the ion exchange mechanism that occurs between sample ions, mobile phase ions and stationary phase ionic groups. This technique is used for the separation of both inorganic and organic ions (anions and cations). Thus, when an ion from the ion exchange resin is replaced by an ion contained in the sample, this ion remains retained for a while and then is eluted according the mobile phase passes through the chromatographic column. The different ions present in the sample remain retained for different periods within the column, according to the affinity of each ion with the stationary and mobile phase, which provides adequate separation of the analytes. In this sense, for the separation of anions, ionic species to which halogens and sulfur are commonly determined, the stationary phase is usually composed of quaternary amino groups (WEISS, 2016).

Among the types of detectors used in IC, the detection by conductivity is presented as a universal detector, and measures the conductivity of ionic species after separation in the chromatographic column. In this sense, the presence of cations in the solution to be analysed can directly affect the determination of anions, considering that both ions will present conductivity and cations tend to present substantially higher conductivity than the anions (COLLINS; BRAGA; BONATO, 2006). In this way, a suppression system can be used in order to remove cations present in the sample solution, converting them into their respective acids, and, consequently, improving the sensitivity for the detection of anions by conductivity measurements (HADDAD, 2010).

However, the main disadvantages of IC are related to interference with the elution of analytes at retention times similar or close to other (interfering) species, in addition to the low sensitivity for analytes that are generally in low concentrations in samples, such as Br and I (MELLO; NOVO; COELHO JUNIOR; SCAGLIONI *et al.*, 2020; NOVO; MELLO; RONDAN; HENN *et al.*, 2019; RONDAN; HARTWIG; NOVO; MORAES *et al.*, 2018). Although IC is preferentially used for the determination of halogens and sulfur, other forms of separation, such as size-exclusion chromatography, reversed-phase chromatography, as well as their combination can also be used to determine organic species of these elements, for example.

2.2.1.2 Size-exclusion chromatography

Size-exclusion chromatography is a technique based on the separation of the analytes according their hydrodynamic volume. The hydrodynamic volume of analytes in a certain solvent is determined by their molar mass, molecular configuration, and conformation. This technique is usually applied for the separation of large molecules, such as peptides and proteins. The separation by SEC is performed according to the size of the molecules present in the sample solution, which are eluted by the mobile phase and pass through the column, which is composed of a porous packed material. Because of their inability to permeate the pores, large molecules are excluded from the packed bed and elute first. Medium molecules will be able to penetrate the pores to varying

degrees depending on their size, and smaller molecules diffusing further into the pore structure elute last (STRIEGEL, 2009).

Unlike other chromatography modes, SEC depends on the absence of any interaction between the analyte and the stationary phase packed in the column. However, it is important to mention that some factors, such as particle sizes, pore size distribution, temperature, and viscosity can influence the separations by SEC (LUBOMIRSKY; KHODABANDEH; PREIS; SUSEWIND *et al.*, 2021; STRIEGEL, 2009). Each size exclusion column has a molecular weight range for separation. Thus, the pore size of the packing particles determines the molecular size range within which separation occurs. It should be noted that particles of the same or comparable size may not be efficiently separated by SEC.

2.2.1.3 Reverse-phase chromatography (RP)

Reverse-phase chromatography is the most popular and used technique among the liquid chromatographic separation modes. In RP-HPLC technique, the stationary phase is nonpolar, frequently composed by C_4, C_8 or C_{18} alkyl chains bonded to the surface of silica or closely comparable materials, while the mobile phase is polar, composed by water or combinations of water with polar water-miscible solvents, such as methanol and acetonitrile. Elution can be performed under isocratic circumstances, in which the concentration of organic solvent remains constant, or under gradient elution, in which the quantity of organic solvent is gradually raised over time. As a result, the solutes are eluted in increasing order of molecular hydrophobicity. Due to a variety of features, such as excellent resolution that can be achieved for closely related as well as structurally disparate substances under a large variety of chromatographic conditions; the ease of improving chromatographic selectivity by modifying the characteristics of the mobile phase; as well as the suitable reproducibility of repetitive separations performed during over a long period of time, RP-HPLC is a very powerful tool for analysing a varied range of compounds (AGUILAR, 2004).

2.2.2 Mass spectrometry

A mass spectrometer is basically composed by an ion source, a mass analyser, a detector, and a vacuum system, according shown in Figure 2. The mass spectrometry is based on the generation of charged ions (atoms or molecules) in an ion source, by the removal or addition of an electron or proton. The charged species are separated in a mass analyser according their *m/z* ratios, and the ion current promoted by the species is measured by the detector, amplified and displayed in the form of a mass spectrum. The first two steps are performed under high vacuum, allowing ions to move freely in space without colliding or interacting with other species. Collisions can cause molecular ion fragmentation and the formation of non-specific reaction products. These processes lead to reduced sensitivity, increased measurement ambiguity and decreased resolution in the results obtained (HOFFMANN; STROOBANT, 2007).

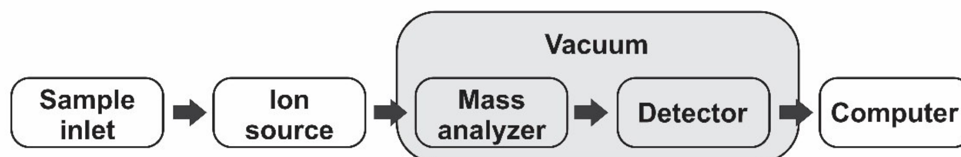

Figure 2. Basic components of a mass spectrometer.

Mass spectrometry has important applications in almost all areas of science. This characteristic is due to the high level of molecular specificity, detection sensitivity, and availability of ionization techniques for all classes of compounds. Among the most used ionization sources, it can be highlighted inductively coupled plasma (ICP) ionization, which has currently assumed a more prominent role in the field of elemental and isotopic analysis, and atmospheric pressure ionizations (API), such as electrospray ionization (ESI), which is considered a soft ionization and thus is commonly used for the ionization of large biomolecules, resulting in the formation of their molecular ions and, little fragmentation is observed (HO; LAM; CHAN; CHEUNG *et al.*, 2003; PITT, 2009). Despite that, in some cases ESI ionization is also used for elemental analysis (MELLO; NOVO; COELHO JUNIOR; SCAGLIONI *et al.*, 2020; MESKO;

BALBINOT; SCAGLIONI; NASCIMENTO *et al.*, 2020; MESKO; TORALLES; COELHO JUNIOR; RONDAN *et al.*, 2020).

Although different mass analyser instruments have been coupled to an ICP source, the quadrupole remains the preferred type of mass spectrometer for this combination because of its obvious ability to easily integrate atmospheric pressure devices (DASS, 2007). In the same way, ESI source with a quadrupole mass analyser is the most successful ESI-MS combination, whereas the combination with high resolution mass analyser, such as ESI-Orbitrap also became an effective system. These ESI-MS combinations are being used for the analysis of a wide range of compounds (DASS, 2007).

The quadrupole mass analyser operates by applying a direct current (DC) and an alternating current (AC) in its two pairs of metallic bars. By selecting the ideal AC/DC ratio on each pair of bars, ions of a selected mass pass through the bars towards the detector, while other unstable ions are ejected from the quadrupole. That is, the separation of ions occurs according to their *m/z* ratio. Ion oscillations along the quadrupole system provide a signal that is transformed into an electrical pulse (THOMAS, 2001). Quadrupole analysers can be arranged as a simple linear or triple system, with the first module allowing for targeted mass selection, the second for fragmentation, and the third for detection. This arrangement is commonly used in tandem mass spectrometry analysis.

2.2.2.1 Tandem mass spectrometry

Tandem mass spectrometry (MS^n) is a multistep analysis that consists of producing different and successive MS scans separated by an event that allows a dissociation process and results in the appearance of product ions from a precursor ion selected in a previous MS scan event. This technique is primarily intended for identification, structural elucidation, and quantification with high sensitivity and reliability. A tandem mass spectrometer can be thought of in two ways: performing tandem mass spectrometry in space, by coupling two physically distinct instruments, or in time, by applying an appropriate sequence of events in an ion storage device (DASS, 2007).

In tandem mass spectrometry in space, mass analysers in succession serve as a scan filter to select precursor ions, a fragmentation collision cell, and a detector to acquire product ion signals. The triple quadrupole is the most common tandem mass spectrometry in space apparatus, with the second quadrupole operating in radiofrequency mode and conducting fragmentation of chosen precursor ions. Tandem mass spectrometry in time may also be accomplished, by using a few analysers like as ion traps and orbitrap, which are configured such that the different processes are carried out sequentially in the same instrument (HOFFMANN; STROOBANT, 2007).

The orbitrap is a very high-resolution analyzer, reaching 1,000,000 for some instruments. Orbitrap works as an electrostatic ion trap in a pulsed mode. An orbitrap is made up of two electrodes: an axial spindle-like central electrode and a coaxial barrel-like outer electrode. The trapped ions rotate around the central electrode and oscillate harmonically along its length. The *m/z* values of the trapped ions are related to the frequencies of their harmonic oscillations. Ion frequencies are obtained from the image current induced between the axial halves of the outer electrode and converted to mass spectra. An orbitrap has also been combined with a quadrupole linear ion trap (LIT) to provide a hybrid tandem mass spectrometry system (YATES; COCIORVA; LIAO; ZABROUSKOV, 2006). In addition, a tribrid tandem mass spectrometry system is reported in the literature, which is composed by three mass analysers: quadrupole, orbitrap and LIT. In this sense, because of its unique architecture, orbitrap mass analysis may be significantly parallelized with the operation of the mass choosing quadrupole, the ion routing multipole (used for both accumulating ions and fragmentation), and the ion trap (KIYONAMI; PEAKE; YOKOI; MILLER, 2016).

2.2.3 Hyphenated techniques

The term hyphenated technique is referred to an online combination of a separation technique, generally a chromatographic technique, with a sensitive and element-specific detector, such as mass spectrometry. For the choice of the hyphenated technique, it is necessary to take into account the research objective as well as the compatibility in the coupling between the separation technique

and the detection techniques. The combination between analytical techniques has effectively improved the sensitivity and selectivity for elemental and speciation studies. In this sense, it is possible to combine different types of LC techniques for separation, and mass spectrometry, using different ion sources, as detector.

The separation technique will be essential for the improvement of selectivity, especially in cases in which the physicochemical properties of target analytes are similar. While the detection technique will be essential for the improvement of sensitivity, especially when analytes concentration present in a sample is very small and low limits of detection are required.

Since HPLC and its variations are widely used for the analysis of the most diverse types of compounds, this technique is also the most used between the separation techniques in hyphenated techniques. Meanwhile, due to the versatile of ICP-MS, allowing the detection of almost 40 elements sequentially with high sensitivity and selectivity, this technique is one of the most used detectors in hyphenated techniques. In this sense, the hyphenation of both techniques is a powerful tool for speciation studies. However, the application of HPLC-ICP-MS as successful hyphenated technique for speciation studies would not have been possible without compatibility between mobile phase flow rate through chromatographic column and liquid flow rate of nebulizer, which allows to work from 0.1 mL min^{-1} to 1.5 mL min^{-1} depending on the column diameter used (DASS, 2007).

As mentioned in previous items, ICP-MS presents some limitation, mainly regarding the polyatomic interference and matrix effects for complex matrices, such as seaweed. In this sense, the use of a chromatographic column before detection allows not only analytes species separation, as well as distinguish them from the matrix components. In addition, since speciation studies are performed using at least two stages (sample preparation and analysis), this also contributes to the minimization of interference during analysis.

Even though HPLC-ICP-MS is an excellent combination for the determination of several compounds, the use of other ionization sources can also be used with mass spectrometry in order to obtain not only elemental ions information, but also molecular ions data by using, for example, LC-ESI-MS. Using this technique, it is possible to operate both ionization modes, positive and

negative, in order to obtain protonated or deprotonated ions, respectively. In addition, the hyphenation of techniques by using two detectors is also possible, which allows analytes to be read by two detection modes in a single analysis. This is very useful in terms of all halogens and sulfur quantification using a simple quadrupole mass spectrometer, since by the coupling of LC using conductimetric detection (CD) to MS it is possible to quantify the analytes in their anionic forms (F^-, Cl^-, SO_4^{2-}) by CD, and for those elements which CD presents low sensitivity, such as Br and I, their detection is performed by the MS detector (MELLO; NOVO; COELHO JUNIOR; SCAGLIONI *et al.*, 2020; MESKO; BALBINOT; SCAGLIONI; NASCIMENTO *et al.*, 2020; MESKO; TORALLES; COELHO JUNIOR; RONDAN *et al.*, 2020).

As for the subsequent determination of the total concentration, for speciation studies it is generally necessary to carry out a sample preparation step. Sample preparation is performed in order to make the sample compatible with the determination technique, which often needs to be analysed in the form of a solution containing the analytes. In this sense, choosing the appropriate preparation method for each type of analysis is extremely important to obtain reliable results.

2.3 Sample preparation for further halogens and sulfur determination

The sample preparation step is considered the most critical step during the analytical sequence, once it is during the sample preparation that the main errors related to contamination and loss of analytes can occur, especially when the determination of halogens and/or sulfur is intended. These possible errors culminate directly in obtaining inaccurate results, compromising the entire analysis. In addition, this stage usually takes longer and is more costly (FLORES, 2014; KRUG; ROCHA, 2016).

Therefore, if the sample is solid and mostly organic (such as seaweed) and the analytes are bound to the matrix components, it is necessary to destroy the organic fraction so that the analytes are disposed in a suitable solution for further analysis. Another alternative is the use of extraction methods, in which the analytes are extracted from the sample matrix using dilute solutions (or water) and the application of a stirring, heating or even alternative energy

sources, such as microwave and ultrasound. As for the determination technique, the choice of sample preparation method must take into account some factors, such as the nature of the sample, the analytes to be determined and their concentrations, the desired precision and accuracy, as well as the determination technique to be used (FLORES, 2014; KRUG; ROCHA, 2016). In this way, it is evident that there is no universal method of sample preparation and the selection of the most convenient method for the desired purpose will be left to the analyst's discretion. In this context, considering the continuous technological advance in relation to analytical techniques, it is also necessary to develop new methods that allow the determination of elements in increasingly smaller concentrations and in different matrices (KRUG; ROCHA, 2016; MESTER; STURGEON, 2003).

2.3.1 Sample preparation for further total content determination

Although most of the methods used during sample preparation are based on the use of acids for sample digestion, this is not feasible when targeting the subsequent determination of some non-metals, such as halogens and sulfur. Thus, the preparation of samples aiming at the determination of these elements becomes a challenge, since in an acid medium, these elements tend to form volatile species, which can cause their loss by volatilization (ANTES; DOS SANTOS; LOURENÇO GUIMARÃES; GOTTFRIED PANIZ *et al.*, 2011). Furthermore, the use of concentrated acids makes it necessary to carry out successive dilutions before the analysis, in order to reduce the acidity of the medium and avoid compatibility problems with some determination techniques (FLORES; BARIN; PANIZ; MEDEIROS *et al.*, 2004; MELLO; BARIN; DUARTE; BIZZI *et al.*, 2013).

Additionally, to minimize problems related to the occurrence of interferences during the analyses, it is desirable that the sample matrix be completely eliminated. Thus, in order to avoid or minimize the occurrence of the mentioned problems, the use of some alternative conditions to the use of acid digestion is addressed in the literature, such as the use of closed systems, the use of alkaline solutions, as well as methods that guarantee the total sample decomposition, such as methods based on combustion (FLORES; BARIN; PANIZ; MEDEIROS *et al.*, 2004; NÓBREGA; SANTOS; DE SOUSA; CADORE

et al., 2006). In this sense, the use of alkaline solutions has been widely described in the literature as an advantageous alternative (NÓBREGA; SANTOS; DE SOUSA; CADORE *et al.*, 2006; NOVO; MELLO; RONDAN; HENN *et al.*, 2019; RONDAN; HARTWIG; NOVO; MORAES *et al.*, 2018). On the other hand, when alkaline solutions are used in extraction-based methods, the efficiency of the method may be compromised due to the non-quantitative extraction of the analytes, as well as the possibility of extracting other compounds, which may act as interferents during the analysis (KRUG; ROCHA, 2016).

In this context, methods based on the combustion of samples become an interesting alternative. In these methods, the organic matter present in the sample is completely oxidized and converted into their respective combustion products (CO_2 and H_2O), which minimizes the risk of interference occurring during the determination of the analytes (FLORES; BARIN; MESKO; KNAPP, 2007). Combustion bomb, oxygen flask, and microwave-induced combustion (MIC) are examples of closed system combustion methods. Among these methods, MIC deserves to be highlighted because it has several advantages over conventional methods, such as the high throughput and the efficiency in digest a wide range of matrices (FLORES, 2014).

Due to its numerous advantages, MIC is one of the most prominent sample preparation methods since it was proposed in 2004 (FLORES; BARIN; PANIZ; MEDEIROS *et al.*, 2004; FLORES, 2014). The MIC combines the advantages of microwave-assisted wet digestion and combustion in closed systems, being a highly robust method for preparing samples with high carbon contents. The combustion process, in MIC, occurs through the incidence of microwave radiation on a filter paper moistened with an ignition solution, usually solutions of nitrate salts (PEREIRA; BIZZI; SCHMIDT; MESKO *et al.*, 2015). The interaction of microwaves with the ignition solution results in an exothermic redox reaction, which releases enough energy to start the combustion process, given that the process takes place in a pressurized environment with oxygen. Thus, as the combustion process occurs, the analytes present in the sample are released and, thus, are absorbed by an adequate solution (FLORES, 2014). The system used for the MIC method is almost the same used for microwave-assisted wet digestion in a closed system. The only modification made is the introduction of a

quartz support, on which the filter paper and the sample are placed. Solid samples are generally introduced into the system in the form of tablets or wrapped in low-density polyethylene films. In Figure 3, the main components of the system used for the decomposition of samples through the MIC are demonstrated.

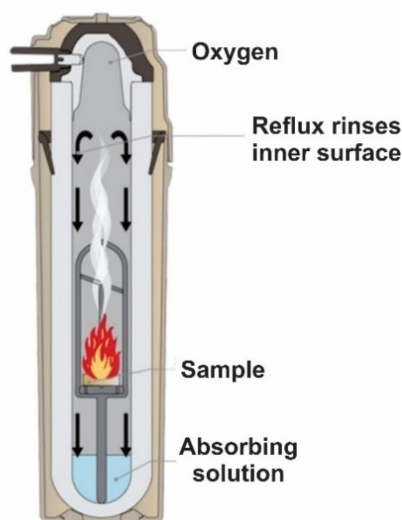

Figure 3. Commercial system used for the decomposition of samples by MIC, demonstrating the burning of a sample and the reflux of the absorbing solution promoted by microwave heating (adapted from Microwave Reaction System for Sample Preparation, Anton Paar, http://anton-paar.com).

2.3.2 Sample preparation for further speciation analysis

Speciation analysis is carried out in three steps, including sample preparation, separation of chemical species, and their identification and possibly quantification. The main purpose of speciation analysis, as well as the most challenging aspect of sample preparation, is to extract analytes from a sample without changing its species. Digestion procedures, which are commonly applied for further determination of the total elemental concentration, are unsuitable since they modify the original species (MATUSIEWICZ, 2017; SZPUNAR, 2000).

Trace elements may be present as distinct species (free anions or bound to simple or complex metabolites) in a complex biological matrix, such as seaweed. Thus, through speciation analysis can identify and quantify one or

more compounds associated to trace elements present in a sample. The large majority of analytical methods for speciation analyses are applicable to liquid samples. However, when the sample is solid, an extraction from the matrix is mandatory. In this sense, the solid samples stand out among the sample types submitted for speciation analysis, since variability in the composition of this type of matrix generally requires adjusting the extraction conditions for each situation. In cases which the composition, in terms of chemical species, is little known or even unknown, it is recommended to use selective extractions, varying the type of solvent (SZPUNAR; ŁOBIŃSKI, 2003).

Although solid-liquid extraction can be done without any external action (agitation, temperature or pressure), these extraction methods tend to be time-consuming. On the other hand, long extraction durations increase the possibility of species degradation and interconversion, as well as losses and/or contamination. Thus, despite the relatively high efficiency of traditional solid-liquid extraction with mechanical or magnetic agitation, numerous alternatives have been proposed, such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (ultrasound-assisted extraction (UAE), accelerated solvent extraction (ASE) and supercritical fluid extraction (SFE), aiming to improve extractions in terms of time, efficiency, sample mass amount, among other factors (KRUG; ROCHA, 2016).

The extracting solvent is selected based on the characteristics of the sample matrix and the species to be extracted. In many cases, water, dilute acids or bases, organic solvents, as well as their mixtures can be used. Another very common option is enzymatic extraction, using mainly proteases, lipases, trypsin, pepsin or mixtures of these. Enzymatic extraction is commonly used due to the many advantages, such as not requiring high temperatures (usually 35 – 50 °C) and work in biological range of pH, controlled by using buffer solutions (KRUG; ROCHA, 2016; MESTER; STURGEON, 2003; SZPUNAR; ŁOBIŃSKI, 2003). Moreover, as enzymes only act on certain chemical bonds, enzymatic hydrolysis is a valuable tool for differentiating between fractions of elements bound to different matrix components (BERMEJO; CAPELO; MOTA; MADRID *et al.*, 2004).

Furthermore, it should be noted that many species cannot be successfully extracted using only one extractant. In this way, sequential extraction

approaches have been developed in this context. Sequential extraction provides total element content and individual species present in solvent fractions. In these methods, a series of extractants (water, dilute salt solutions, enzymes, organic solvents, acids and bases) are applied to the same sample in order to subdivide the total element content and specific species in different solvent fractions (URE; DAVIDSON, 2008). Thus, it can be seen that there are several methods of sample preparation based on extraction that can be applied in order to characterize a sample in terms of its chemical composition.

3 Chapters

The results that are part of this thesis are divided into two chapters and presented in the form of an article and a manuscript. The Introduction, Materials and Methods, Results and Discussion, and References sections are found in the article and manuscript, representing this study in its entirety. The studies are structured according to the rules of scientific journals with some modifications.

Chapter 1

Article

The results of this chapter of the thesis are presented in the form of a scientific article, which is organized in this way. The items Introduction, Materials and Methods, Results and discussion and References can be found in the article. This scientific article was submitted to the journal Environmental Science & Technology.

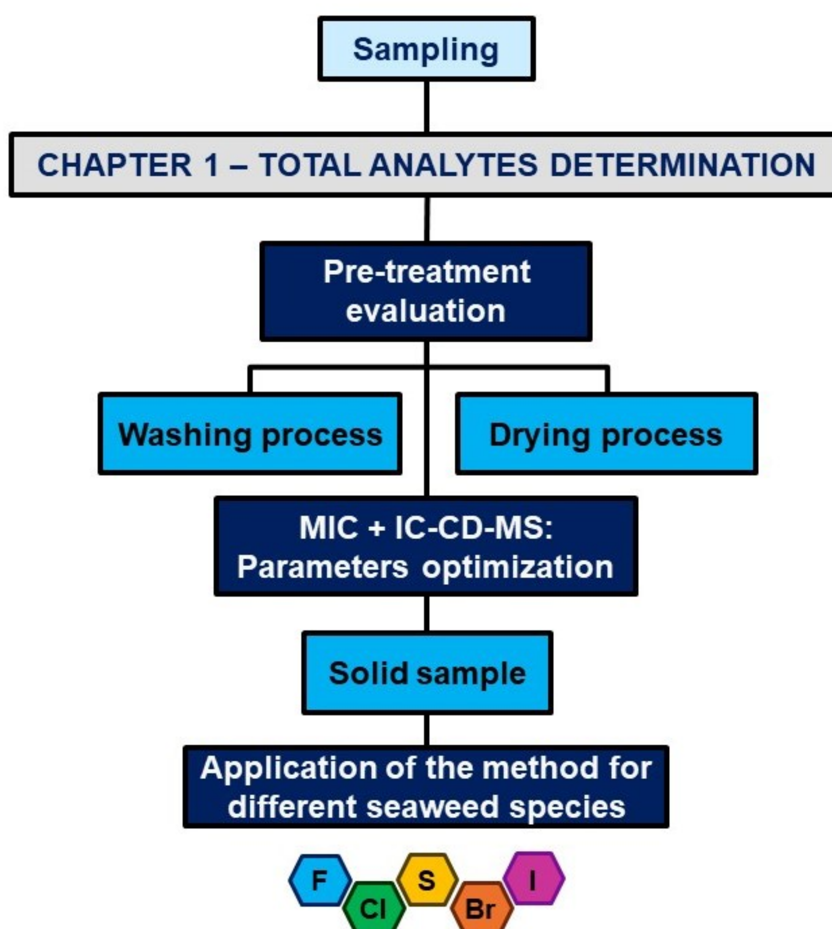

Figure 1. Flowchart showing the content of the article in relation to the method developed for the total determination of halogens and sulfur in seaweed from the Antarctic.

New strategy for single analysis of Antarctic seaweed for halogens and sulfur determination by IC-CD-MS

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Abstract

In this study, a method for halogens and sulfur determination in seaweed from the Antarctic continent was developed. Microwave-induced combustion was optimized for the sample preparation, and the determination of halogens and sulfur was performed using ion chromatography with conductivity detection coupled to mass spectrometry in a single analysis. Up to 1000 mg of sample were satisfactorily digested in a closed system pressurized with 2 MPa of oxygen. Using 150 mmol L^{-1} NH_4OH, recoveries from 96% to 106% were obtained for halogens and sulfur, with relative standard deviations lower than 8%. Additionally, through the analysis of the certified reference material BCR 060, no statistical difference was observed for bromine, chlorine, fluorine, and sulfur values. Limits of quantification (LOQ) of 0.07, 12.3, 1.6, 0.10, and 4.1 mg kg^{-1} for bromine, chlorine, fluorine, iodine, and sulfur, respectively, were obtained. The proposed method was applied to four seaweed species and with exception of fluorine concentration (below the LOQ), the concentrations of the analytes varied in a wide range (bromine: 126 to 517 mg kg^{-1}; chlorine: 552 to 1758 mg kg^{-1}; iodine: 64 to 490 mg kg^{-1}; sulfur: 6542 to 63811 mg kg^{-1}), showing the relevance of this development for further environmental studies.

Keywords: Macroalgae; Non-metals; Ion chromatography; Mass spectrometry; MIC.

Introduction

In the last decade, several climatic changes have been observed in the Antarctic continent, such as the increase in atmospheric temperature and changes in the frequency of snow precipitation [1-3]. Consequently, glaciers and frozen soils have been deteriorating over time in the Antarctic continent. Thus, considering that the Antarctic is the main thermal regulator of the planet, acting mainly in the southern hemisphere, any change in this continent will directly reflect the climatic conditions in the rest of the world [4]. Although anthropogenic activities, such as the operation of the Antarctic stations, scientific research, the logistic transport in the region, as well as tourism are pointed as the main causes of these changes, natural sources, such as the seawater, volcanic activities, and emission of compounds through metabolic processes of seaweed, can contribute to such changes [5].

Seaweed can incorporate some elements from the environment in which they develop due to the presence of different functional groups and proteins in their structures [6]. This ability is directly affected by some parameters, such as the family, the genus, the species, and the geographic origin of the seaweed, as well as the time of exposure to the elements [6-8]. Thus, among the various other applications, seaweed has been widely used as biomarker in aquatic environments [9-11].

Among the elements absorbed, metabolized, and emitted by seaweed, halogens (bromine, chlorine, fluorine, and iodine) and sulfur can be highlighted since they are capable of altering some climatic characteristics [5]. When halogens are released into the atmosphere, they tend to react with ozone molecules, in a continuous cycle, resulting in the depletion of this gas, which acts in the absorption of part of the ultraviolet radiation incident on the planet [12]. The halogens atoms themselves (bromine, chlorine, and iodine,

mainly) and their oxides (ClO, BrO, and IO) are the main reactive halogens species responsible for ozone layer depletion [13].

Although sulfur is not an element that causes depletion of the ozone layer, it indirectly contributes to the ozone depletion cycle, since sulfur released into the atmosphere can be oxidized through chemical reactions, forming particulate sulfate. This formation makes the compounds containing sulfur to be sources of condensation nuclei, favoring the formation of polar stratospheric clouds (PSCs). The main reactions that convert non-reactive halogenated species into reactive species occur on the surface of PSCs [5]. Additionally, the presence of this element in the atmosphere favors an increase in the acidity of rains, which can promote several environmental problems [14].

Despite the problems involved in the high presence and release of these elements from marine seaweed, most of the studies involving elementary determination, reported in the literature, perform only the determination of metals or metalloids [7, 15-17]. Beyond that, if the use of seaweed species present in the Antarctic continent is considered, the representativeness of metals determination is even greater [9-11, 18]. Therefore, the monitoring of non-metals is highly relevant, since there are little data in the literature regarding this group of elements, especially concerning the Antarctic continent. This is probably linked to the difficulty in determining non-metals, mainly halogens, in solid samples with complex compositions, such as seaweed. Thus, reliable and adequate sample preparation methods aiming subsequent determination of halogens and sulfur in complex matrices, such as the seaweed, must be developed to quantify these elements appropriately.

In this sense, the microwave-induced combustion (MIC) method can be considered a suitable alternative for the preparation of seaweed for further determination of halogens and sulfur, since, among other advantages, this method allows the use of alkaline

solutions for the absorption of the elements, which are suitable for this purpose [19-21]. Concerning the determination of halogens and/or sulfur in different matrices, chromatographic or spectrometric techniques are generally used [19, 20, 22-24]. In this sense, ion chromatography with conductivity detection coupled to mass spectrometry (IC-CD-MS) unites the advantages of both techniques, allowing the determination of halogens and sulfur in a single analysis [25-28]. Besides, by using a separation technique and two forms of detection, the sensitivity of the technique is improved [25]. Therefore, the present study aimed to develop, for the first time, a method for the determination of the total concentration of bromine, chlorine, fluorine, iodine, and sulfur in seaweed from the Antarctic continent in a single analysis.

Materials and methods

Instrumentation

For MIC procedures, high-pressure quartz vessels (internal volume: 80 mL; maximum operating pressure: 8 MPa; maximum operating temperature: 280 °C) and a microwave oven (model Multiwave 3000, Anton Paar, Austria) were used. An analytical balance (model AY220, Shimadzu, Philippines), with a maximum load of 220 g and a resolution of 0.0001 g was used to weigh samples and reagents.

Bromine, chlorine, fluorine, iodine, and sulfur were determined using an ion chromatograph (ICS-5000, Dionex/Thermo Fisher Scientific, USA) equipped with an IonPac AS11-HC analytical anion exchange column (4 µm, 2 x 250 mm) and an IonPac AG11-HC guard column (4 µm, 2 x 50 mm), at a controlled temperature of 36 °C. An eluent source EGC 500 KOH generator cartridge with continuously regenerated anion-trap column (CR-ATC), a Dionex ERS 500 anion electrolytically regenerated suppressor (2 mm, using the auto suppression external water mode at 0.20 mL min^{-1} and current

from 2 to 80 mA), and an AS-AP autosampler, were also used. The mobile phase used for the elution of analytes was a potassium hydroxide gradient from 1 to 90 mmol L^{-1}, at a flow rate of 0.28 mL min^{-1}, and the injection volume used was 50 µL. A conductivity cell, at a controlled temperature (36 °C), was used for chloride, fluoride, and sulfate detection. While for bromide and iodide determination, IC coupled to a mass spectrometer (MSQ Plus Single Quadrupole Mass Spectrometer, Thermo Fisher Scientific), with negative mode electrospray ionization (ESI), was used. The ESI-MS parameters were 132 V of cone voltage, 4.20 kV of needle voltage, and 600 °C of the temperature probe. Through the use of IC-CD coupled to MS, all analytes were determined in a single chromatography run by peak area integration. Data acquisition and instrument control were performed using Chromeleon 7.0 software (Thermo Fisher Scientific). The parameters used for halogens and sulfur analysis by IC-CD-MS were selected according to the previous study [25]. Ultrapure water obtained from a purification system (model Simplicity UV, Millipore Corp., France) was used to prepare solutions, make dilutions, as well as to generate the eluent used in IC-CD-MS.

For discs of filter paper and low-density polyethylene (LDPE) films decontamination, an ultrasonic bath (model USC-2800A, Unique, Brazil) was used. Samples, salts, and laboratory glassware were dried in a conventional oven (model 400/2ND, De Leo, Brazil), while filter papers and LDPE films were dried in class 100 laminar flow hood (model CSLH-12, Veco, Brazil). For grinding the samples, a household coffee grinder (B55, Botini, Brazil) was used.

Reagents and Chemicals

Ultrapure water with a resistivity of 18.2 MΩ cm was used to prepare all solutions used in this study. All reagents used were of analytical grade or higher purity. The decontamination of the MIC system was carried out in two steps using 6 mL of

concentrated nitric acid (Neon, Brazil), and 6 mL of ultrapure water, respectively, under a microwave-heating program: *i*) 1000 W/10 min; *ii*) 0 W/20 min. Laboratory materials in general, such as the LDPE films, the filter papers, as well as the flasks for solution storage were decontaminated with ethanol (96%, Synth, Brazil) after double distillation. The igniter solution for MIC, 6 mol L^{-1} of ammonium nitrate solution, was prepared by the dissolution of solid NH_4NO_3 (Merck, Germany) in water. Oxygen with a purity of 99.5% (Oxigeo, Brazil) was used for the pressurization of the MIC system.

Discs of filter paper (12 mg, 15 mm of diameter, Unifil, Germany) with low ash content (90 µg) were used to contain the ignition solution for the combustion process. They were previously decontaminated by immersion in nitric acid 10% (v v^{-1}) for 10 min and subsequently immersed in double-distilled ethanol for 10 min. These processes were performed in an ultrasonic bath and, in the end, the papers were rinsed with ultrapure water and dried in class 100 laminar flow hood. The same procedure was applied for LDPE films (KL Embalagens, Brazil) decontamination.

Solutions of ammonium hydroxide, used as absorbing solutions in the MIC method, were prepared from concentrated solution (27% NH_3 in water, Merck), while the solution of ammonium carbonate also used as an absorbing solution, was prepared from respective salt (Merck).

Calibration solutions for IC-CD-MS were prepared by dilution of stock standard solutions in water. These stock standard solutions were prepared by the respective dissolution of potassium chloride (KCl, Merck), sodium sulfate (Na_2SO_4, Merck), sodium fluoride (NaF, Merck), potassium bromide (KBr, Merck), and potassium iodide (KI, Merck) in water. These same solutions were also used in the recovery tests performed for the choice of the absorbing solution. Before each IC-CD-MS analysis, the

solutions were always filtered through a PTFE syringe filter (pore size 0.22 µm, Chromafil®Xtra PTFE-20/25, Macherey-Nagel, Germany).

Samples and certified reference material

Four species of seaweed from different regions of the Antarctic were used in this work, as shown in Table 1 and Figure 2. The samples were collected manually during periods of low tide between January and February 2018. After collecting the samples, solid waste, such as stones and some invertebrate animals, were removed with tweezers and the samples were washed with fresh water to remove part of the seawater. The samples were stored in polyethylene packages and kept refrigerated in an ultra-freezer (at -80 ºC) onboard the research ship until they arrived in Brazil, and then were stored in a conventional freezer at -18 ºC until the pre-treatment of the samples.

Table 1. Seaweed species collected in the Antarctic regions and their respective coordinates.

Seaweed	Taxonomic group	Region	Coordinates of sampling sites
Desmarestia anceps	Phaeophyta	Demay	62º13’19.3” S and 58°26’36.6” W
Desmarestia anceps	Phaeophyta	Half Moon	62º38’30.2” S and 60°22’09.7” W
Gigartina skottsbergii	Rhodophyta	Punta Plaza	62°05’25.0” S and 58°24’31.0” W
Iridaea cordata	Rhodophyta	Punta Plaza	62°05’25.0” S and 58°24’31.0” W
Palmaia decipiens	Rhodophyta	Snow	62º44’07.0” S and 61º13’45.0” W

D. anceps species (from the Demay region) was chosen for the method optimization since it presents an adequate size for obtaining high sample mass and, for this reason, large quantities are usually also collected. A certified reference material (CRM) of an aquatic plant from the Community Bureau of Reference (BCR 060), which

presents certified values for bromine, chlorine, fluorine, and sulfur, was used for accuracy evaluation of the proposed method.

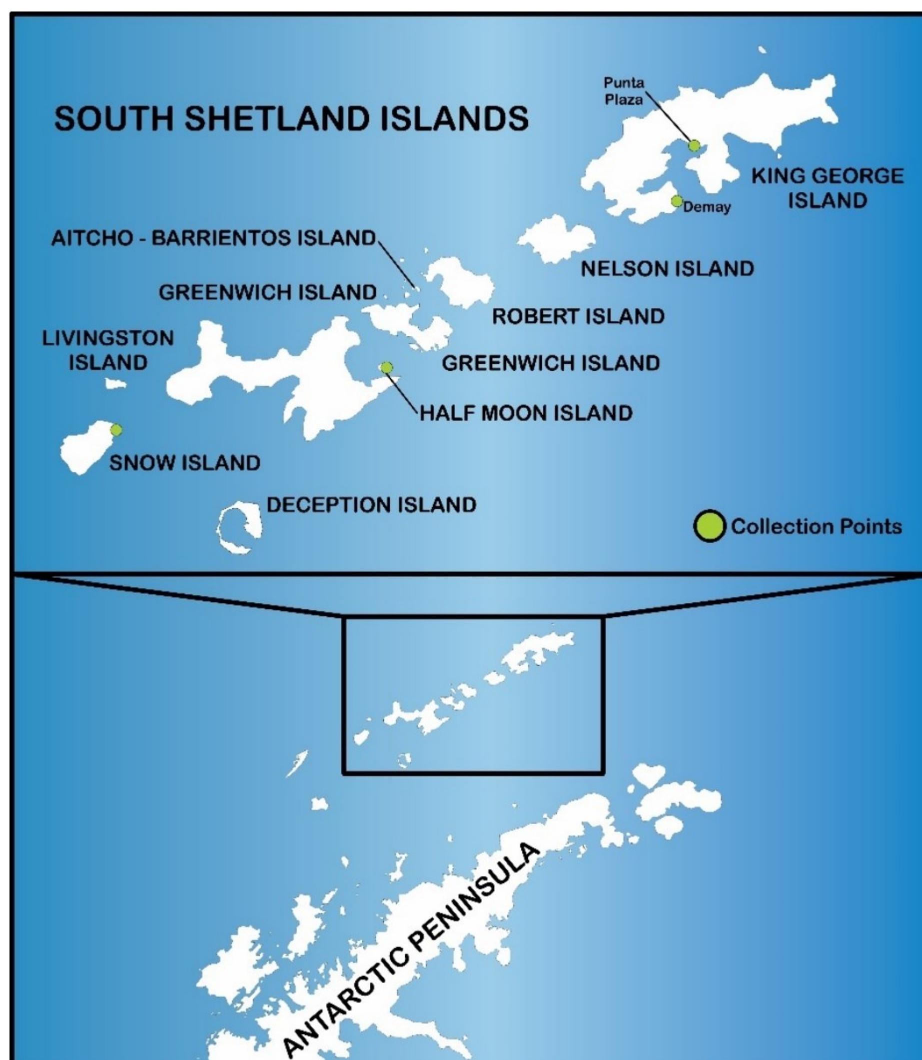

Figure 2. Antarctic (South Shetland Islands) map indicating the collection points of seaweed used in this study.

Pre-treatment evaluation

To evaluate possible losses of analytes from the samples during the pre-treatment step, different conditions in the washing and drying steps were evaluated, such as different water volumes and different drying methods (Figure 3), respectively. For this, *D. anceps* (Demay) sample was thawed at room temperature, cut with scissors, to obtain

equally representative portions of the sample, and separated into three portions. Each portion was washed three times sequentially, by immersion, using different water ratios, to remove the salt present in the sample. Each of the three washes was performed in about 30 s to avoid extracting analytes from the sample. Three water volumes were evaluated, 3, 5, and 10 mL of water for each 1 g of sample (wet weight).

Regarding the drying step, after washing the samples, oven-drying (OD, using 50 ºC and 100 ºC) and freeze-drying (FD) were evaluated. Each portion of samples was submitted to different drying processes, which were carried out for 24 h and 48 h, respectively, for oven-drying and freeze-drying. For the freeze-drying process, the sample was previously frozen in an ultra-freezer at -80 ºC for 24 h. Lastly, the samples were ground, prepared, and analyzed.

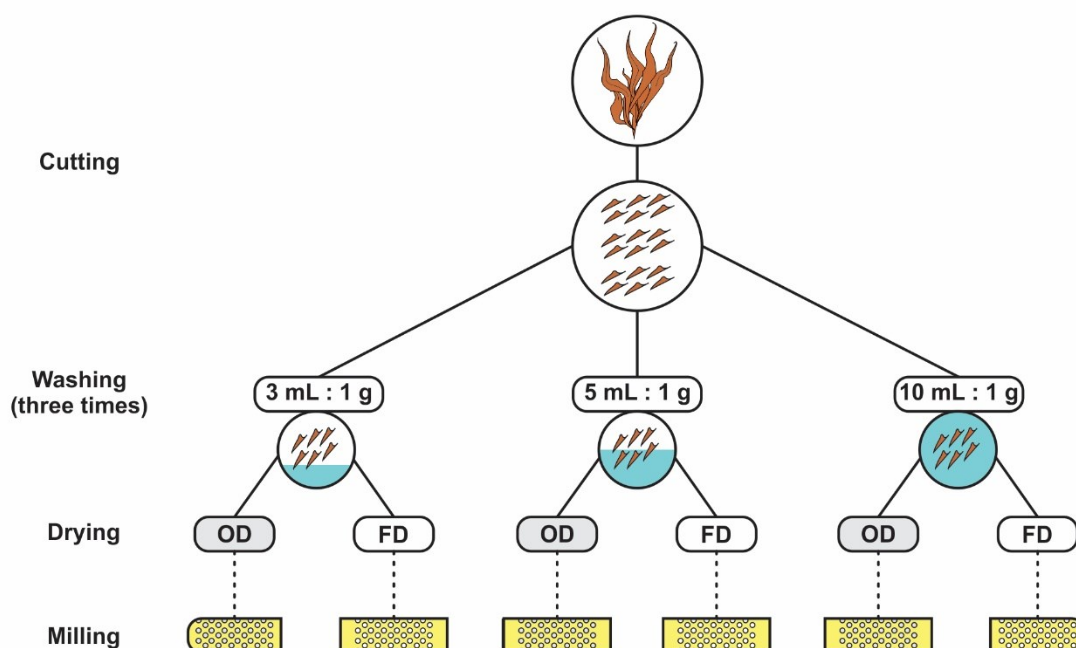

Figure 3. Flowchart demonstrating each step of sample pre-treatment evaluation.

Digestion by microwave-induced combustion

For digestion of seaweed by MIC, the samples were introduced in the form of wrappers in the system [24, 29]. After the pre-treatment step, the ground samples were weighed on LDPE films, sealed by heating, and the LDPE excess was discarded. Samples were placed on quartz holders containing a disc of filter paper previously moistened with 50 µL of 6 mol L^{-1} NH_4NO_3 solution, which is the igniter solution of combustion. The holders were placed inside quartz vessels containing 6 mL of absorbing solution (50, 100, or 150 mmol L^{-1} NH_4OH, or 150 mmol L^{-1} $(NH_4)_2CO_3$ or ultrapure water). After that, the vessels were closed with PTFE lids, inserted in polyetheretherketone (PEEK) covers, fixed in the rotor, and pressurized with 2 MPa of O_2. Then, the MIC process was performed using the following microwave irradiation program, which consists of two steps: *i)* 1400 W for 5 min (combustion and reflux steps); *ii)* 0 W for 20 min (cooling step).

Some parameters, such as the volume and concentration of ignition solution, the volume of absorbing solution, the irradiation program, as well as the pressure of oxygen, used in this study, are considered standard to the MIC method [23, 29, 30]. After the sample preparation, the digests were transferred to volumetric flasks and diluted to 25 mL with ultrapure water for further chlorine, fluorine, and sulfur determination by IC-CD and bromine and iodine determination by IC-MS, in the same analysis.

Recovery tests were performed for the evaluation of the absorbing solution. These tests consisted of adding a standard solution, containing a known concentration of the evaluated analytes (bromine, chlorine, fluorine, iodine, and sulfur), to the seaweed sample, previous to digestion. About 50% of the concentrations of the analytes previously determined were added to the samples, by adding 100 µL of a standard solution containing all analytes evaluated. Since fluorine was below the LOQ in the sample, a

concentration representing twice the limit of quantification value for this analyte was added. The final solutions obtained were also analyzed by IC-CD-MS.

Evaluation of the sample mass

For the evaluation of the sample mass, it was used ultrapure water as the absorbing solution and was evaluated sample masses from 100 mg to 1100 mg. It is important to emphasize that during the sample mass evaluation, the internal pressure of the quartz vessel was monitored. Initially, it was started using the lowest mass (100 mg) to be evaluated. After verifying that the sample was completely digested and the pressure did not increase excessively during the evaluation, it was proceeded to the next test using a higher value mass, and so on (up to 1100 mg).

Analytical parameters

The limits of quantification (LOQ) were calculated from the results obtained in the analysis of ten replicates of the blanks, using the standard deviation and the mean values of the analyte measurements [31], also considering the final volume of the digests (25 mL) and the sample mass (500 mg) used.

The solutions used for IC-CD-MS calibration, with concentrations ranging from 1 to 10 mg L^{-1} for Cl^- and SO_4^{2-}, from 10 to 250 µg L^{-1} for F^-, and from 2.5 to 100 µg L^{-1} for Br^- and I^- were prepared by dilution of stock standard solutions with water.

Results and discussion

Pre-treatment evaluation

The sample pre-treatments, including washing, drying, and grounding, are very important steps when the objective is to carry out elemental determination, which in most

cases their influence on element concentration is not evaluated in method development. As seaweed present a high content of salt from seawater, it is important to remove all analytes that can be present to not quantify analytes that do not come from the samples. For the evaluation of this step, different washing conditions (3, 5, and 10 mL of ultrapure water for each 1 g of sample), and drying process (oven-drying at 50 ºC and freeze-drying) were used. The results obtained for different conditions evaluated are shown in Figure 4. It is important to emphasize that only the concentrations of bromine, chlorine, iodine, and sulfur were monitored to evaluate the influence of the pre-treatment steps on the analyte concentration since fluorine concentration was below the LOQ.

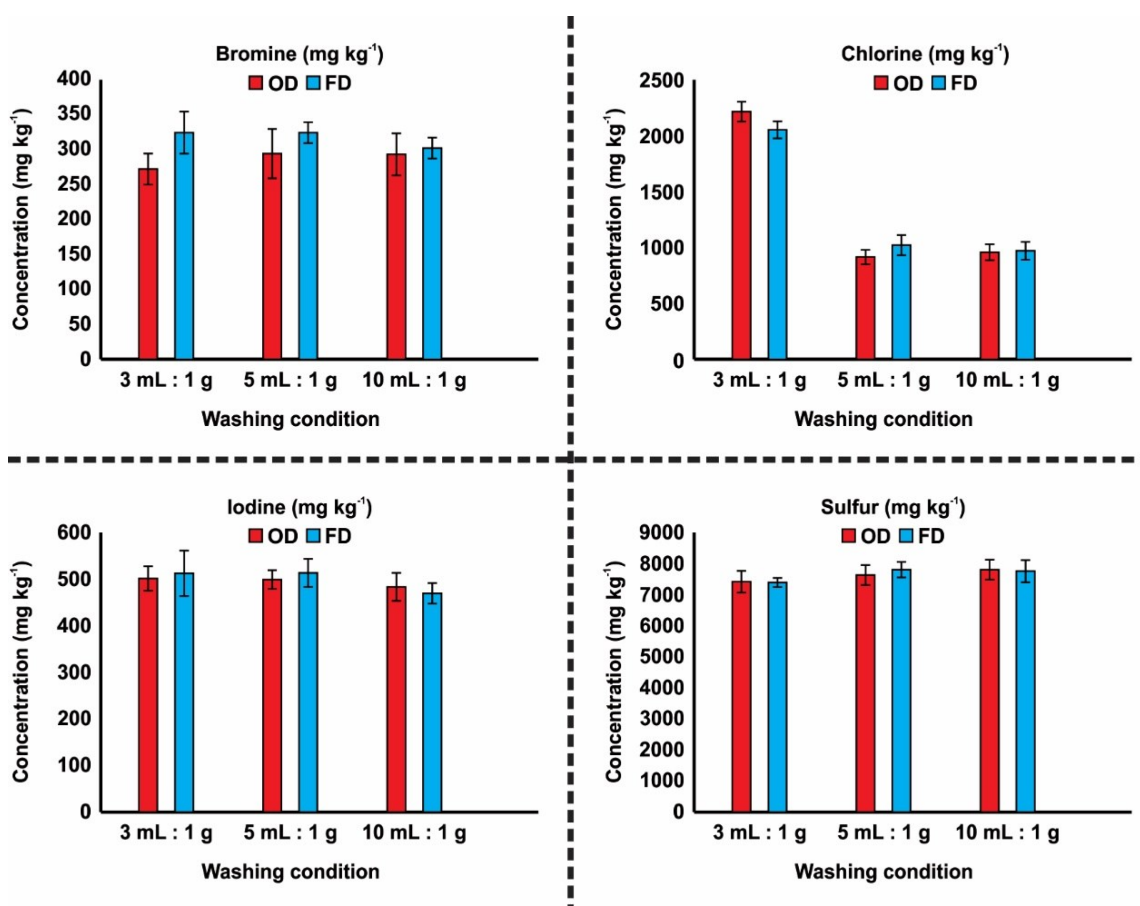

Figure 4. Halogens and sulfur concentrations obtained in *D. anceps* seaweed after evaluation of different conditions in pre-treatment processes (n = 3).

As can be seen in the results presented in Figure 4, when the 3 mL : 1g ratio was used during the washing step, the results obtained showed a statistical difference for chlorine concentration (ANOVA/Tukey test, 95% confidence level) when compared to other two volumes of water (5 mL : 1g, and 10 mL : 1 g). This result was expected, given a large amount of sodium chloride present in the samples due to seawater. Thus, it can be observed that the 3 mL : 1 g ratio was not sufficient to remove all the sea salt from the samples. On the other hand, for the other analytes, the results did not present statistical differences (ANOVA/Tukey test, 95% confidence level). Therefore, given the results obtained, the washing ratio chosen as a condition of the method was 5 mL : 1 g.

In turn, the different drying processes (oven-drying at 50 ºC, and freeze-drying) did not present statistical difference (Student's t-test, 95% confidence level) for all analytes evaluated, demonstrating that both processes can be used for the drying of Antarctic seaweed aiming at the subsequent total determination of halogens and sulfur, without the loss of analytes in the pre-treatment step. In this way, the oven-drying process was again evaluated using the washing ratio chosen and two drying temperatures (50 ºC and 100 ºC). The results obtained are shown in Figure 5.

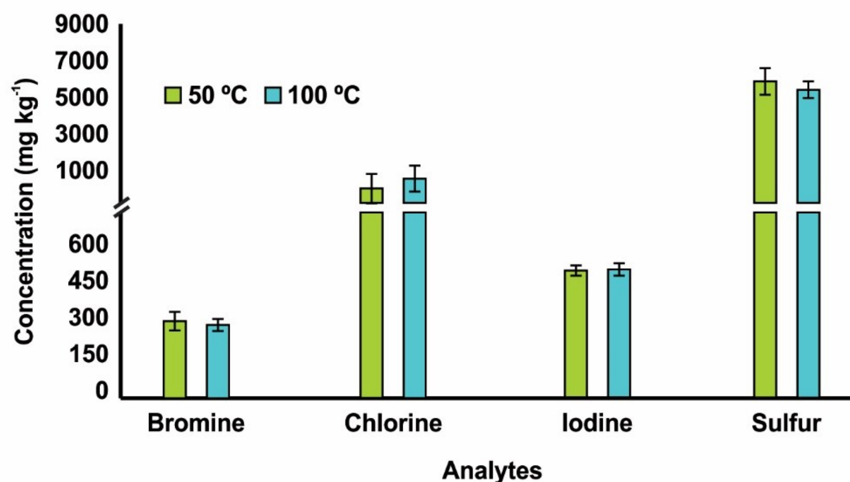

Figure 5. Halogens and sulfur concentrations in *D. anceps* seaweed after oven-drying process using 50 ºC and 100 ºC (n = 3).

As can be observed in Figure 5, the results obtained using both temperatures did not present statistical differences (Student's *t*-test, 95% confidence level) for bromine, chlorine, iodine, and sulfur. Thus, based on the results observed the oven-drying at 100 ºC was chosen as a condition of the method, considering that this process does not need the freezing step and also because it completes drying in less time (about 24 h) than other methods evaluated (oven-drying, 50°C: 48 h; Freeze-drying: 48 h considering the freeze step).

Evaluation of sample mass and suitability of absorbing solutions

Although the MIC method has demonstrated, since it was proposed, its suitability for the preparation of various samples in different states (solid, semi-solid, and liquid) and with different compositions [19, 26, 27, 32], the evaluation of some parameters must always be carried out during the development of a new method, to assure the best performance of the system, accurate results, and security for the analyst. Two essential parameters to be evaluated during the development of a method using MIC are: (i) the

sample mass used for the preparation, taking into account the concentration of the analytes, as well as the complete digestion of the sample; and (ii) the most suitable solution to absorb the analytes. This is because the behavior of the combustion varies according to the sample composition, and in some cases, it may present the formation of intense flames, capable of damaging components of the MIC system, such as PTFE lids. In addition, the choice of solutions that are not capable of absorbing all species of analytes formed during the sample digestion results in non-quantitative recoveries, as will be discussed below.

Evaluation of the sample mass

Taking into account that there is no information about halogens and sulfur concentrations in seaweed from the Antarctic and that the sample mass used in the method is inversely proportional to the LOQs, a study to determine the largest sample mass that can be digested by the MIC method was carried out. Therefore, masses from 100 mg to 1100 mg of *D. anceps* sample (Demay), using ultrapure water as the absorbing solution, were evaluated.

The increase in sample mass was proportional to the increase in the internal pressure of the system; however, it is important to emphasize that, during the combustion, the higher pressure reached corresponded to less than 50% of the maximum work pressure recommended (8 MPa) by the microwave oven manufacturer. In this sense, the pressures reached can be considered adequate, even using high sample masses, from the point of view of operating safety using closed vessels.

After the digestion of each mass of *D. anceps* sample, using 2 MPa of O_2, clear solutions were obtained when masses up to 1000 mg were combusted (Figure 4a). From this evaluation, it was possible to observe that the use of mass between 800 mg and

1000 mg resulted in increasingly intense flames. During the evaluation, the flames did not reach the lids. However, the use of high sample mass is not indicated, as this may result in the melting and/or deformation of the lids, which are made of PTFE.

When a higher sample mass (1100 mg) was used, the digests presented a yellow coloration, as well as material not completely combusted at the bottom of the quartz vessel, demonstrating that the process was incomplete (Figure 6b). Figure 6 shows the difference in the aspect of the solutions considered suitable (a) and not suitable(b).

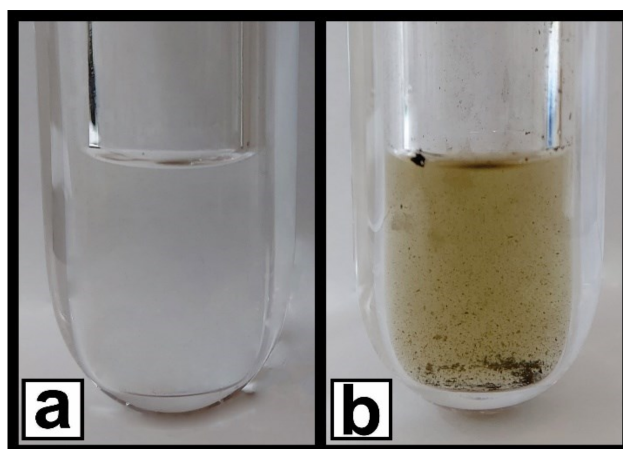

Figure 6. Aspects of the solutions after sample preparation by MIC using (a) from 100 to 1000 mg and (b) 1100 mg of *D. anceps* sample.

As can be observed, Figure 6a demonstrates a clear solution, without the presence of residues at the end of the digestion process. On the other hand, Figure 6b shows a yellow solution, with residues at the bottom of the vessel, typical characteristics of incomplete combustion.

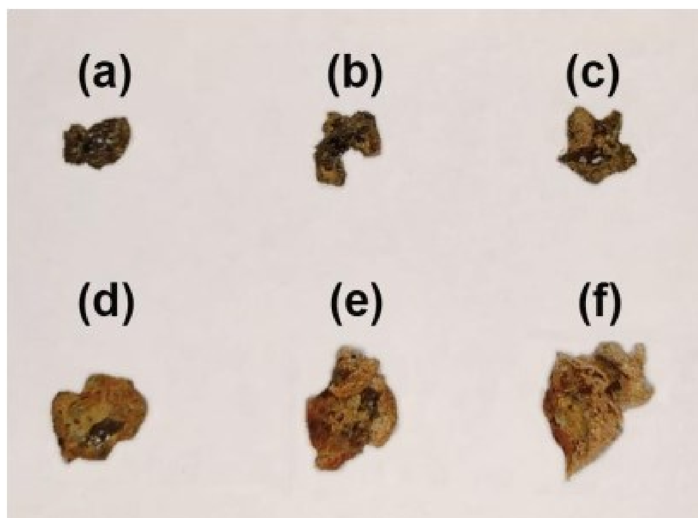

Figure 7. Residues resulting from the decomposition by MIC of (a) 500 mg, (b) 600 mg, (c) 700 mg, (d) 800 mg, (e) 900 mg, and (f) 1000 mg of *D. anceps* sample.

It is important to emphasize that at the end of each digestion, the presence of solid residues at the base of the quartz holder was observed, which apparently increased proportionally to the sample mass (Figure 7). To verify whether the release of analytes from the sample was not affected by the presence of residues at the end of combustion, the intermediate (500 mg) and the maximum (1000 mg) masses of the sample were digested and analyzed. However, to perform this evaluation, it was necessary first to choose the most suitable solution to absorb the analytes before setting the sample mass.

Influence of the absorbing solution on halogens and sulfur recoveries

After obtaining some results regarding the conditions used for digestion of different sample masses, evaluations regarding the absorbing solution to be used in the MIC method were carried out. These evaluations were made to choose the best condition for the absorption of bromine, chlorine, fluorine, iodine, and sulfur, as well as to select the sample mass to be used as a condition of the method.

For that, water and alkaline solutions were evaluated for the absorption of the analytes. In this study, ultrapure water, different concentrations of NH_4OH (50, 100, and 150 mmol L^{-1}) and $(NH_4)_2CO_3$ 150 mmol L^{-1} were used. This last solution was evaluated considering that it was suitable for the absorption of halogens and sulfur in previous studies for commercialized edible seaweed species [29]. The other solutions were evaluated for being alkaline or slightly alkaline solutions, which are suitable for the absorption of halogens and/or sulfur using the MIC method [20, 23, 24].

In this sense, to choose the most suitable solution to analyte absorption, the sample (500 mg) was digested by MIC with each of the mentioned solutions. Subsequently, the final solutions were analyzed by IC-CD-MS. The concentrations determined after the sample digestion using all evaluated solutions are shown in Table 2.

As can be seen in Table 2, when ultrapure water was used as an absorbing solution, the concentrations of bromine, chlorine, and iodine were lower and differed statistically (ANOVA/Tukey test, 95% confidence level) when compared to the values obtained using the other evaluated solutions. This result indicates that water may not be able to efficiently absorb the analytes originally present in the sample. Also, it can be seen that it was not possible to quantify fluorine in the sample, regardless of the solution used.

Table 2. Concentrations of bromine, chlorine, fluorine, iodine, and sulfur in *D. anceps* sample analyzed by IC-CD-MS after sample preparation by MIC using different absorbing solutions (mean ± standard deviation, n=3).

Absorbing solution	Concentrations (mg kg^{-1})				
	Br	Cl	F	I	S
Ultrapure water	299 ± 31^{a}	410 ± 43^{a}	nd	280 ± 52^{a}	8875 ± 704^{a}
50 mmol L^{-1} NH_4OH	360 ± 44^{b}	560 ± 63^{b}	nd	328 ± 39^{a}	8778 ± 655^{a}
100 mmol L^{-1} NH_4OH	381 ± 29^{b}	576 ± 46^{b}	nd	481 ± 29^{b}	8909 ± 622^{a}
150 mmol L^{-1} NH_4OH	379 ± 29^{b}	572 ± 44^{b}	nd	490 ± 19^{b}	8923 ± 600^{a}
150 mmol L^{-1} $(NH_4)_2CO_3$	386 ± 28^{b}	581 ± 39^{b}	nd	492 ± 23^{b}	8851 ± 587^{a}

nd = not detected; Equal letters for the same element do not show a statistically significant difference by ANOVA/Tukey test, 95% confidence level.

In this sense, to confirm the results and evaluate the efficiency of each solution for the retention of analytes quantitatively, recovery tests were performed. The results obtained from recovery tests are shown in Figure 8.

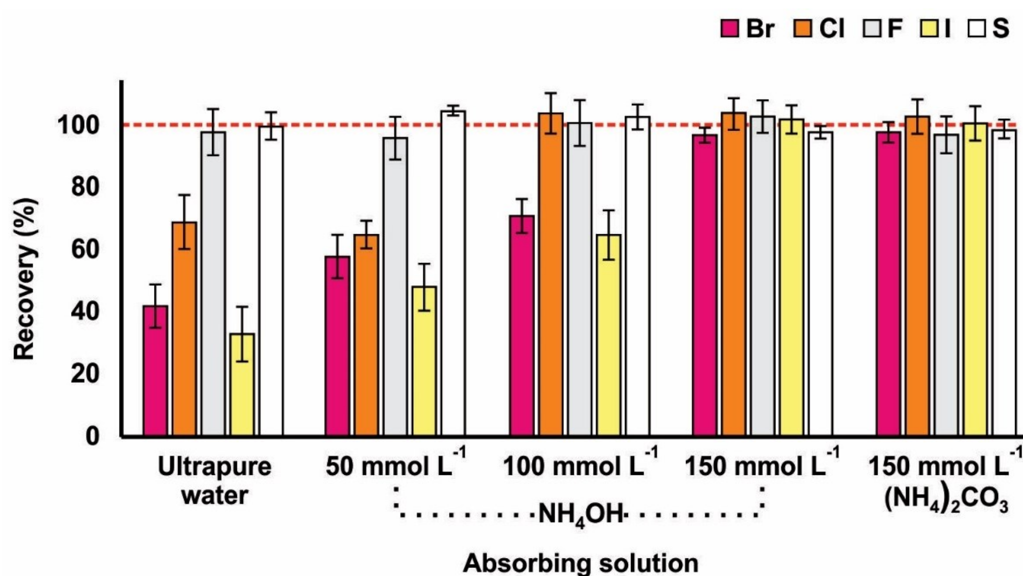

Figure 8. Recoveries obtained from spike tests for bromine, chlorine, fluorine, iodine, and sulfur in *D. anceps* sample after sample preparation by MIC using different absorbing solutions and analysis by IC-CD-MS (n=3).

In Figure 8, the coloured bars represent the mean of the recoveries and the error bars represent the standard deviation (SD) of the measurements. As can be seen, when ultrapure water was used as an absorbing solution, only fluorine and sulfur were recovered quantitatively (98% and 100%, respectively). The same was observed when 50 mmol L^{-1} NH_4OH was used, obtaining recoveries of 96% and 105%, respectively, for fluorine and sulfur. On the other hand, when 100 mmol L^{-1} NH_4OH was used, in addition to fluorine (101%) and sulfur (103%), chlorine was also quantitatively recovered (104%). The low recoveries for bromine, chlorine, and iodine may be linked to the low pH values observed in the digests obtained when using ultrapure water, 50 mmol L^{-1} and 100 mmol L^{-1} NH_4OH (2, 3, and 5, respectively). This is because acidic media favor the formation of volatile species of these elements. In this sense, only when more concentrated solutions (150 mmol L^{-1} NH_4OH and 150 mmol L^{-1} $(NH_4)_2CO_3$) were used, all analytes were recovered efficiently. The pH values determined in these solutions were around 8. Furthermore, when these solutions were used, the relative standard deviations (RSDs) obtained were always less than 8%. As well as for the absorption of halogens and sulfur in edible marine algae [24, 29], the solution of 150 mmol L^{-1} $(NH_4)_2CO_3$ proved to be efficient for the absorption of these analytes in seaweed from the Antarctic. As both solutions demonstrated suitability for the absorption of the analytes, as well as similar chromatograms (Figure 8) and blank values, the 150 mmol L^{-1} NH_4OH was chosen. This choice can be justified considering that the carbonate ion has a retention time close to sulfate and bromide retention times and depending on the concentration of the analyte in the sample, the carbonate ion could act as interference during the analysis. Figure 9 shows the separation and quantification of the analytes after sample preparation with the addition of fluorine (twice the LOQ) by MIC using both suitable solutions.

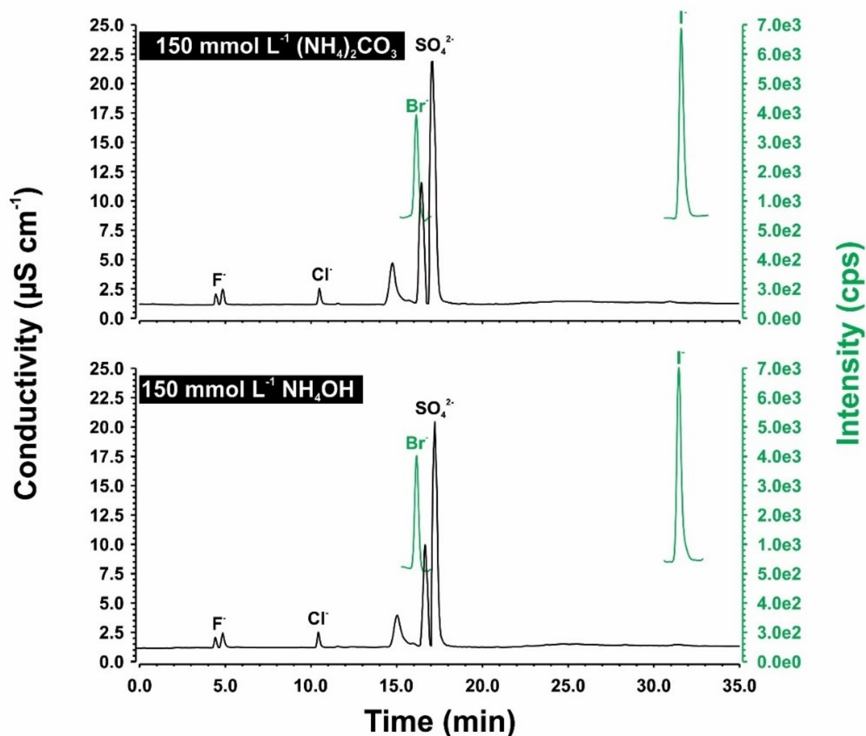

Figure 9. Chromatograms obtained by IC-CD-MS after analysis of *D. anceps* seaweed prepared by MIC, with the addition of twice the LOQ for fluorine, using 150 mmol L^{-1} $(NH_4)_2CO_3$ and 150 mmol L^{-1} NH_4OH as the absorbing solution.

From the choice of the most suitable absorbing solution for the retention of the analytes, the study was resumed to define the sample mass. This test was carried out to verify whether the analytes were made available in the same way for the absorbing solution, regardless of the sample mass used. Then, the analysis of the digests obtained after the digestion of 500 mg and 1000 mg of *D. anceps* sample was carried out, using 150 mmol L^{-1} NH4OH as the absorbing solution. After this evaluation, it was possible to observe that the results showed no significant difference for all determined analytes (Student's *t*-test, 95% confidence level). In addition, it was observed that using 500 mg of sample it was already possible to quantify all analytes, except fluorine. However, even using 1000 mg of sample, this element could not be quantified in the samples evaluated. Thus, based on the results obtained during the studies, and considering that from 800 mg

of sample resulted in more intense flames during the combustion process, 500 mg of sample was selected as a condition to be applied to other seaweed samples.

The accuracy of the proposed method was evaluated using the CRM BCR 060 (aquatic plant). No statistic differences were observed (Student's t-test, 95% confidence level) between certified and found values for bromine, chlorine, fluorine, and sulfur, which presented an agreement with the reference values of 98 ± 4%, 104 ± 7%, 97 ± 5%, and 102 ± 4%, respectively. Moreover, the iodine recovery in this evaluation was 101 ± 3%. Using the optimized method, which associates MIC with IC-CD-MS, low values of blanks were obtained, which, associated with the high sample mass possible to be used, culminated in obtaining low values of LOQ (bromine: 0.07 mg kg^{-1}, chlorine: 12.3 mg kg^{-1}, fluorine: 1.6 mg kg^{-1}, iodine: 0.10 mg kg^{-1}, and sulfur: 4.1 mg kg^{-1}) for all analytes.

Determination of halogens and sulfur in seaweed from the Antarctic

After optimizing and defining the best conditions (500 mg of sample, 150 mmol L^{-1} NH_4OH as the absorbing solution) among the evaluated parameters, the proposed method was applied for other three seaweed species from the Antarctic (*Iridaea cordata*, *Gigartina skottsbergii,* and *Palmaria decipiens*) and *D. anceps* collected in Half Moon region. The results obtained for bromine, chlorine, fluorine, iodine, and sulfur in all samples analyzed are shown in Table 3.

Table 3. Halogens and sulfur concentrations in different seaweed species from the Antarctic determined by IC-CD-MS after MIC method using 500 mg of sample and 150 mmol L^{-1} NH_4OH as the absorbing solution (mean ± standard deviation, n=3).

Seaweed species	Concentration (mg kg^{-1})				
	Bromine	Chlorine	Fluorine	Iodine	Sulfur
*Desmarestia anceps**	379 ± 29	572 ± 44	< 1.6	490 ± 19	8923 ± 600
*Desmarestia anceps***	282 ± 11	1061 ± 30	< 1.6	264 ± 8	6542 ± 310
Gigartina skottsbergii	517 ± 40	1129 ± 25	< 1.6	257 ± 9	63784 ± 1679
Iridaea cordata	126 ± 3	552 ± 12	< 1.6	64 ± 2	63811 ± 1478
Palmaria decipiens	235 ± 9	1758 ± 57	< 1.6	64 ± 3	39986 ± 2969

*Demay; **Half Moon

As can be seen in Table 3, the developed method allowed the quantification of all analytes, except fluorine, in all samples evaluated in this study. Although fluorine was not quantified in the samples, this study demonstrated, through recovery tests and CRM analysis, that it is possible to quantify this analyte using the developed method.

The results demonstrate the suitability of the LOQs obtained and also show the low standard deviations (always < 8%), demonstrating the precision of the method. The concentration ranges varied in different proportions for each analyte. Although *Gigartina skottsbergii* and *Iridaea cordata* seaweed were collected in the same region, the concentrations of bromine, chlorine, and iodine showed significant differences (Student's *t*-test, 95% confidence level) between each sample. In the same way, *D. anceps* samples, which were collected in different regions, also presented significant differences for all analytes (Student's *t*-test, 95% confidence level), suggesting that the region can influence the analyte concentration in the same seaweed species.

Although sulfur concentration for *Iridaea cordata* and *Gigartina skottsbergii* did not differ statistically (Student's *t*-test, 95% confidence level), each seaweed species presented the maximum concentration of a specific analyte. Except for the *D. anceps* seaweed from the Demay region, the analyte concentrations were present in the samples, in increasing order of values, as follows: iodine, bromine, chlorine, and sulfur.

These variations among the analytes and samples can be attributed to the composition of the cell wall of each species, allowing the accumulation of trace elements in different proportions [6, 33]. In addition, several other factors can influence such variations, such as geographic location, color, size, among others characteristics of each seaweed, and the environment where they grow [6, 34, 35].

Although many studies demonstrate a great nutritional and environmental contribution when performing the determination of metals present in edible and inedible seaweed, there is no information about the concentration of all halogens and sulfur, which are as important as metals. In addition, the development of a method that provides reliable results, with adequate precision and accuracy, for the determination of all halogens and sulfur in seaweed from the Antarctic, brings unprecedented information to the literature, which can collaborate with several areas of study not started due, most likely, to the lack of information in the literature. Thus, it is worth mentioning that the method developed is the first analytical method that uses MIC for the digestion of seaweed from the Antarctic continent allowing the multi-element determination of bromine, chlorine, fluorine, iodine, and sulfur by IC-CD-MS in a single analysis.

CRediT author statement

Filipe Soares Rondan: Conceptualization, Investigation, Methodology, Formal analysis, Validation, Figures design, Writing – original draft. **Rodrigo Mendes Pereira:**

Conceptualization, Writing - review & editing. **Álisson Aline da Silva:** Methodology, Writing - review & editing. **Priscila Tessmer Scaglioni:** Conceptualization, Writing - review & editing. **Pio Colepicolo:** Project administration, Funding acquisition, Writing - review & editing. **Márcia Foster Mesko:** Conceptualization, Project administration, Writing- review and editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 2

Manuscript

The main results of this chapter of the thesis are presented in the form of a manuscript, which is thus organized. The items Introduction, Experimental, Results and discussion, and References can be found in the manuscript.

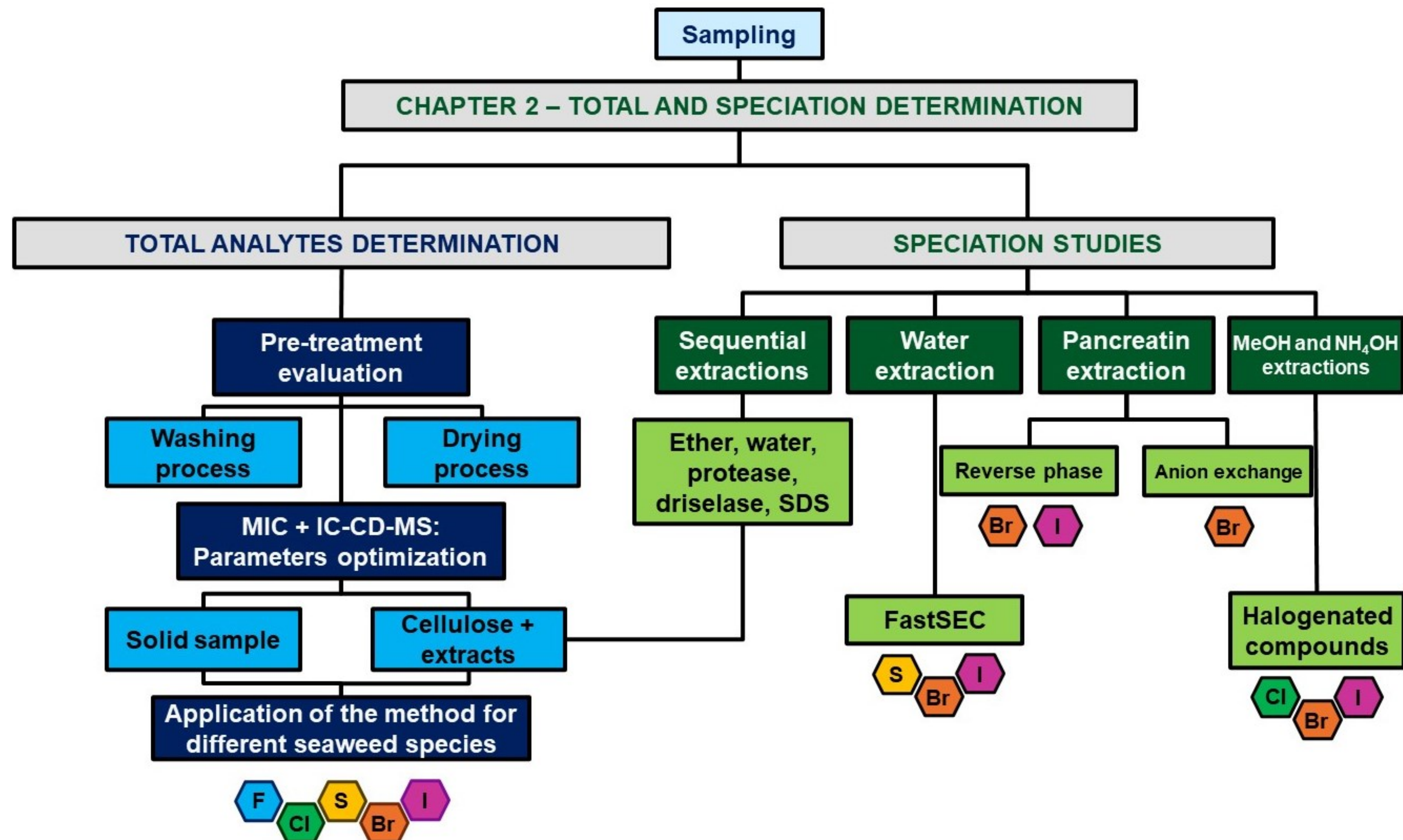

Figure 1. Flowchart showing the content of the manuscript in relation to the methods optimized and used for the total determination and species of halogens and sulfur in seaweed from the Antarctic.

Fractionation and speciation analysis for halogens and sulfur in seaweed from Antarctic using a multi-technique approach

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Abstract

In this study, different sample preparation methods, based on extraction and combustion, were used to characterize seaweed from the Antarctic on halogens and sulfur and their species. For this purpose, a multi-technique approach was used, combining highly selective and sensitive chromatographic and spectrometric techniques. By using optimized methods, it was possible to determine total halogens and sulfur content, the distribution of bromine, iodine, and sulfur in different classes of species (lipids, water-soluble, proteins, carbohydrates, and residue), as well as the identification of iodinated amino acids (MIT and DIT) and thiols (Cys, GSH, HCys, GluCys, and Pen) in ten brown and red seaweed. Bromate and iodate were not detected in the samples, which presented only bromide and iodide species in their composition. Additionally unknown bromine, iodine, and sulfur species were observed in different extracts evaluated. Furthermore, 25 halogenated polyphenols were identified in seaweed, of which only four were reported in the literature. The total concentrations for all analytes varied in a wide range, except for fluorine, which was below the limit of quantification (1.6 mg kg^{-1}) for all analyzed samples. After pancreatin extraction, the extraction efficiencies obtained ranged from 31 to 107% for bromine and from 23 to 105% for iodine, while the chromatographic recoveries for bromine and iodine ranged from 69 to 109% and from 72 to 92%, respectively. The results obtained in this study comprise unprecedented data in the literature on species of halogens and sulfur present in seaweed from the Antarctic.

Keywords: Algae, HPLC-ICP-MS, IC-CD-MS, sample preparation

1. Introduction

Antarctic seaweed are photoautotrophic organisms that play essential functions in the marine polar environment. In addition to being primary producers in the Antarctic ecosystem, representing an important food source for herbivores and detritivores, they also provide habitats and breeding areas for various animal species (Gómez and Huovinen 2020). Among 151 species have been identified on the continent, of which 41 are endemic (Pellizzari et al. 2017). These species under extreme conditions, such as low temperatures and short periods of exposure to the sun light, that influenced their chemical composition and metabolism (Carreto, Carignan, and Montoya 2005; Becker et al. 2011; Becker, Graeve, and Bischof 2010; Teixeira et al. 2019).

The synthesis of compounds by seaweed is directly associated to the incorporation of elements from the environment in which they develop (Farías et al. 2002), such as halogens (bromine, chlorine, fluorine, and iodine) and sulfur, which are present in seawater (Al-Adilah et al. 2020; Giordano et al., 2008). Besides, halogens can be accumulated by brown and red seaweed (Saenko et al. 1978; Kupper et al., 2013), and the reduced forms of these elements act as antioxidants involved in superoxide detoxification and other processes (Küpper et al. 2013). While sulfur is present in several important compounds in seaweed, such as proteins, peptides, polysaccharides, among others (Schaumlöffel et al., 2007; Wang et al., 2007; Barahona et al., 2021). Seaweed also release halogenated and sulfur compounds into marine environments and to the atmosphere, where they can contribute to the depletion of the ozone layer in polar regions, such as in the Antarctic (Al-Adilah et al. 2020; von Glasow et al., 2004; Saiz-Lopez et al., 2012). These metabolic characteristics make seaweed to be used as bioindicators of environmental changes and pollution, which are important considering

that the environmental variations in the Antarctic continent are reflected in the rest of the planet.

Studies about the compositions of the Antarctic seaweed are mainly focused on organic compounds and metals (Pereira et al. 2017; Barahona et al. 2021; Berneira et al. 2021; Farías et al. 2002; Picoloto et al. 2017; Mesko et al. 2015), and information about nonmetals is extremely scarce in the literature, limited to total selenium content and volatile halogenated organic compounds (Laturnus 2001; Laturnus et al. 1997; Laturnus, Wiencke, and Klöser 1996; Montone et al. 2001; Guilherme et al. 2020). Recently, our research group developed a method for the total halogens and sulfur determination in seaweed from the Antarctic (see Chapter 1), demonstrating that these elements vary in a wide range of concentrations in these organisms. Therefore, based on previous results, in the present work different sample preparation methods based on extraction and combustion were used to obtain a characterization and provide comprehensive information on the content of halogens and sulfur and their species in brown and red seaweed from the Antarctic using a multi-technique approach.

2. Experimental

2.1. Instrumentation

An analytical balance (model AY220, Shimadzu, Philippines), with a maximum load of 220 g and a resolution of 0.0001 g was used to weigh samples and reagents. A ball mill (MM 200, Retsch, Germany) equipped with zirconium containers and balls was used for sample milling. A centrifuge (model 5415R, Eppendorf, Germany) was used for the centrifugation of samples after different sample preparation methods.

For microwave-induced combustion (MIC) procedures, a microwave oven (model Multiwave 3000, Anton Paar, Austria) equipped with high-pressure quartz vessels was

used. An ultrasonic bath (35 kHz, Bandelin, Sonorex, Germany) was used for ultrasound extractions. A shaking water bath (SBS40, Stuart, United Kingdom) was used for solution incubations during enzyme extractions.

Total halogens and sulfur concentrations were determined using an ion chromatograph (ICS-5000, Dionex/Thermo Fisher Scientific, USA) equipped with an IonPac AS11-HC analytical anion exchange column (4 µm, 2 x 250 mm) and an IonPac AG11-HC guard column (4 µm, 2 x 50 mm). An eluent source EGC 500 KOH generator cartridge with continuously regenerated anion-trap column (CR-ATC), a Dionex ERS 500 anion electrolytically regenerated suppressor, and an AS-AP autosampler, were also used. The mobile phase used for the elution of analytes was a potassium hydroxide gradient. The conductivity detector was used for chloride, fluoride, and sulfate detection. While for bromide and iodide determination, IC coupled to a mass spectrometer (MSQ Plus Single Quadrupole Mass Spectrometer, Thermo Fisher Scientific), with negative mode electrospray ionization (ESI), was used.

For HPLC-ICP-MS analysis, a Dionex HPLC UltiMate® 3000 LC Quaternary Analytical pump (Dionex, USA) was used. FastSEC chromatographic separations were performed with an Acquity UPLC Protein BEH SEC Column (125Å, 1.7 µm, 4.6 mm x 150 mm). The HPLC system was coupled to an ICP-MS (7700 series ICP-MS, Agilent Technologies, Japan) for analysis of organic and inorganic halogenated compounds. While for sulfur compounds determination, the HPLC system was coupled to an ICP-MS (NexION 5000, PerkinElmer, USA) using a cell-based Reaction mode (in the Universal Cell) with oxygen as reaction gas in order to monitor isotope $^{48}SO^{+}$. Reverse phase chromatographic separations were performed on a Zorbax SB C18 (4.6 mm × 150 mm i.d.) analytical column (Agilent, Palo Alto, CA, USA). Anion exchange chromatographic separations were performed with an anion exchange HPLC column

PRP-X100 (250 mm × 4.1 mm i.d., Hamilton, USA) coupled to an PRP-X100 (Hamilton) guard column. A pH-meter (SevenCompact, Mettler Toledo, USA) with a combined electrode (InLab Expert Pro-ISM, Mettler Toledo) was used for pH measurements. Solutions were filtered through 0.45 µm cellulose acetate syringe filters (25 mm diameter; Chromafil® Xtra CA-45/25) or 0.22 µm H-PTFE syringe filters (25 mm diameter; Unifil, Brazil) and, when necessary, centrifugal filters (10 kDa or 30 kDa, Merck) were used before injection in chromatographic systems.

Analysis of halogenated polyphenol compounds was carried out using an Ultimate 3000 RSLC system (ThermoFisher Scientific, Germany) coupled with an Orbitrap Fusion Lumos Tribrid mass spectrometer (ThermoFisher Scientific, USA) operated in positive and negative mode. Data-treatment for the structurally intelligent annotation of compound structures was carried out using Compound Discoverer 3.2™ (ThermoFisher Scientific). The separation of compounds was performed on an Acquity UPLC BEH C18 column (150 x 2.1 mm, 1.7 µm, 130 Å) (Water, France) at 40 °C. The parameters used for HPLC-ICP-MS and IC-CD-MS analysis are detailed in Tables 1 and 2.

Table 1. HPLC-ICP-MS parameters used for analysis.

FastSEC-HPLC-ICP-MS	
Column	ACQUITY UPLC Protein BEH SEC
Mobile phase	10 mmol L^{-1} ammonium acetate
Flow	0.3 mL min^{-1}
Injection volume	5 µL
RP-HPLC-ICP-MS	
Column	ZORBAX SB C18
Mobile phase	A : 0.01% FA in H_2O B : 0.01% FA in MeOH
Flow	0.5 mL min^{-1}
Gradient	0-4 min 5% B 4-10 min 50% B 10-15 min 75% B 15-20 min 75% B 21 – 27 min 5% B
Injection volume	5 µL
AE-HPLC-ICP-MS	
Column	PRP-X100
Mobile phase	A: 20 mmol L^{-1} acetic acid / 10 mmol L^{-1} triethylamine B: 40 mmol L^{-1} acetic acid / 20 mmol L^{-1} triethylamine
Flow	1.0 mL min^{-1}
Gradient	0-3 min 10% B 3-15 min 100% B 15-35 min 100% B 35-36 min 10% B 36 – 40 min 10% B
Injection volume	50 µL

RP-HPLC-ICP-MS – halogenated polyphenols compounds	
Column	Acquity UPLC BEH C18
Mobile phase	A: 0.1 % formic acid in ACN B: 0.1 % formic acid in H_2O
Flow	0.4 mL min^{-1}
Gradient	0-2 min, 10 % B 2-16 min 90 % B 16-21 min, 90 % B 21-23 min, 10 % B 23-25 min, 10% B
Injection volume	15 µL

Table 2. Parameters used for IC-CD-MS analysis.

IC-CD	
Column	IonPac AS11-HC
Guard column	IonPac AG11-HC
Mobile phase	Potassium hydroxide
Flow	0.28 mL min^{-1}
Gradient	0.0 – 7.5 min (1 mmol L^{-1}) 7.5 – 8.0 min (4 mmol L^{-1}) 8.0 – 22 min (20 mmol L^{-1}) 22 – 30 min (90 mmol L^{-1}) 30 – 33 min (1 mmol L^{-1})
Injection volume	50 µL
MS	
Probe temperature	600 °C
Needle voltage	4.2 kV
Cone voltage	132 V
Isotopes	^{81}Br, ^{127}I

2.2.Reagents and Chemicals

Ultrapure water, with resistivity of 18 MΩ cm, obtained from a water purificator (Direct-Q 3UV, Millipore Corp., USA) was used for preparation of all solutions in this study. Methanol, acetonitrile, and formic acid (Merck, Germany), ammonium acetate, and triethylamine (Sigma-Aldrich, Germany) were used for mobile phases preparation. The igniter solution used for MIC procedures was prepared from ammonium nitrate (Merck) salt. Ammonium hydroxide solution 27% (w/v) and petroleum ether used for extractions procedures were from Merck.

Ammonium carbonate, trizma® base, and sodium dodecyl sulfate (Sigma-Aldrich) were used for buffer solutions preparation. Pancreatin from porcine pancreas, agarase from *Pseudomonas atlantica*, driselase from *Basidiomycetes sp*., and protease from *Streptomyces griseus* (Sigma-Aldrich) were used for enzymatic extractions.

Bromide, bromate, chloride, fluoride, iodide, iodate, and sulfate stock standard solutions, 1000 mg L^{-1}, were prepared from potassium bromide, potassium bromate, potassium chloride, potassium fluoride, potassium iodide, potassium iodate, and potassium sulfate (Merck), respectively. Standard stock solutions of 500 mg L^{-1} of 3-iodo-L-tyrosine (MIT) and 3,5-diiodo-L-tyrosine dihydrate (DIT) were prepared by dissolving the amino acids (Sigma–Aldrich) in water. L-glutathione (GSH), L-Cysteine (Cys), DL-homocysteine (HCys), penicillamine (Pen), and γ-L-Glutamyl-L-cysteine (γ-Glu-Cys) from Sigma-Aldrich were prepared in water.

2.3. Samples

Ten seaweed species (Brown: *Ascoseira mirabilis*, *Desmarestia anceps*, *Desmarestia antarctica*, *Himantothallus grandifolius*, and *Phaeurus antarcticus;* Red: *Curdiea racovitzae*, *Gigartina skottsbergii*, *Iridaea cordata*, *Myriograme mangini*, and

Palmaria decipiens) from different regions of the Antarctic (Figure 2) were used in this study, as described in Table 3. The appearance of the samples is shown in Figure 3. The samples were collected manually during periods of low tide between November and December 2017 and 2018. The samples were washed three times with ultrapure water, using 5 mL for each 1 g of seaweed, dried in a conventional oven at 100 °C for 24 h, and milled for further analysis, according to protocol previously developed (Chapter 1).

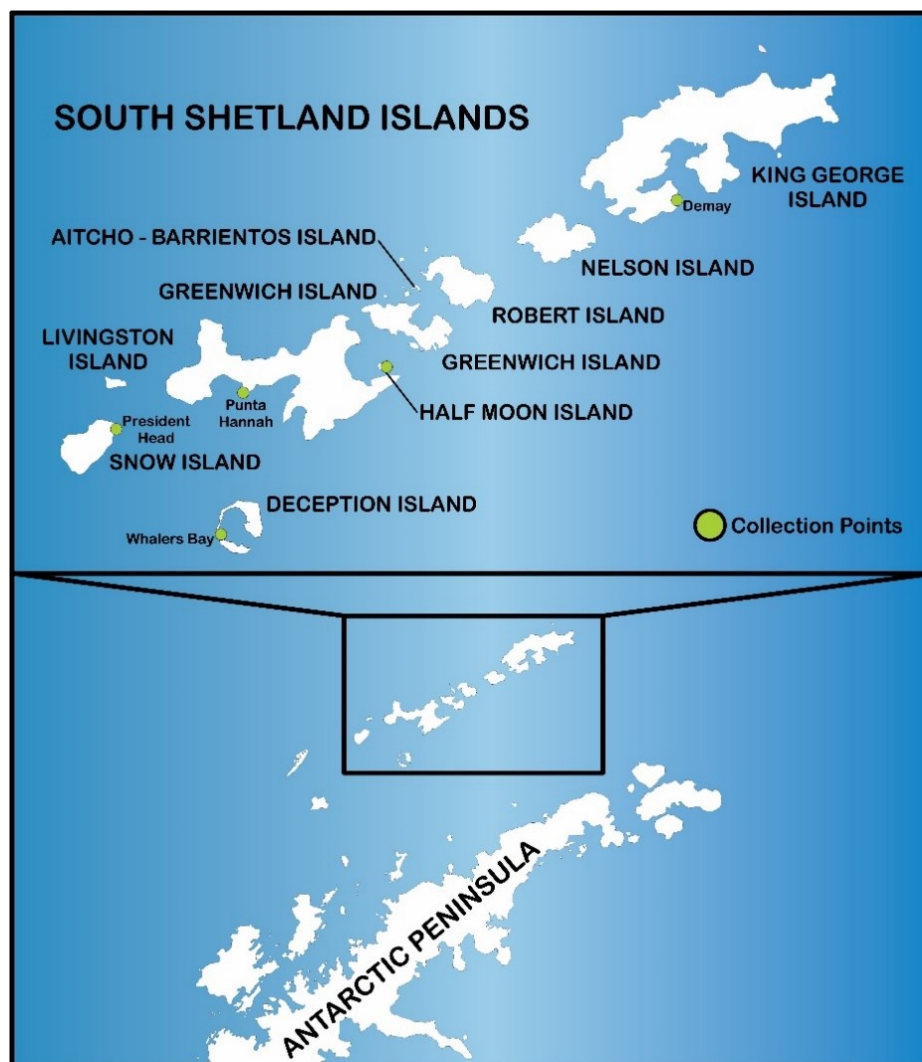

Figure 2. Antarctic (South Shetland Islands) map indicating the collection points of seaweed used in this study.

Table 3. Identification of brown (yellow shading) and red (red shading) seaweed from the Antarctic used in this study.

Seaweed (initials)	**Taxonomic group**	**Region**	**Year of collection**	**Coordinates of sampling sites**
Ascoseira mirabilis (AM)*	Phaeophyta	Snow – President Head	2018	62º44’07.0” S and 61º13’45.0” W
Desmarestia anceps (DA)*	Phaeophyta	Half Moon	2018	62º38’30.2” S and 60°22’09.7” W
Desmarestia antarctica (DT)*	Phaeophyta	Snow – President Head	2018	62º44’07.0” S and 61º13’45.0” W
Himantothallus grandifolius (HG)*	Phaeophyta	King George - Demay	2017	62º13’19.3” S and 58°26’36.6” W
Phaeurus antarcticus (PA)*	Phaeophyta	King George - Demay	2017	62º13’19.3” S and 58°26’36.6” W
Curdiea racovitzae (CR)*	Rhodophyta	Half Moon	2018	62º38’30.2” S and 60°22’09.7” W
Gigartina skottsbergii (GS)	Rhodophyta	Livingston - Punta Hannah	2018	62°39’00.5” S and 60°35’57.7” W
Iridaea cordata (IC)	Rhodophyta	Snow – President Head	2018	62º44’07.0” S and 61º13’45.0” W
Myriograme mangini (MM)	Rhodophyta	Livingston - Punta Hannah	2018	62°39’00.5” S and 60°35’57.7” W
Palmaria decipiens (PD)	Rhodophyta	Deception – Whalers Bay	2018	62º44’07.0” S and 61º13’45.0” W

*Endemic species

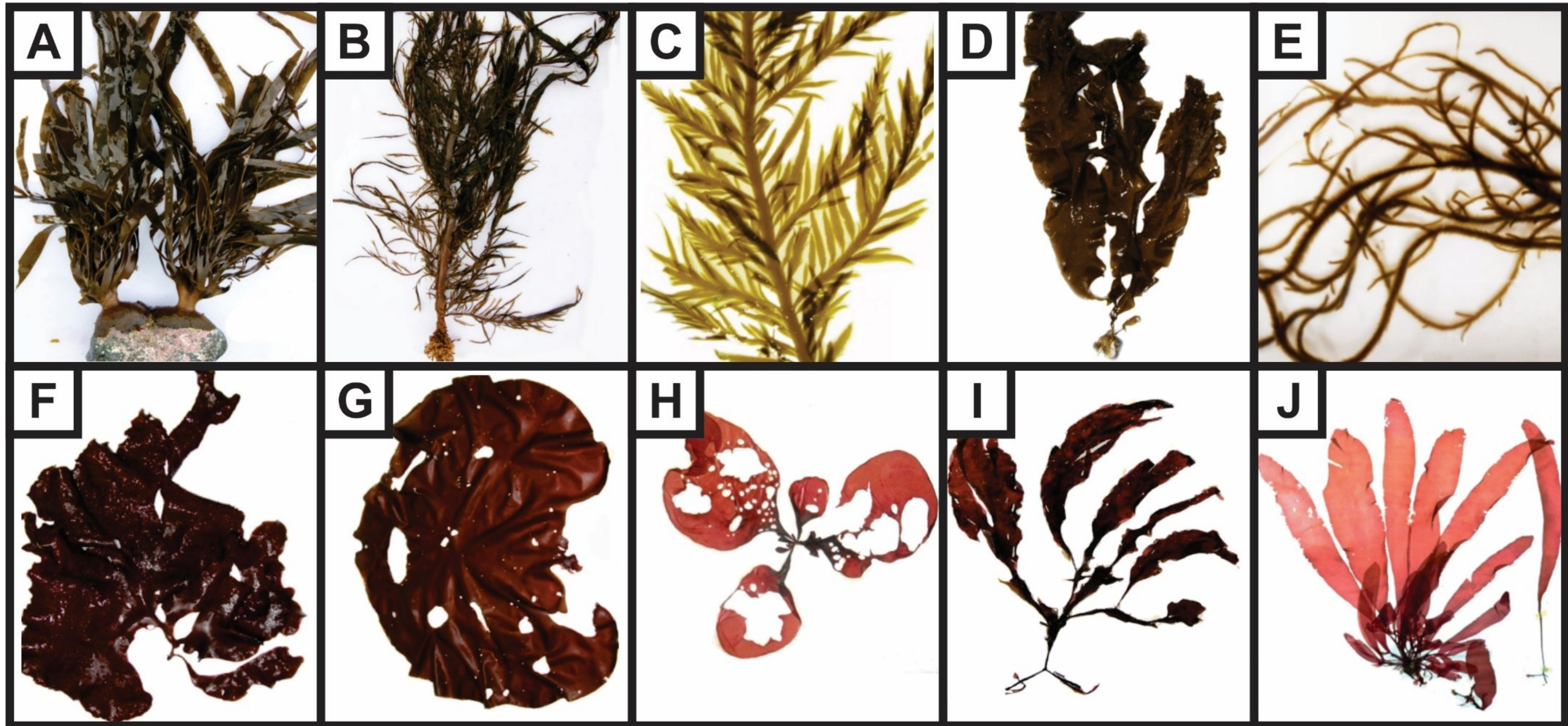

Figure 3. Antarctic seaweed species used in the present study: (A) *Ascoseira mirabilis*, (B) *Desmarestia anceps*, (C) *Desmarestia antarctica*, (D) *Himantothallus grandifolius*, (E) *Phaeurus antarcticus*, (F) *Curdiea racovitzae*, (G) *Gigartina skottsbergii*, (H) *Iridaea cordata*, (I) *Myriograme mangini*, (J) *Palmaria decipiens*. Adapted from: MEDEIROS, 2013.

2.4. Microwave-induced combustion for further determination of total halogens and sulfur

For digestion of Antarctic seaweed samples by MIC, the method previously developed (presented in Chapter 1) was used. For this, samples were wrapped on low-density polyethylene (LDPE) films, and sealed by heating. The wrappers were placed on the base of quartz holders containing a disc of filter paper with 50 µL of 6 mol L^{-1} NH_4NO_3 solution. The holders were transferred into quartz vessels containing 6 mL of 150 mmol L^{-1} NH_4OH solution. The quartz vessels were pressurized with 20 bar of O_2 and then, the samples were submitted to the following microwave irradiation program: *i*) 1400 W for 5 min (combustion and reflux steps); *ii*) 0 W for 20 min (cooling step). Final solutions were diluted with water up to 25 mL, filtered (using syringe PTFE filter membrane 0.22 µm) for further determination of bromine, chlorine, fluorine, iodine, and sulfur by ion chromatography with conductivity detection coupled to mass spectrometry (IC-CD-MS).

For sample preparation of each extract obtained from seaweed using different solutions and conditions (see item 2.6), the wraps were prepared containing 300 mg of cellulose (used as a combustion aid and also to retain the liquid sample), 400 µL of extract, and 100 µL of the igniter solution (6 mol L^{-1} NH_4NO_3). The other conditions used for the MIC method were the same as previously described.

2.5. Pancreatin extraction

For pancreatin extraction, an adapted method from Romarís-Hortas, Bermejo-Barrera, and Moreda-Piñeiro (2013) was used. For this, 0.04 g of sample and 1.5 mL of solution (30 mmol L^{-1} Tris/HCl buffer at pH 7.5) containing 9 mg of pancreatin were used. The samples were submitted to the water bath at 50 °C for 12 h, centrifuged (13200

rpm for 15 min) and the supernatant was filtered (using syringe cellulose acetate filter membrane 0.45 µm), diluted, and analyzed. This procedure was applied to all ten species of seaweed, and it was performed in triplicate.

2.6. Sequential extractions of halogens and sulfur species

In order to verify the distribution of bromine, chlorine, iodine, and sulfur in different fractions of samples among different classes of species, a sequential extraction was performed for each seaweed sample, using petroleum ether, water, driselase, protease, and sodium dodecyl sulfate (SDS) to target lipid, water-soluble, polysaccharide, and protein fractions, respectively (Ayouni et al. 2007; Ruiz Encinar et al. 2003). For these experiments, initially, 0.025 g of each seaweed species were weighted in 2 mL tubes and sequentially extracted from steps 1 to 5, using 1 mL of different solutions and conditions (Table 4). After each step, samples were centrifuged for 15 min at 13200 rpm, the supernatant was collected, and the residues were submitted to the next step. All procedures were performed in triplicates.

Table 4. Conditions used for sequential extractions.

Extraction sequence	Extracting solution (1 mL)	Conditions
Step 1	Petroleum ether	Vortex for 1 min
Step 2	Ultrapure water	Ultrasonic bath for 1 h at room temperature
Step 3	30 mmol L^{-1} $(NH_4)_2CO_3$ at pH=7.5 containing 0.4% (w/v) of protease enzyme	Incubation at 37 °C overnight in a water bath
Step 4	20 mmol L^{-1} $NH_4CH_3CO_2$ at pH=5 containing 2% (w/v) of driselase enzyme	Incubation at 37 °C overnight in a water bath
Step 5	4% (w/v) SDS solution in water	Ultrasonic bath for 1 h at room temperature

2.7. Agarase extraction

For this, after water extraction (Table 4), in a second step, agarase solution (0.6 mg of agarase for each 1 mL of 2 mol L^{-1} ammonium acetate buffer at pH 6) was added to the supernatant collected from water extraction (Kazlowski et al. 2008). The samples were incubated in a bath at 43 °C for 24 h. After that, the samples were centrifuged (13200 rpm for 15 min using a centrifugal filter - 30 kDa), diluted and analyzed. This procedure was applied to all red seaweed, and it was performed in duplicate.

2.8. Methanol extraction

For this procedure, each seaweed was prepared by using 0.075 g of sample and 1.5 mL of MeOH/H_2O (1:1) in 2 mL tubes, which were submitted to the ultrasonic bath for 30 min. After that, the samples were centrifuged (13200 rpm for 15 min) and the supernatant was collected, diluted five times in the mobile phase and analysed. Samples which presented a gelled final solution were centrifuged using centrifugal filters (30 kDa). This procedure was applied to all ten species of seaweed, and it was performed in triplicate.

2.9. Ammonium hydroxide extraction

For alkaline extraction, each seaweed was prepared using 0.075 g of sample and 1.5 mL of 0.1 mmol L^{-1} NH_4OH. The samples were submitted to the ultrasonic bath for 30 min. After that, the samples were centrifuged (13200 rpm for 15 min) and the supernatant was collected, diluted five times in mobile phase and analyzed. Samples which presented a gelled final solution were centrifuged using centrifugal filters

(30 kDa). This procedure was applied to all ten species of seaweed, and it was performed in triplicate.

3. Results and discussion

3.1. Total halogens and sulfur determination in samples

The total bromine, chlorine, fluorine, iodine, and sulfur concentrations obtained for the Antarctic seaweed studied are shown in Table 5; the fluorine content was below the LOQ (1.6 mg kg^{-1}) for all samples. Bromine concentrations varied in a wide range, about 60-fold comparing the lowest (*P. antarcticus* – 10 mg kg^{-1}) and the highest (*D. antarctica* – 614 mg kg^{-1}) concentrations. The variation observed in bromine content was higher in brown seaweed (10 to 614 mg kg^{-1}) than in red ones (137 to 327 mg kg^{-1}). The chlorine concentrations varied in a smaller range (about 18-fold, from 630 to 11 353 mg kg^{-1}, for *P. decipiens* and *H. grandifolius*, respectively). It was observed that, except for *P. antarcticus* sample, the brown seaweed species evaluated always presented higher chlorine contents than the red ones. For iodine concentrations, the variation of values was lower (about 10-fold, varying from 44 to 455 mg kg^{-1} for *P. antarcticus* and *D. anceps*, respectively). In turn, sulfur concentrations also varied in a wide range, about 12-fold, from 4 570 to 56 350 mg kg^{-1}. Sulfur concentrations in brown seaweed were lower (from 4 570 to 10 710 mg kg^{-1}) than in red seaweed (from 11 600 to 56 350 mg kg^{-1}). Probably it is related to the high sulfated polysaccharides content in red seaweed, such as agar and carrageenans (Torres et al., 2019).

Table 5. Total halogens and sulfur concentration in seaweed from the Antarctic.

Seaweed species	Concentrations (mg kg^{-1})				
	Bromine	Chlorine	Fluorine	Iodine	Sulfur
Ascoseira mirabilis	101 ± 4	2 657 ± 79	< 1.6*	215 ± 8	8 418 ± 116
Desmarestia anceps	438 ± 5	2 328 ± 70	< 1.6*	455 ± 8	10 344 ± 255
Desmarestia antarctica	614 ± 15	2 446 ± 10	< 1.6*	373 ± 6	4 586 ± 234
Himantothallus grandifolius	533 ± 27	11 353 ± 445	< 1.6*	375 ± 11	5 474 ± 430
Phaeurus antarcticus	10 ± 2	852 ± 25	< 1.6*	44 ± 1	10 710 ± 187
Curdiea racovitzae	327 ± 27	1 790 ± 108	< 1.6*	411 ± 34	18 534 ± 1562
Gigartina skottsbergii	137 ± 11	941 ± 88	< 1.6*	54 ± 3	56 350 ± 1 237
Iridaea cordata	208 ± 20	977 ± 21	< 1.6*	343 ± 9	31 423 ± 438
Myriograme mangini	216 ± 7	1 893 ± 20	< 1.6*	192 ± 15	34 096 ± 1 626
Palmaria decipiens	171 ± 10	630 ± 33	< 1.6*	54 ± 2	11 603 ± 163

*Limit of quantification.

Since halogens and sulfur determination in Antarctic seaweed is not reported in the literature, the obtained results cannot be compared with other seaweed from the Antarctic. Given the extreme environmental conditions in which these organisms developed (Gómez and Huovinen 2020), it was expected to have different variations on composition in seaweed from the Antarctic compared to other seaweed species. However, the bromine, chlorine, iodine, and sulfur concentrations obtained in this study are within the range related in the literature for different brown and red species – without taking into account the different regions and environmental conditions of each study – from 7 to 59 260 mg kg^{-1} for bromine (Rossbach and Nguyen 1993; Hou and Yan 1998; Gómez-Ordóñez, Alonso, and Rupérez 2010; Malinowski et al. 2022; Mesko et al. 2014; Romarís-Hortas, Moreda-Piñeiro, and Bermejo-Barrera 2009; El Zokm, Ismail, and El-Said 2021), from 7 to 135 000 mg kg^{-1} for chlorine (Hou and Yan 1998; Coelho Junior et al. 2017; Rossbach and Nguyen 1993; Gómez-Ordóñez, Alonso, and Rupérez 2010; Malinowski et al. 2022; El Zokm, Ismail, and El-Said 2021), from 3 to 11 630 mg kg^{-1} for iodine (Hou and Yan 1998; Stévant et al. 2018; Nunes et al. 2019; Malinowski et al. 2022; Yeh, Hung, and Lin 2014; Al-Adilah et al. 2020; Mesko et al. 2014; Romarís-Hortas, Moreda-Piñeiro, and Bermejo-Barrera 2009; El Zokm, Ismail, and El-Said 2021), and from 4 430 to 80 700 mg kg^{-1} for sulfur (Rupérez, 2001; Rupérez, 2002; McDermid, 2003; Gómez-Ordonez, 2010).

Furthermore, despite the LOQ for the present study being lower than those reported in the literature, fluorine concentrations in other brown and red seaweed could be quantified in other studies, with values varying from 26 to 29 000 mg kg^{-1} (Rossbach and Nguyen 1993; Gómez-Ordóñez, Alonso, and Rupérez 2010; Al-Adilah et al. 2020; El Zokm, Ismail, and El-Said 2021; Malinowski et al. 2022). Once little is known about fluorine accumulation in seaweed (Al-Adilah et al. 2022), probably the difference in the

results is related to different seaweed species, as well as the environmental extreme conditions where Antarctic seaweed developed (Gómez and Huovinen 2020; Farías et al. 2002).

For all samples evaluated (except for *H. grandifolius*), sulfur concentrations were always the highest among the analytes determined. Additionally, two distributions of halogens concentrations were observed for the analysed samples, [Cl] > [I] > [Br] > [F] for *A. mirabilis*, *D. anceps*, *P. antarcticus*, *C. racovitzae*, and *I. cordata* samples, and [Cl] > [Br] > [I] > [F] for *D. antarctica*, *H. grandifolius*, *G. skottsbergii*, *M. mangini*, and *P. decipiens* samples. However, no correlations with collection regions or time could be observed probably because of the low number of samples, as well as to the different species collected in each point, which are not enough to perform any statistical evaluation.

Total element studies are crucial for providing a mass balance utilizing additional data about chemical species and for determining how elements are distributed in samples. However, the information on the total element content is not sufficient for the evaluation of its environmental cycling and biological functions (Kot and Namieśnik 2000). Thus, considering that speciation studies carried out for nonmetals in Antarctic seaweed were focused on the produced volatile halogenated species determination, such as low-molecular weight halogenated C_1 to C_4 hydrocarbons (Laturnus 2001; Laturnus et al. 1997; Laturnus, Wiencke, and Klöser 1996; Laturnus et al. 2000), it is necessary to perform analysis in order to obtain information on the distribution of halogens and sulfur in seaweed composition.

3.2. Element fractionation among different classes of species

The first step in evaluating the species present in a sample can be conveniently performed by a fractionation approach, allowing the identification of groups of species of interest for a more detailed assessment in relation to the halogenated and sulfur species present in seaweed. A number of procedures has been published for the fractionation of elemental species in biological samples (Ayouni et al. 2007; Ruiz Encinar et al. 2003), which were adapted to allow the fractionation of halogens and sulfur in seaweed from the Antarctic.

In the present work, the experiments carried out for the sequential extractions aimed to obtain the distribution of halogens and sulfur among fractions containing (i) lipids (extracted with petroleum ether), (ii) water-soluble species, (iii) proteins (dissolved with protease and SDS) and (iv) carbohydrates (dissolved with cellulase and pectinase enzymes). The results of the total bromine, iodine, and sulfur contents in individual fractions are presented in Figure 4. The fluorine content was below the LOQ (cf. Table 5), and the high blank values (about 3 mg L^{-1}) for chlorine (probably derived from the reagents used) did not allow to determine this analyte in none of the fractions. The difference between the total element concentration in the samples and the sum of concentrations obtained for the different fractions was considered as a residue.

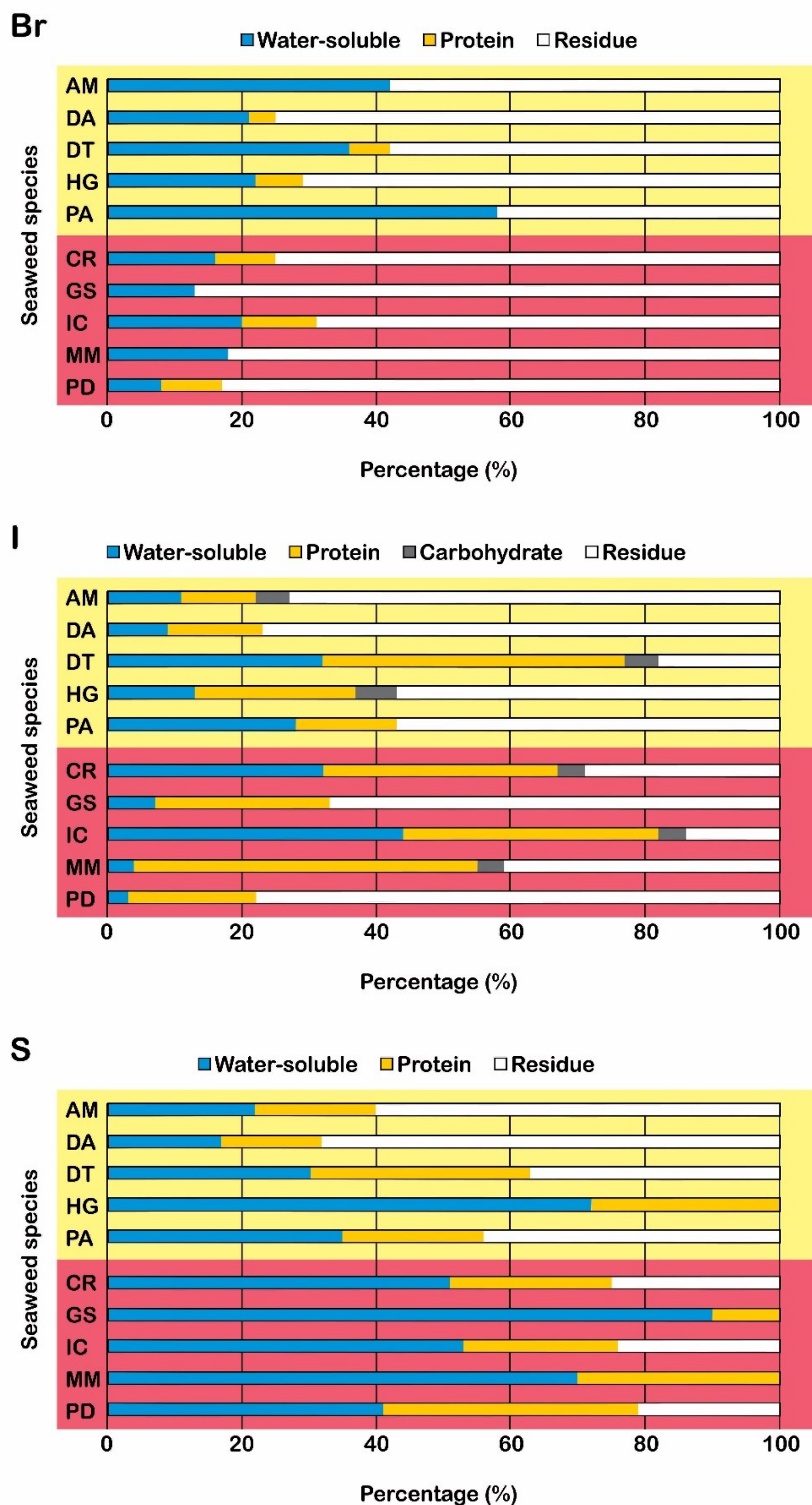

Figure 4. Distribution of bromine, iodine, and sulfur in different fractions of Antarctic seaweed (*A. mirabilis* – AM, *D. anceps* – DA, *D. antarctica* – DT, *H. grandifolius* – HG, *P. antarcticus* – PA, *C. racovitzae* – CR, *G. skottsbergii* – GS, *I. cordata* – IC, *M. mangini* – MM, and *P. decipiens* – PD).

As can be seen in Figure 4, neither bromine and iodine nor sulfur were found in lipid fraction. This can be attributed to low lipid content in Antarctic seaweed (1 – 7%) (Peters et al. 2005). All seaweed contain water-soluble bromine, iodine, and sulfur species. For bromine they presented a higher content in brown (from 21% to 58%) than in red seaweed samples (from 8% to 20%). For iodine, the concentration range in water-soluble fraction was similar for brown (from 9% to 42%) and red (from 3% to 44%) seaweed, which were lower than the values for iodine found in the water-soluble fraction by GÓMEZ-JACINTO; ARIAS-BORREGO; GARCÍA-BARRERA; GARBAYO *et al.* (2010) in microalgae (about 66%). For sulfur, except for *H. grandifolius* (72%) the concentrations in water-soluble fraction were lower in brown seaweed (from 17% to 35%) than in red seaweed (41% to 91%). The water-soluble bromine, iodine, and sulfur species were studied in detail by different HPLC techniques coupled to both elemental and molecular MS detectors. The results are presented in section 3.3.

Despite the importance of halides in biology, very little is known about their binding to proteins, with a notable exception of iodine for which abundant literature data exist (Al-Adilah et al. 2020; Hou et al. 1999; Hou et al. 2009). Nevertheless, it can be expected that negatively charged anions of Br^-, Cl^-, F^-, and I^- would be attracted and bound to the positively charged protein side chains. In terms of amino acid residues, which compose the binding sites, a strong preference for positively charged amino acid residues was found for all four halides with arginine (Arg), the most universal anchor for halide binding via its guanidinium moiety (Skitchenko et al. 2020). On the other hand, the presence of bromine bound to amino acids is reported in a few works, such as bromotyrosines and bromohistidines that are present in tips of crabs and in jaws of marine worms - in the composition of resistant structures (Birkedal et al. 2006; Schofield

et al. 2009). Despite being of potential interest, protein-bound bromine is still unexplored and, consequently, little information is reported, which makes its research relevant.

In this way, as expected, bromine concentration in protein fraction was very low, in most cases below 10% of the total Br content, and significant in the case of iodine, from 11% to 35% for brown, and from 19 to 51% for red seaweed. The iodine content in the protein fraction for Antarctic seaweed was lower than that observed by HOU; YAN e CHAI (2000) in a seaweed from China (about 65%). Sulfur in protein fraction varied from 15% to 32% for brown, and from 11% to 38% for red seaweed. It is important to mention that the sulfur content in protein fraction was considered only with protease extraction, since extraction with SDS makes the determination of sulfur unfeasible.

Contrary to bromide, iodine was detected in carbohydrate fraction of some studied seaweed: in *A. mirabilis*, *D. antarctica*, and *H. grandifolius* from brown, and *C. racovitzae*, *I. cordata*, and *M. mangini* from red seaweed. For sulfur, the high values of blanks (about 120 mg L^{-1}) made it impossible to quantify this analyte in the carbohydrate fraction. Even though the seaweed studied showed a significant difference in the carbohydrate content (from 0.4% to 7% for brown and from 22% to 55% for red) (Peters et al. 2005), similar iodine concentration in this fraction was obtained for both types of seaweed. Moreover, for *C. racovitzae*, *I. cordata*, *A. mirabilis*, *D. antarctica*, and *P. antarcticus* samples the order of iodine concentrations was [water-soluble] ≥ [protein] ≥ [carbohydrate], whereas for *G. skottsbergii*, *M. mangini*, *P. decipiens*, *D. anceps*, and *H. grandifolius* was [protein] > [water-soluble] > [carbohydrate].

High residue fraction for bromine was observed for all seaweed studied. However, there is no clear information in the literature about which other species of bromine could be present in seaweed, consequently none of the extraction methods used

was able to extract most of the bromine species. In order to identify which bromine and iodine species are present in Antarctic seaweed, speciation studies were carried out for all the samples and the results are presented in the next sections.

3.3. Speciation of bromine, iodine, and sulfur in Antarctic seaweed

3.3.1. Bromine speciation in Antarctic seaweed after pancreatin extraction

Although the results obtained from the analysis of bromine in fractionation study presented a low content of this element in all fractions evaluated, the information about the presence of bromine in seaweed is very important in order to identify which species are contained in the samples, even more in samples whose halogens composition is totally unknown in the literature. Thus, the use of the same method, optimized for iodine speciation, was attempted for the speciation of bromine. However, the analysis of seaweed extracts after pancreatin extraction by RP-HPLC-ICP-MS was problematic due to the coelution of bromide and bromate (Figure 5), which did not allow to quantify inorganic bromine. Therefore the extracts were analysed by AE-HPLC-ICP-MS (Figure 6), which allowed to obtain the separation of these two brominated species, making it possible to confirm that in all the samples evaluated only bromide was present. However, the chromatograms obtained by RP-HPLC-ICP-MS, in addition to unresolved bromine/bromate peak showed also unidentified organic bromine species, which could be quantified. In this way, using an external calibration, quantitative results were obtained from the integration of the peaks corresponding to each species. For this, it was necessary to correct the results according to the suppression observed for the analytes, which occurred due to the increase in MeOH concentration during the elution of the mobile phase used in the elution gradient. The quantitative results are presented in Table 6.

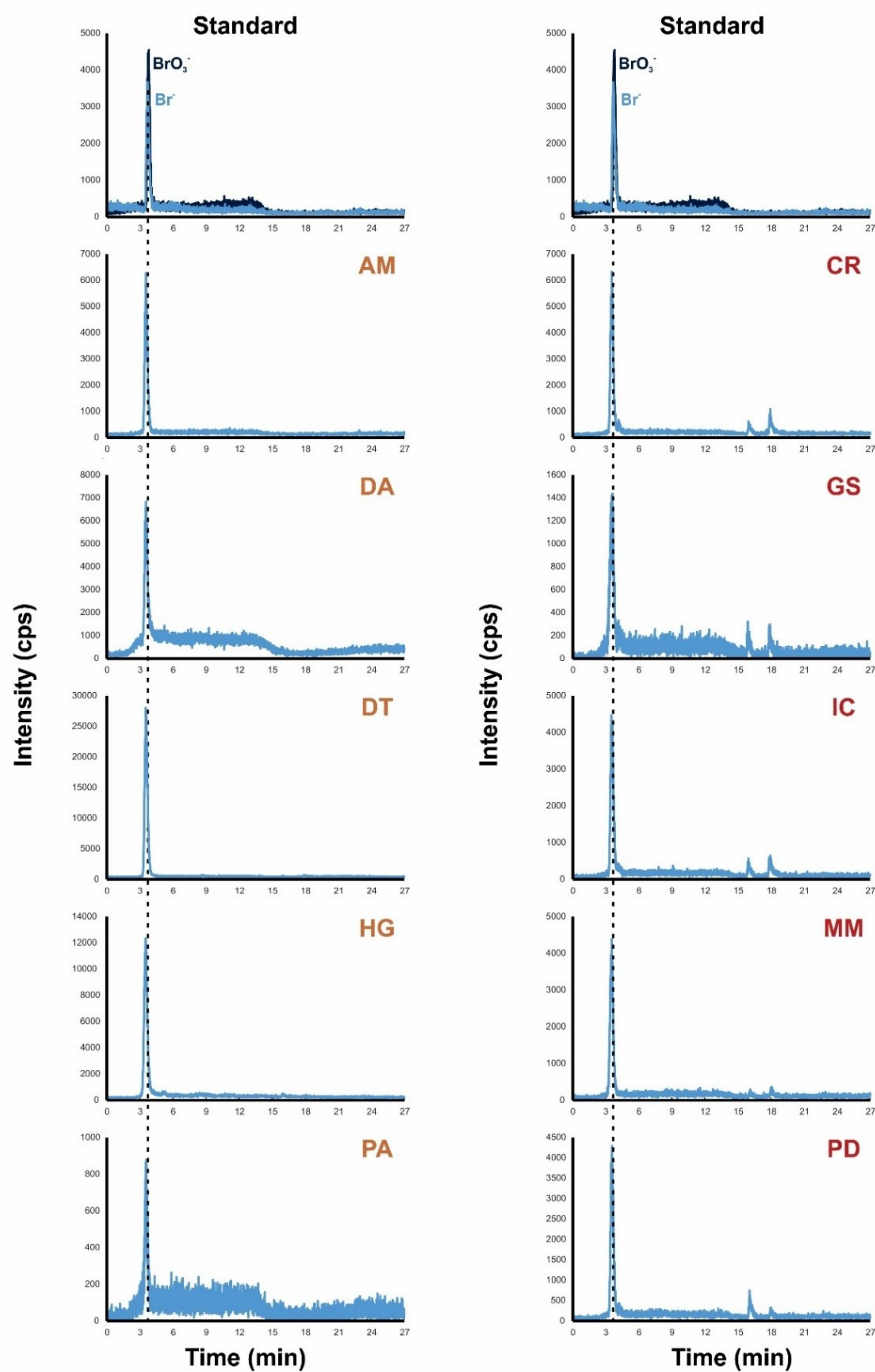

Figure 5. Bromine species in Antarctic seaweed obtained by RP-HPLC-ICP-MS after pancreatin extraction.

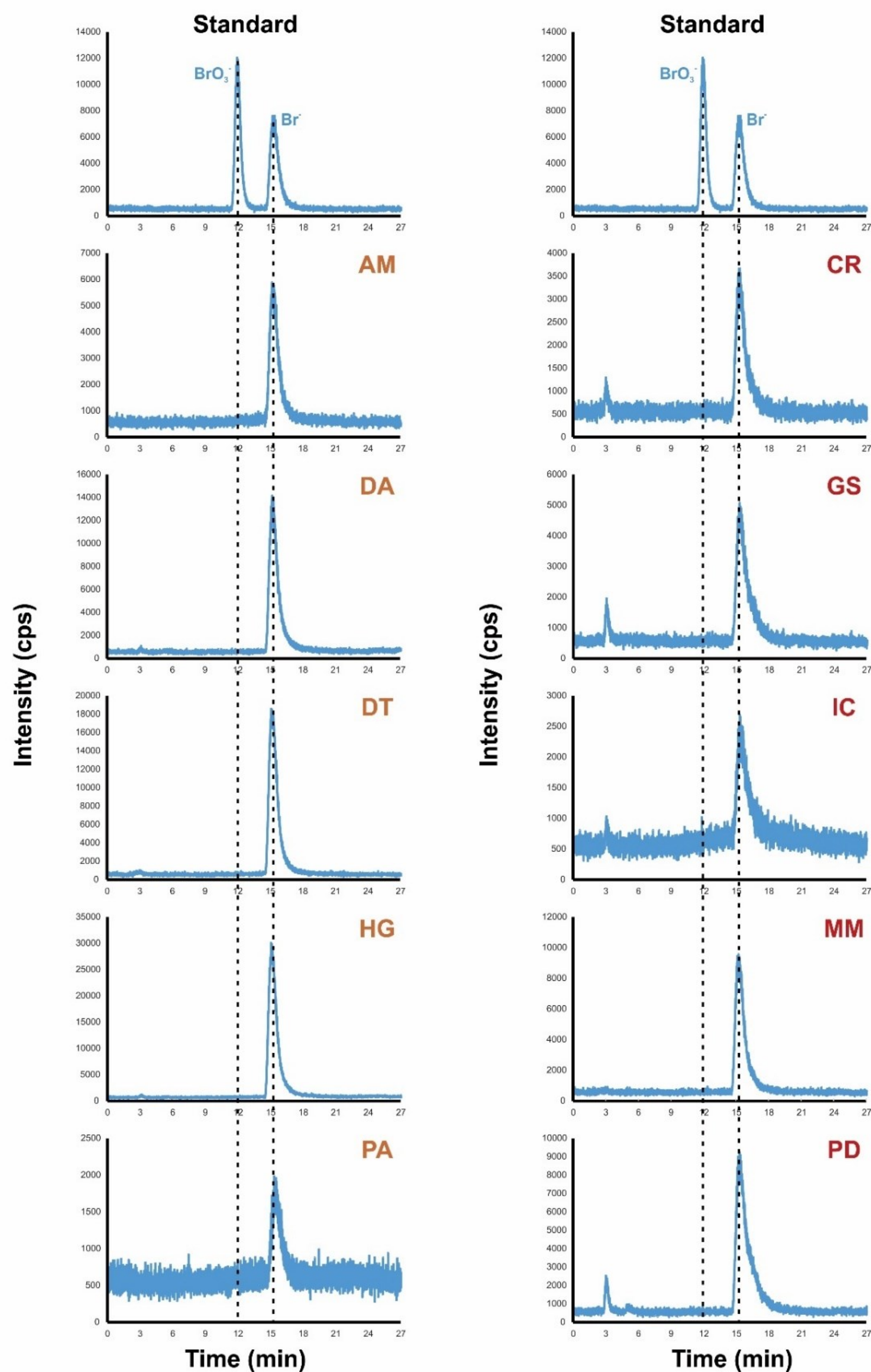

Figure 6. Bromine species obtained by AE-HPLC-ICP-MS after pancreatin extraction.

All the samples contained bromide, and for all red seaweed it was observed two unknwon peaks (RT = 15.9 min and 17.8 min) which were not observed for any brown species. Except for *P. antarcticus* sample (13 mg kg^{-1}), bromide concentrations in

studied samples are within the concentration range related in the literature, varying from 16 mg kg^{-1} to 795 mg kg^{-1} (Wang and Jiang 2008; Romarís-Hortas, Bermejo-Barrera, and Moreda-Piñeiro 2012).

3.3.2. Iodine speciation in Antarctic seaweed after pancreatin extraction

The presence of significant amounts of iodine in the form of iodinated amino acids (MIT and DIT) for different seaweeds species was reported in the literature (Romarís-Hortas, Bermejo-Barrera, and Moreda-Piñeiro 2013; Wang et al. 2019; Peng et al. 2018; Han et al. 2012; Sun et al. 2015). In order to quantify these species, enzymatic digestion of the different sample fractions is necessary to hydrolyse them and release organic and inorganic iodinated species. In this study, pancreatin extraction was used for Antarctic seaweed, once this enzyme presented a better performance over other enzymes evaluated in releasing MIT, DIT, and inorganic iodine species from seaweed (Romarís-Hortas, Bermejo-Barrera, and Moreda-Piñeiro 2013). The quantitative results are shown in Table 6, and the chromatograms in Figure 7.

By using RP-HPLC-ICP-MS, it was possible to separate four iodine standard species: I^-, IO_3^-, MIT and DIT, as demonstrated in Figure 7. As can be seen in Table 6, all seaweed evaluated contained iodide and MIT in the extracts, varying from 7.4 mg kg^{-1} (*P. decipiens*) to 261 mg kg^{-1} (*C. racovitzae*), and from 3.3 mg kg^{-1} (*P. antarcticus*) to 50 mg kg^{-1} (*M. mangini*), respectively. DIT was observed in all red seaweed, varying from 1.3 mg kg^{-1} (*P. decipiens*) to 16 mg kg^{-1} (*M. mangini*), but only one brown seaweed contained this iodine species (*D. antarctica* – 18 mg kg^{-1}). The higher concentrations of MIT and DIT found in red seaweed are associated with higher protein content in red than in brown seaweed (FLEURENCE; MORANÇAIS; DUMAY, 2018).

Table 6. Quantitative data for brominated and iodinated species in seaweed from the Antarctic after pancreatin extraction.

Seaweed species	Bromide	Unknown Br species	Sum of Br species (%)*	Iodide	MIT	DIT	Unknown I species	Sum of I species (%)*
Ascoseira mirabilis	79 ± 4	-	79 (78.2)	104 ± 8	6.3 ± 0.4	-	-	110 (51.3)
Desmarestia anceps	96 ± 5	30 ± 1	126 (28.7)	24 ± 3	7.0 ± 0.7	-	46 ± 4	77 (16.9)
Desmarestia antarctica	386 ± 19	33 ± 2	419 (68.2)	210 ± 17	31 ± 2	18 ± 2	15 ± 1	274 (73.4)
Himantothallus grandifolius	188 ± 9	35 ± 2	223 (41.8)	72 ± 6	14 ± 1	-	70 ± 5	156 (41.6)
Phaeurus antarcticus	13 ± 1	-	13 (100)	23 ± 2	3.3 ± 0.2	-	2.7 ± 0.3	29 (65.9)
Curdiea racovitzae	166 ± 8	61 ± 4	227 (69.4)	261 ± 21	45 ± 4	10 ± 1	14 ± 1	330 (80.3)
Gigartina skottsbergii	42 ± 2	25 ± 1	67 (48.9)	4.5 ± 0.5	13 ± 1	3.6 ± 0.2	5.3 ± 0.3	26 (48.8)
Iridaea cordata	91 ± 5	48 ± 3	139 (66.8)	249 ± 20	39 ± 2	7.9 ± 0.8	11 ± 1	307 (89.5)
Myriograme mangini	66 ± 3	27 ± 2	93 (43.0)	16 ± 1	50 ± 3	16 ± 1	9 ± 1	91 (47.4)
Palmaria decipiens	81 ± 4	31 ± 2	112 (65.5)	7.4 ± 0.9	8.7 ± 1.1	1.3 ± 0.1	2.2 ± 0.1	20 (36.9)

* Percentage of the sum of species in relation to the total content in the sample.

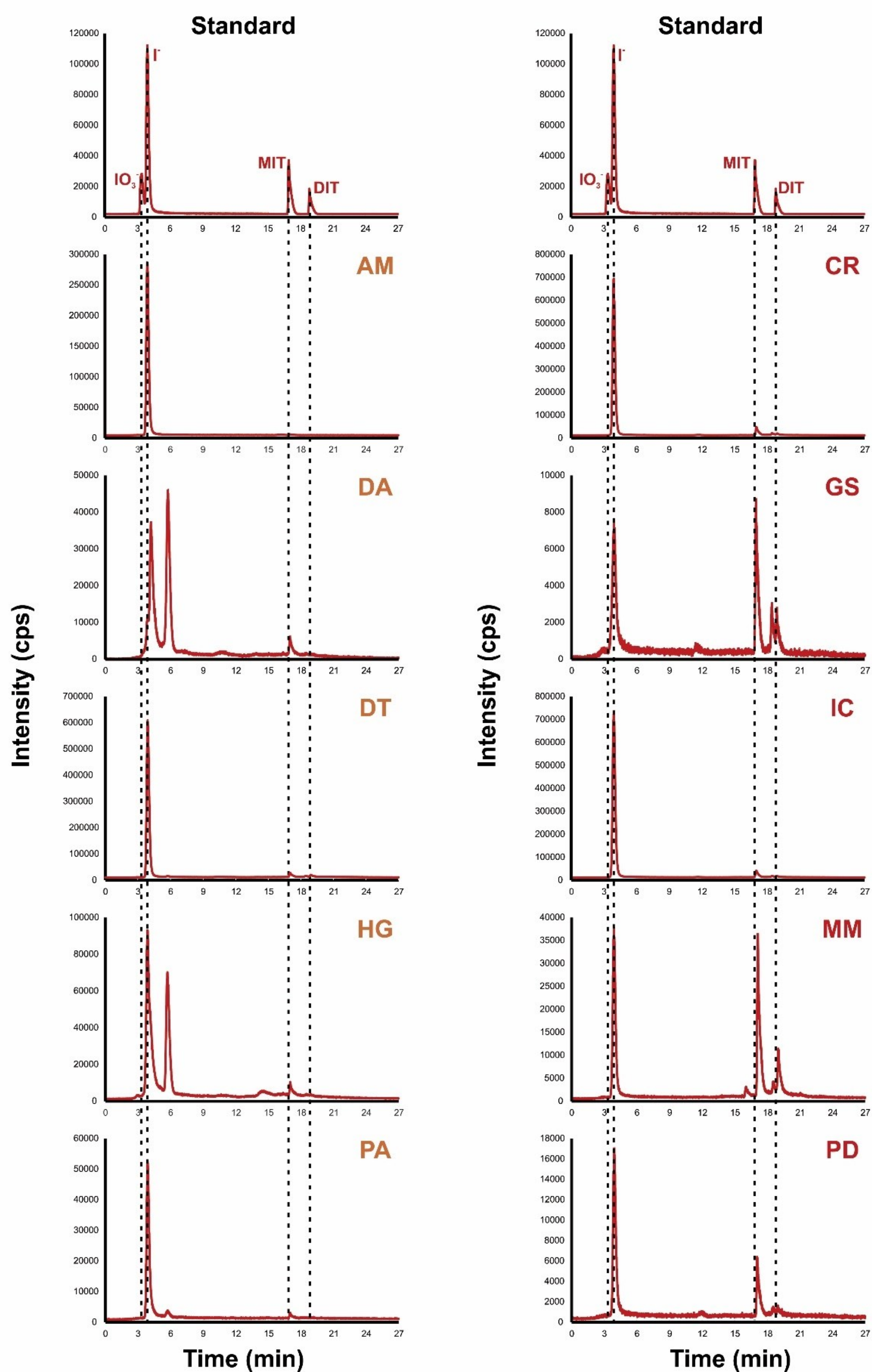

Figure 7. Iodine species in Antarctic seaweed obtained by RP-HPLC-ICP-MS after pancreatin extraction.

Except for the *A. mirabilis* sample, brown seaweed presented an unknown iodine signal (RT= 5.7 min). For red seaweed, an unknown signal was observed (RT= 16 min) only for *M. mangini* sample, whereas all red seaweed presented an unknown iodine signal (RT = 18.5 min) between MIT and DIT, which was also observed by Romarís-Hortas, Bermejo-Barrera, and Moreda-Piñeiro (2013) in edible seaweed. The sum of unknwon iodine species varied from 2.7 mg kg^{-1} to 46 mg kg^{-1} for brown seaweed and from 2.2 mg kg^{-1} to 14 mg kg^{-1} for red seaweed (Table 5).

As observed in other studies in the literature, no iodate was detected in the studied seaweed (Wang and Jiang 2008; Han et al. 2012; Romarís-Hortas, Bermejo-Barrera, and Moreda-Piñeiro 2012; Sun et al. 2015). In general, red seaweed presented more organic iodine than brown species. Iodide and DIT concentrations obtained in this study are within the concentration ranges reported in the literature for other seaweed species, varying from 0.7 mg kg^{-1} to 3 940 mg kg^{-1} for iodide (Shah et al. 2005; Wang and Jiang 2008; Han et al. 2012; Romarís-Hortas, Bermejo-Barrera, and Moreda-Piñeiro 2012; Sun et al. 2015; Peng et al. 2018), and from 0.07 mg kg^{-1} to 59 mg kg^{-1} for DIT (Han et al. 2012; Romarís-Hortas, Bermejo-Barrera, and Moreda-Piñeiro 2012, 2013; Sun et al. 2015; Peng et al. 2018; Wang et al. 2019). Except for M. mangini sample (50 mg kg^{-1}), MIT concentrations in the studied seaweed are also within the concentration range reported in other studies, varying from 0.12 mg kg^{-1} to 42 mg kg^{-1} (Han et al. 2012; Romarís-Hortas, Bermejo-Barrera, and Moreda-Piñeiro 2012, 2013; Sun et al. 2015; Peng et al. 2018; Wang et al. 2019). For most samples studied the distribution of iodine species concentration was $[I^-] \geq [MIT] > [DIT]$, which was also observed for most samples studied in the literature (Romarís-Hortas, Bermejo-Barrera, and Moreda-Piñeiro 2012, 2013; Sun et al. 2015; Peng et al. 2018).

Analytical parameters related to the pancreatin extraction method, such as extraction efficiencies and chromatographic recoveries are presented in Table 7. The extraction efficiency was calculated by the relationship between the analyte concentration in the solid sample and the total analyte concentration obtained by flow injection analysis (FIA) in the extracts after pancreatin extraction. The chromatographic recoveries were calculed by comparing the sum of analytes species in the extracts after pancreatin extraction with the analytes concentration obtained after analysis of extracts by FIA.

As can be seen, extraction efficiencies for bromine and iodine were from 31 to 107%, and from 23 to 105%, respectively. Whereas the chromatographic recoveries varied from 69 to 109% for bromine, and from 72 to 92% for iodine. Although the extraction efficiencies for iodine in some samples were slightly lower than those reported by Romarís-Hortas et al. (2013) – from 76 to 96% –, by using the method adapted in the present study, it was also possible to obtain suitable recoveries for bromine, in addition to the fact that ten species of seaweed were used, with unique characteristics, which justifies the large variation in recoveries and efficiencies obtained.

It can be seen that the method used was not very efficient for the extraction of bromine, however, based on the results regarding the chromatographic recovery, it can be observed that the method of analysis was satisfactory, since recoveries from 69 to 109% were obtained for this analyte. In this sense, new methods must be evaluated to allow a better extraction efficiency of this element in seaweed from the Antarctic.

Table 7. Total bromine and iodine concentration in seaweed samples after microwave-induced combustion and after pancreatin extraction; and bromine and iodine extraction efficiencies and chromatographic recoveries after pancreatin extraction.

Seaweed species	Concentration (mg kg^{-1})									
	Total Br	Extraction efficiency (%)	Total Br after pancreatin extraction	Chromatogr. recovery (%)	Sum of Br species*	Total I	Extraction efficiency (%)	Total I after pancreatin extraction	Chromatogr. recovery (%)	Sum of I species*
A. mirabilis	101 ± 4	75	76 ± 4	103	79 ± 3	215 ± 8	56	121 ± 6	92	111 ± 6
D. anceps	438 ± 5	31	138 ± 7	91	126 ± 4	455 ± 8	23	105 ± 5	73	77 ± 3
D. antarctica	614 ± 15	99	609 ± 30	69	419 ± 14	373 ± 6	101	378 ± 19	72	273 ± 9
H. grandifolius	533 ± 27	54	286 ± 14	78	223 ± 7	375 ± 11	56	212 ± 11	73	156 ± 4
P. antarcticus	13 ± 2	107	14 ± 1	93	13 ± 1	44 ± 1	90	40 ± 2	73	29 ± 1
C. racovitzae	327 ± 27	82	269 ± 13	85	228 ± 6	411 ± 34	105	435 ± 22	76	330 ± 11
G. skottsbergii	137 ± 11	51	70 ± 4	94	66 ± 2	54 ± 3	55	30 ± 1	87	26 ± 1
I. cordata	208 ± 20	72	149 ± 7	93	139 ± 4	343 ± 9	101	347 ± 17	89	307 ± 10
M. mangini	216 ± 7	43	93 ± 5	100	93 ± 3	192 ± 15	60	116 ± 6	78	91 ± 2
P. decipiens	171 ± 10	60	103 ± 5	109	112 ± 3	54 ± 2	44	24 ± 1	83	20 ± 1

* Sum of species obtained after pancreatin extraction.

3.3.3. Sulfur speciation in Antarctic seaweed

Considering that a significant part of the total sulfur concentration in the samples was present in the protein fraction, which was already expected due to the various sulfur-containing molecules present in seaweed, as well as taking into account the different compounds observed in the water-soluble fractions, studies are needed to enable a more precise identification of such species.

3.4. Speciation of low molecular weight (LMW) water-soluble species

3.4.1. Molecular weight-based profiling of water-soluble bromine, iodine, and sulfur species using fastSEC-ICP-MS

More precise information about the water-soluble bromine, iodine, and sulfur species was obtained by their molecular weight-based profiling using size-exclusion chromatography. The UPLC chromatographic column used allowed the separation from 1 to 80 kDa and fast analysis. This technique gives an estimation of the molecular size of the species including the high molecular ones that cannot be analysed using other techniques. The column recoveries were generally higher than 80%, which is a satisfactory proof of good control of possible on-column effects and carry-over. The chromatograms obtained by fastSEC-ICP-MS analysis for seaweed water extracts and the standard solutions are presented in Figures 8, 9, and 10 for bromine, iodine, and sulfur, respectively.

As shown in Figure 8, with exception of *A. mirabilis*, all brown seaweed showed an unknown peak signal, with low intensity (RT= 3.3 min) in addition to bromide, whereas only *D. anceps* and *H. grandifolius* brown seaweed samples showed more bromine unknown peaks (between 4 and 6 min). For red species, only *G. skottsbergii* sample presented an unknown signal (also in RT = 3.3 min) in addition to bromide,

which was the only peak observed for all the other red seaweed. However, none of the signals matched with bromate retention time, indicating that this species is not present in extracts of the studied samples. This is probably related to bromide being the main bromine species present in seawater (Al-Adilah et al. 2020). Wang and Jiang (2008) also quantified only bromide specie in a seaweed sample and bromate was below the LOD, whereas Romarís-Hortas, Bermejo-Barrera, and Moreda-Piñeiro (2012) quantified both bromine species (Br^- and BrO_3^-) in edible brown and red seaweed, and in both studies, bromide was always in higher concentration than bromate.

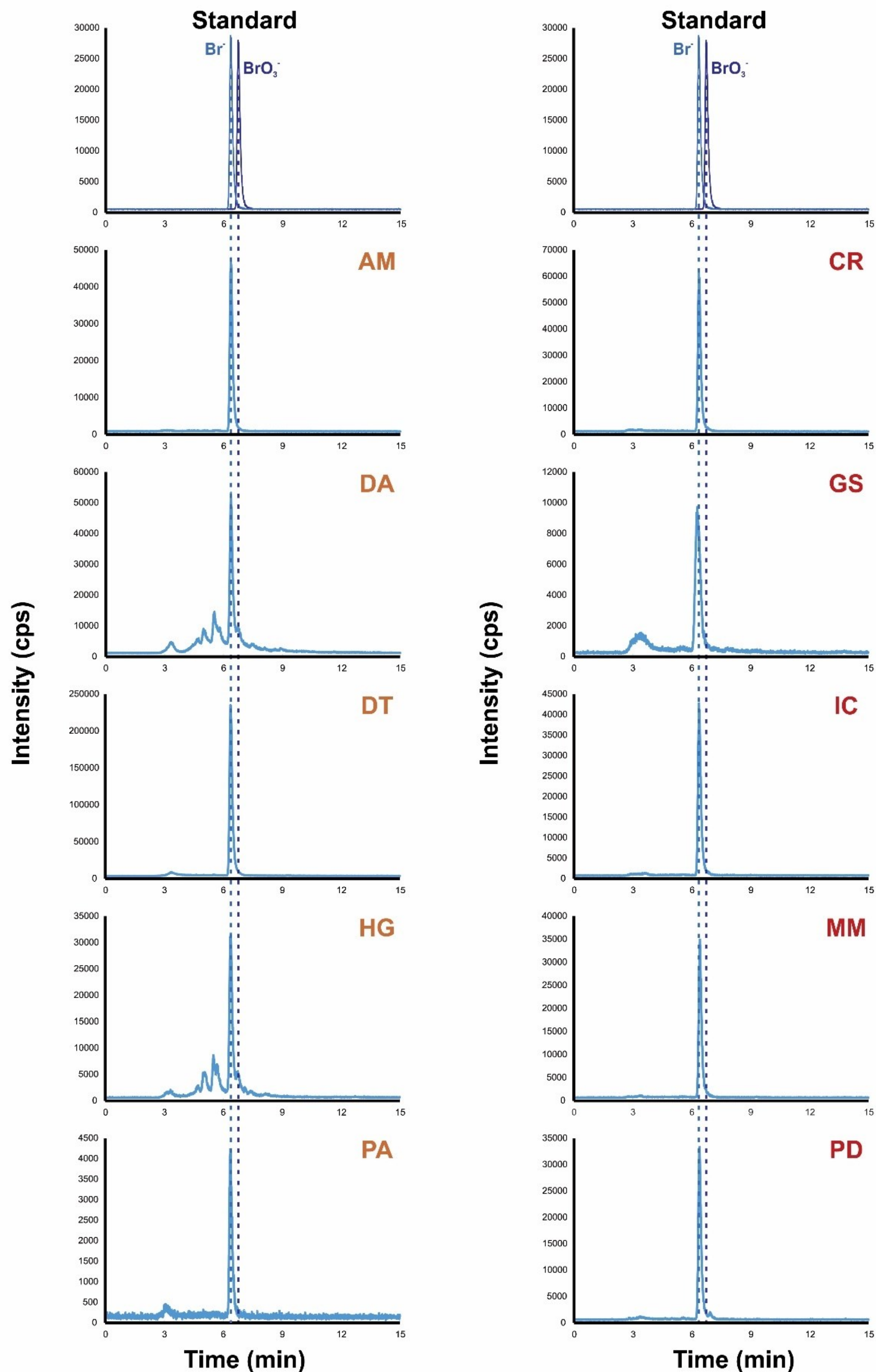

Figure 8. Profiles of LMW water-soluble bromine species in Antarctic seaweed obtained by fastSEC-ICP-MS in water fraction.

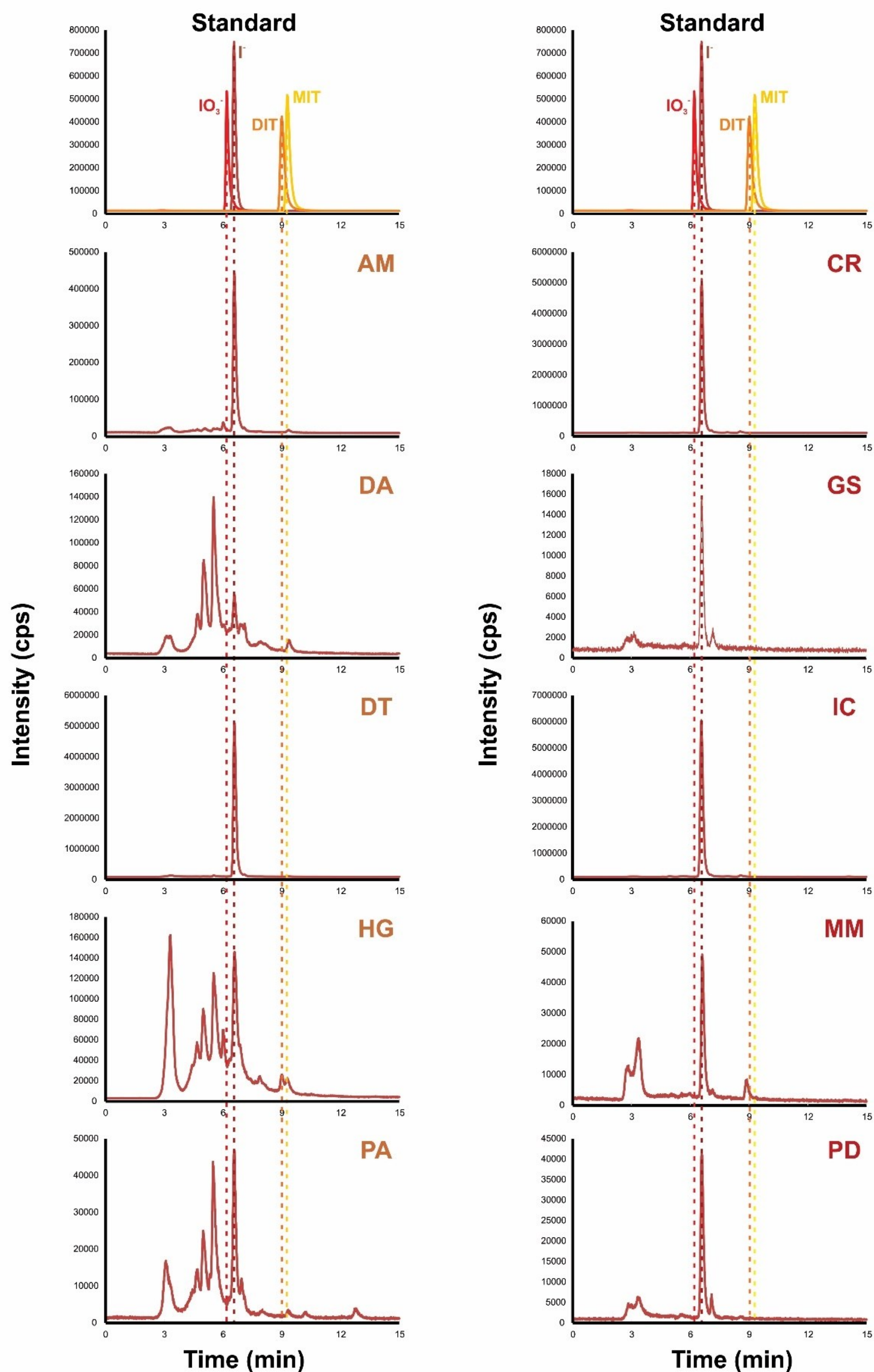

Figure 9. Profiles of LMW water-soluble iodine species in Antarctic seaweed obtained by fastSEC-ICP-MS in water fraction.

As shown in Figure 9, for *A. mirabilis* and *D. antarctica* brown samples only iodide peak is present in the chromatograms, whereas for the other samples unknown peaks are also present. For red seaweed, also two samples (*C. racovitzae* and *I. cordata*) presented only iodide signal, whereas the other red seaweed showed in addition to iodide, also some unknown peaks. Similar to what was observed for brominated species, iodine species are also observed in greater variety for brown than for red seaweed. However, similarly as for bromate, none of the signals matched with iodate retention time, indicating absence of this species. It was proposed that iodate is reduced to iodide in seaweed cell surface for further absorption, what justify the main presence of iodide in seaweed analysed (Carrano et al. 2020). In this sense, most studies in the literature also quantified only iodide specie in seaweed (Wang and Jiang 2008; Han et al. 2012; Romarís-Hortas, Bermejo-Barrera, and Moreda-Piñeiro 2012; Sun et al. 2015), with exception of Shah et al. (2005) that quantified iodate in Wakame seaweed (4.16 mg kg^{-1}) in lower concentration than iodide in the same sample (140 mg kg^{-1}). Finally, the observed bromine and iodine unknown peaks can be related to halogenated polyphenols, generally present in seaweed (Shah et al. 2005).

As can be seen in Figure 10, the sulfur standards analyzed were eluted in three different regions according to their sizes, with sulfate eluting first, followed by Cys, HCys, and Pen, and finally larger molecules – GSH and GluCys. All analyzed brown and red seaweed presented varied patterns of compounds, including signals in the retention times referring to the analyzed standards. Additionally, *M. mangini* sample presented one additional signal in a different retention time. From these results it could be observed that sulfur species probably present in the samples include sulfate, Cys, GluCys, GSH, HCys, and Pen, and more studies should be carried out in order to confirm the presence of these species, as well as quantify them.

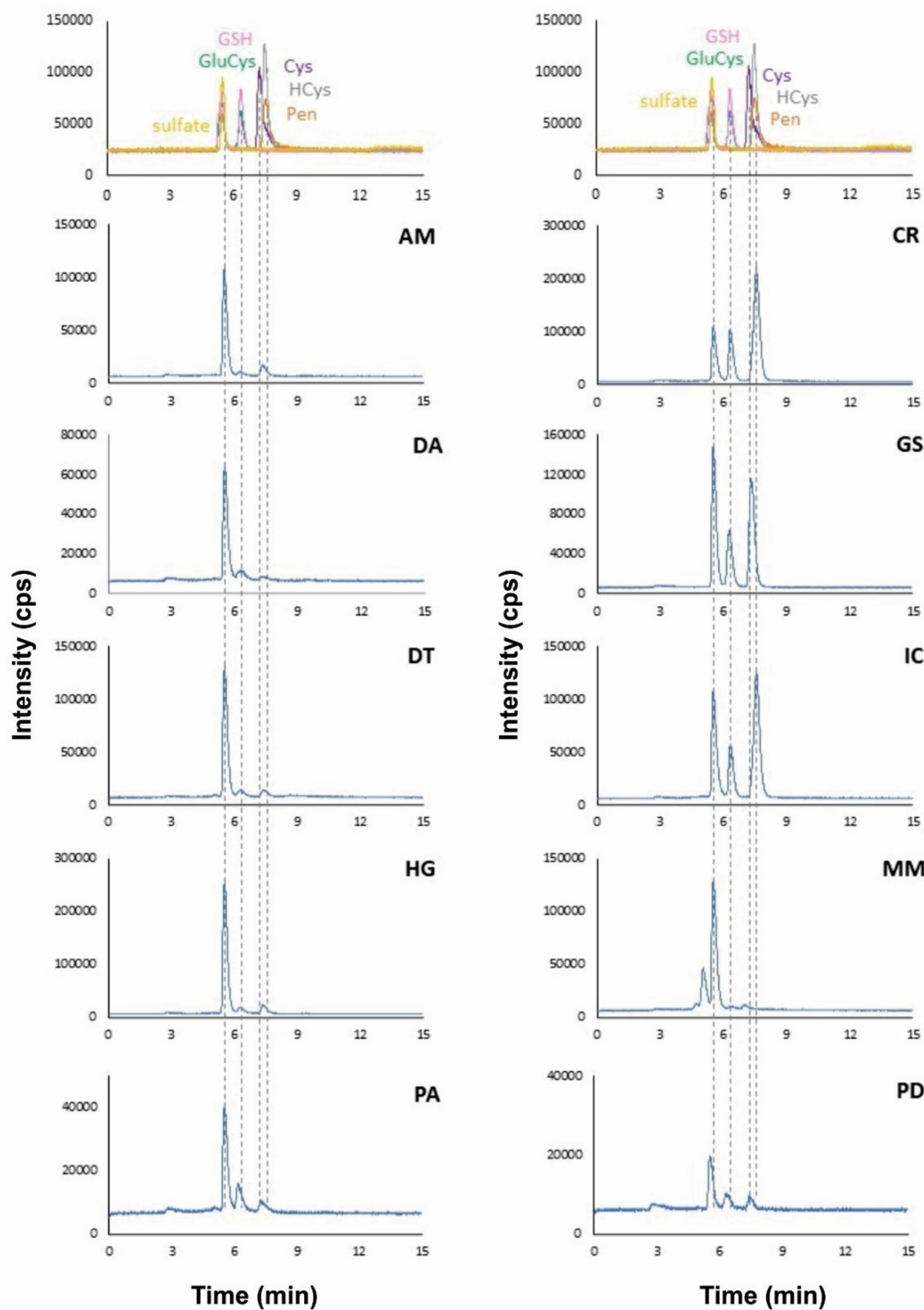

Figure 10. Profiles of LMW water-soluble sulfur species in Antarctic seaweed obtained by fastSEC-ICP-MS in water fraction.

The water extraction of red seaweed samples resulted in gelatinous solutions from the composition of their cell walls that are composed of polysaccharides, such as

agar and carrageenans, which represent up to 40-50% of the seaweed dry mass (Torres, Flórez-Fernández, and Domínguez 2019). An enzymatic treatment with agarase was developed for *C. racovitzae*, *G. skottsbergii*, *I. cordata*, *M. mangini*, and *P. decipiens* samples in order to decompose polysaccharides and fully solubilize jelly extracts and evaluate possible changes in their composition.

It was observed that for all the red seaweed samples (except *M. mangini*) bromine concentrations were higher after performing agarase extraction, whereas for iodine concentrations only *G. skottsbergii* and *P. decipiens* samples presented higher concentrations after enzymatic extraction. However, for both elements no new signal was observed (Figure 11), indicating the agarase efficiency in releasing bromine and iodine from red seaweed polysaccharides. It suggests that part of water-soluble brominated and iodinated species is retained in the gelatinous polysaccharide fraction obtained after water extraction of the red seaweed. On the other hand, different from what was observed for bromine and iodine, for sulfur (Figure 12) several new signals were observed after agarase extraction for red seaweed, indicating that other sulfur species are released from polysaccharide jelly solution.

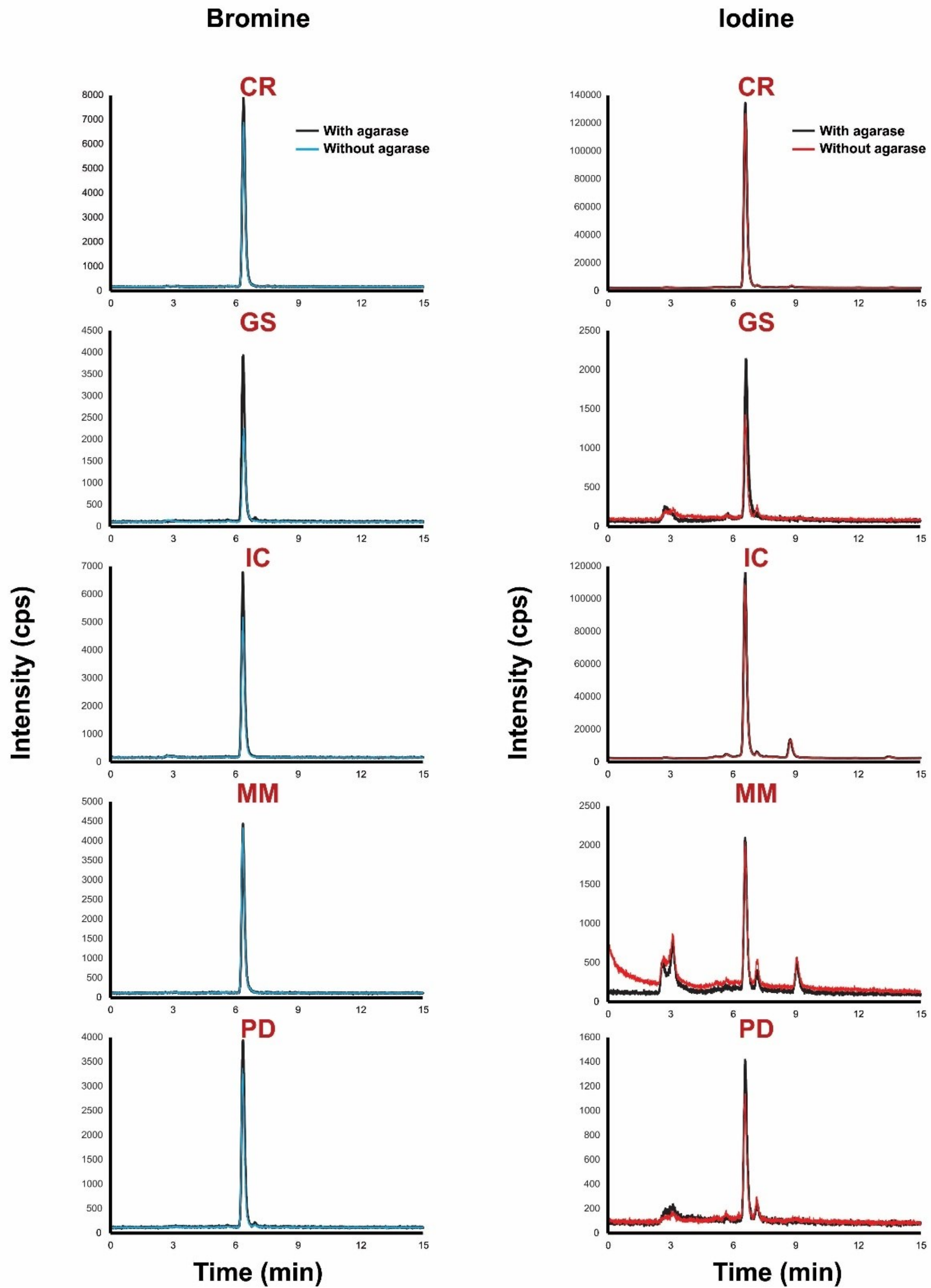

Figure 11. Profiles of LMW water-soluble bromine and iodine species in Antarctic red seaweed obtained by fastSEC-ICP-MS after water and agarase extraction.

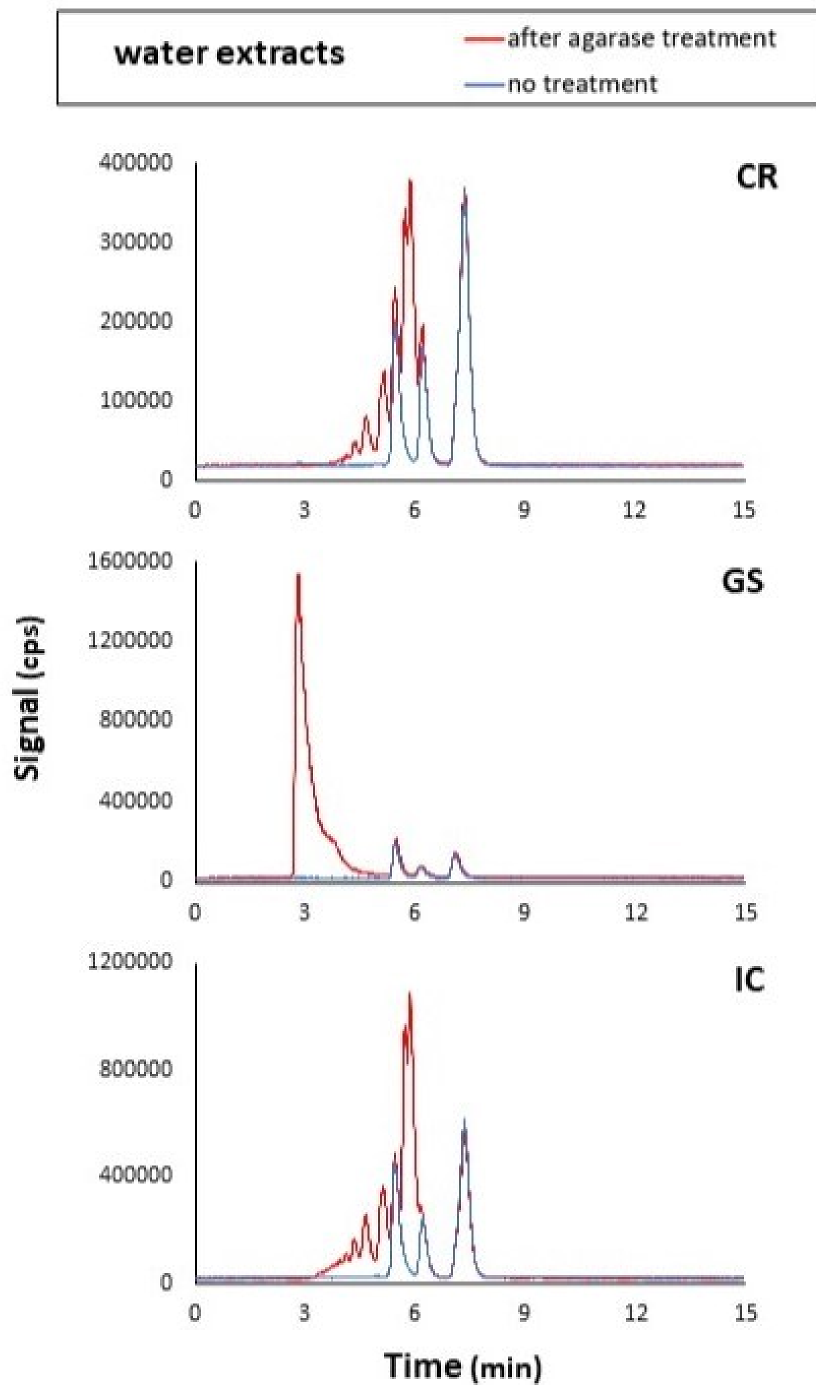

Figure 12. Profiles of LMW water-soluble sulfur species in Antarctic red seaweed obtained by fastSEC-ICP-MS after water and agarase extraction**.**

3.4.2. Halogenated polyphenols

Marine halogenated compounds include a wide range of substances, including peptides, polyketides, indoles, volatile halogenated hydrocarbons, and polyphenolic compounds (Cabrita, Vale, and Rauter 2010). Over the past few decades, scientists have studied the biological effects of halogenated compounds and their findings indicate that these substances have antibacterial, antifungal, antiviral, anti-inflammatory, antifeedant, cytotoxic, among other activities (Blunt et al. 2009). Most of these activities are related to halogenated polyphenols, making them an interesting class of compounds to be analysed. In this sense, the MeOH and NH_4OH extracts from samples were analysed in order to obtain information regarding halogenated polyphenols in Antarctic seaweed. With the exception of sample *P. antarcticus* (brown seaweed), in which no polyphenolic compounds could be detected, the information on the identified compounds is shown in Table 8.

Table 8. Halogenated polyphenol compounds identified in seaweed from the Antarctic.

Compounds	Precursor ion *m/z*	Element composition	Mass error (ppm)	Samples	Fragmentation (%)
1	227.9825	$C_9H_5ClO_5$	0.01	HG	182.9848 (100)
2	237.9435	$C_7H_4Cl_2O_5$	0.07	HG	218.9247 (100); 192.9455 (88); 156.9690 (10)
3	239.9825	$C_{10}H_5ClO_5$	0.07	HG	220.8800 (35); 210.9795 (23); 166.9897 (33); 158.9245 (100)
4	247.9320	$C_7H_5BrO_5$	0.03	DA; HG	228.9134 (100); 202.9339 (28); 80.9223 (3)
5	247.9334	$C_7H_5IO_2$	0.02	AM; CR; DA; DT; GS; IC; MM; PD	-
6	261.9435	$C_9H_4Cl_2O_5$	0.14	HG	216.9457 (100)
7	271.9728	$C_{10}H_5ClO_7$	0.01	HG	226.9745 (100); 182.9846 (5)
8	273.9435	$C_{10}H_4Cl_2O_5$	0.04	HG	244.9410 (10); 236.9589 (28); 200.9510 (29); 192.9692 (100)
9	277.8577	$C_7H_4Br_2O_2$	0.05	DA; DT; IC; MM; PD	276.8493 (54); 78.9182 (35)
10	293.8527	$C_7H_4Br_2O_3$	0.12	CR; DT; PD	248.8545 (100)
11	305.9334	$C_{10}H_4Cl_2O_7$	0.03	HG	260.9354 (100); 216.9457 (4)
12	307.8813	$C_7H_4Cl_4O_5$	0.06	HG	270.8961 (49); 262.8701 (23); 234.9196 (13); 226.9064 (100); 190.9299 (33)
13	315.0509	$C_{13}H_{14}ClNO_6$	0.04	GS	296.0341 (45); 278.0232 (7); 260.0573 (100); 234.0779 (19)
14	315.9218	$C_{10}H_5BrO_7$	0.07	DA; HG	270.9260 (100); 226.9361 (4)

15	**325.8748**	**$C_9H_5BrCl_2O_4$**	**0.06**	**HG**	**288.8901 (100); 244.9001 (6)**
16	**349.8829**	**$C_{10}H_4BrClO_7$**	**0.11**	**HG**	**304.8847 (100); 260.8952 (3)**
17	**363.9081**	**$C_{10}H_5IO_7$**	**0.28**	**DA**	**-**
18	**369.8242**	**$C_9H_5Br_2ClO_4$**	**0.09**	**HG**	**332.8394 (40); 288.8900 (100)**
19	**373.8299**	**$C_7H_4I_2O_2$**	**0.06**	**CR; DT; MM**	**-**
20	**373.8609**	**$C_9H_5Cl_2IO_4$**	**0.12**	**HG**	**-**
21	**383.8219**	**$C_7H_4Cl_3IO_4$**	**0.10**	**HG**	**-**
22	**389.8250**	**$C_7H_4I_2O_3$**	**0.04**	**CR; DA; DT; MM**	**-**
23	**413.7737**	**$C_9H_5Br_3O_4$**	**0.07**	**HG**	**332.8395 (100)**
24	**439.9138**	**$C_{11}H_{11}Cl_3O_{10}S$**	**4.26**	**DA; GS**	**402.9309 (100)**
25	**461.7599**	**$C_9H_5Br_2IO_4$**	**0.04**	**HG**	**-**

As can be seen in Table 4, brominated (9), chlorinated (15), and iodinated (7) compounds were identified, most of them were present in brown seaweed, whereas only 7 compounds (**5**, **9**, **10**, **13**, **19**, **22**, and **24**) were found in red seaweed. Chlorinated compounds were almost exclusively (thirteen out of fifteen) present in *H. grandifolius* sample, which presented the highest chlorine concentration (Table 3). Most of the brominated (seven of nine) and chlorinated compounds (three of seven) were also identified in the *H. grandifolius* sample.

Based on MS2 spectra, it could be noted that for most of compounds the only mass loss was CO_2 (44 Da). According the obtained data, all compounds are halogenated polyphenols, however, there was no possibility to confirm the exact position of the halogens in the molecules only with the information obtained from mass spectra. The general structure of most of identified compounds are presented in Figure 13. The compounds with the same composition as for compounds **5** (2-iodobenzoic acid), **9** (3,5-dibromo-4-hydroxybenzaldehyde), **10** (2,3-Dibromo-4,5-dihydroxybenzaldehyde), and **22** (2-Hydroxy-3,5-diiodobenzoic acid) were also identified in red seaweed species (Li et al. 2008; Parisini et al. 2011; Olsen et al. 2013; Yang et al. 2016). These four compounds were present in *D. antarcticus* brown sample. For the other compounds, when searching Chemspider database by molecular formula, a resultant number of structures hits was obtained for compounds **1** (22), **3** (25), **4** (13), **6** (1), **7** (1), **8** (1), **12** (1), **13** (182), **15** (1), **18** (1), **19** (25), and **23** (1), whereas for the other compounds (**2**, **11**, **14**, **16**, **17**, **20**, **21**, **24**, and **25**) no hits were obtained.

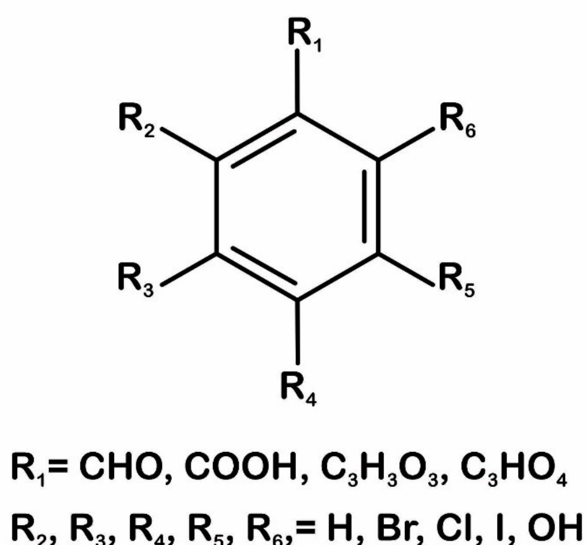

Figure 13. General structure of most halogenated compounds identified in Antarctic seaweed.

4. Conclusion

In this study, it was possible to obtain unprecedented data on halogens and sulfur in brown and red seaweed species from the Antarctic. By using a multi-technique approach involving different sample preparation methods and high sensitivity determination techniques it was obtained comprehensive information about total halogens and sulfur concentration, distribution of bromine, iodine, and sulfur in different seaweed fractions (lipids, water-soluble, proteins, carbohydrates and residue), as well as the quantification of iodinated amino acids (MIT and DIT) in ten seaweed species. Using this combination of methods and techniques it was possible the identification of possible thiols, as well as the separation and identification of inorganic bromine and iodine species in all samples. MIT was present in all studied samples, whereas DIT was quantified in all red species and one brown species, always in lower concentration than MIT. Unknown bromine and iodine species were observed in water and pancreatin extracts. Bromate and iodate were always at levels lower than LOQ for all seaweed samples. Additionally, 25 polyphenols were identified in nine of ten species studied, of which four were already reported in the literature, however, more analysis are needed for confirm the

exact structure of the identified compounds. Finally, speciation studies for sulfur are being carried out in order to confirm the presence of possible thiols observed in the samples, as well as quantify them.

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4 Final remarks

Based on the results obtained during the development of this project, it can be seen that the optimized parameters for the developed method, which employs the MIC, were suitable for the sample preparation of different brown and red seaweed from the Antarctic. Besides, the obtained final digests were compatible with IC-CD-MS, allowing the determination of the total concentrations of bromine, chlorine, fluorine, iodine and sulfur in a single analysis in the samples and in the CRM used to evaluate the method. Using the developed method, low values of blanks were obtained, which together with the high sample masses possible to be used, culminated in the obtaining of low values of LOD and LOQ for all analytes.

In this sense, for all samples evaluated bromine, chlorine, iodine, and sulfur were quantified, while fluorine concentrations were below its LOQ (1.6 mg kg^{-1}). Although fluorine was not quantified in the samples, even using larger sample masses, the results showed through the recovery tests and CRM analysis that it is possible to quantify this analyte using the method. In this sense, it is worth mentioning that the method developed is the first analytical method that uses MIC for the decomposition of seaweed from the Antarctic continent and, it is also the first study that allows the multielemental determination of all halogens and sulfur in these samples in a single analysis. Due to the several advantages presented by the MIC, the developed method has important characteristics, such as decomposing the samples reaching pressures considered safe for the system used, high throughput, low blank values, in addition to allowing the decomposition of high masses (up to 1000 mg) of sample.

Additionally, it can be observed that by using optimized sample preparation methods, aiming at speciation studies, it was possible to obtain important information regarding halogens and sulfur species present in different fractions of seaweed from the Antarctic. This was possible due to the high sensitivity and selectivity presented by analytical determination techniques employed for analysis added to the suitability of sample preparation methods used. Thus, by using extraction methods employing different approaches, such as the use of different enzymes and ultrasound, were obtained data about the species of bromine, iodine, and sulfur, including iodinated amino acids (MIT and DIT) and thiols (Cys, GSH, GluCys, HCys, and Pen), and their

distribution in different fractions of samples (lipids, water-soluble, proteins, and carbohydrates). Additionally, 25 halogenated polyphenols were identified in nine of ten seaweed studied, of which four compounds have already been identified in other seaweed species and reported in the literature. However, more analyses are needed to confirm the exact structure of the identified compounds in the present study.

All results obtained during the development of the present study are unprecedented data in the literature. Thus, using the methods described throughout this study, it was possible to characterize ten seaweed species from the Antarctica in relation to their compositions on halogens and sulfur species, as well as their distribution in different fractions. Therefore, the developed, optimized and applied methods, in addition to providing unprecedented results in the literature, also open the way for new studies aimed at the determination of these elements and their species for different purposes, such as environmental monitoring, as well as analyses for the discovery of new compounds and their possible biological activities.

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