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DEVELOPMENT OF METHODS FOR HALOGEN AND SULFUR DETERMINATION, SPECIATION AND BIOAVAILABILITY ASSESSMENT IN SEAFOOD

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Thesis, presented to the Postgraduate Program in Chemistry of the *Universidade Federal de Pelotas* (UFPel, Brazil), as partial requirement for obtain the Ph.D. in Chemistry (Analytical Chemistry) degree.

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"Estudar não é gasto, é investimento. Aliás, é o melhor, o mais barato e o mais duradouro investimento. Quando você forma alguém, é para sempre. O Brasil vai poder deixar de ser apenas exportador de minério de ferro, de soja e vai virar exportador de conhecimento"

Luiz Inácio Lula da Silva

ABSTRACT

BALBINOT, Fernanda Pitt. **Development of methods for halogen and sulfur determination, speciation and bioavailability assessment in seafood.** 2024. 147p. Thesis (Ph.D. Title in Chemistry) – Chemistry Postgraduate Program, Center for Chemical, Pharmaceutical and Food Sciences, Federal University of Pelotas, Pelotas, 2024.

Seafood is one of the most widely consumed food classes worldwide. Among the elements that can be found in the seafood composition, halogens and sulfur can be mentioned. However, increasing pollution of water bodies harms the safety and quality of these foods, as well change the concentration of nutrients and other elements in them. In this sense, the goal of this thesis was to develop new analytical strategies that make it possible to carry out such analyses on seafood, providing a global view of the presence of these analytes, in which species they can be found in the food, and what fraction of them can effectively be absorbed by the human metabolism. With this in mind, this thesis is divided into three chapters. The first study covers the development and evaluation of parameters for the use of microwave-induced combustion combined with ion chromatography coupled with mass spectrometry for the total determination of Br, Cl, F, I, and S in seafood. In the second chapter, the fractionation and influence of culinary treatments for all analytes, as well as the bioavailable fraction of F, Br and I were evaluated. In the third and final chapter, the results regarding F speciation, more specifically the determination of organofluorine compounds, in the studied samples were be presented. It should be noted that these compounds are emerging global contaminants of great relevance, known as "eternal chemicals" due to their stability, persistence, and ability to accumulate in the environment and organisms. They are increasingly present in foods, such as seafood. As the main results obtained, it can be highlighted that, in the first study, a suitable analytical method was developed for halogen and sulfur determination in several types of seafood, using a single chromatographic run. Recoveries ranged from 92% to 109%, considering spiking experiments and certified reference materials analysis. From the second study it was observed that most of Br, I, and F accumulate in shrimp's shell/head, while most of Cl and S accumulate in shrimp's muscle tissue, as well as different influences of culinary treatments for these analytes depending on the sample matrix. Also, it was verified that up to 63% of Br can be bioavailable from seafood, while most of I remains on the residual fraction (not bioaccessible/bioavailable). In the final study, a method was developed for F speciation, determining PFAS compounds in seafood. Recoveries for 11 compounds ranged from 83 to 115%. Ten PFAS were detected among all samples and four were quantified. Their concentration was also influenced by culinary treatments. After developing this thesis, it was possible to present, in an unprecedented way, an extremely comprehensive view of the relation between elements so important for human metabolism – halogens and sulfur – and a class of food that are part of the diet of almost all cultures around the world.

Keywords: ion chromatography; mass spectrometry; microwave-induced combustion; halogen bioavailability; PFAS speciation.

RESUMO

BALBINOT, Fernanda Pitt. **Development of methods for halogen and sulfur determination, speciation and bioavailability assessment in seafood.** 2024. 147f. Tese (Doutorado em Química) – Programa de Pós-Graduação em Química, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Pelotas, 2024.

Frutos do mar pertencem a uma das classes de alimentos mais consumidas no mundo. Dentre os elementos que podem ser encontrados na composição dos frutos do mar pode-se mencionar os halogênios e enxofre. Entretanto, o aumento da poluição de corpos hídricos prejudica a segurança e qualidade desses alimentos, assim como alterar a concentração de nutrientes e outros elementos nos seus tecidos. Assim, o objetivo dessa tese foi desenvolver novas estratégias analíticas que possibilitem realizar tais análises em frutos do mar, fornecendo a partir disso uma visão global da presença desses analitos, quais formas destes podem ser encontradas no alimento, e qual a sua fração que efetivamente pode ser absorvida pelo organismo humano. Tendo isso em vista, esta tese está dividida em três capítulos. O primeiro abrange o desenvolvimento e avaliação de parâmetros da utilização da combustão iniciada por micro-ondas combinada com a cromatografia de íons acoplada a espectrometria de massa para determinação total de Br, Cl, F, I e S em frutos do mar. No segundo capítulo, são avaliados o fracionamento e a influência de tratamentos culinários para todos analitos, bem como a fração biodisponível de F, Br e I. No terceiro e último capítulo são apresentados os resultados referentes à especiação de F, mais especificamente à determinação de compostos organofluorados, nas amostras estudadas. Ressalta-se que esses compostos são contaminantes globais emergentes de grande relevância, conhecidos como “químicos eternos” pela sua estabilidade, persistência e capacidade de acumulação no ambiente e organismos – e que estão cada vez mais presentes em alimentos, como os frutos do mar. Como principais resultados, destaca-se que, no primeiro estudo, foi desenvolvido um método analítico adequado para determinação de halogênios e enxofre em diversos tipos de frutos do mar, utilizando única análise. As recuperações variaram de 92% a 109%, considerando experimentos de adição de analito e análise de materiais de referência certificados. A partir do segundo estudo observou-se que a maior parte de Br, I e F acumula na casca/cabeça do camarão, enquanto a maior parte de Cl e S acumula no músculo do camarão, bem como diferentes influências dos tratamentos culinários para esses analitos dependendo da matriz da amostra. Além disso, verificou-se que até 63% do Br pode ser biodisponível em frutos do mar, enquanto a maior parte do I permanece na fração residual (não bioacessível/biodisponível). No último trabalho, foi desenvolvido um método para especiação de F, determinando PFAS em frutos do mar. As recuperações para 11 compostos variaram de 83 a 115%. Dez PFAS foram detectados entre todas as amostras e quatro foram quantificados. Após o desenvolvimento desta tese, foi possível apresentar, de maneira inédita, uma visão extremamente abrangente da relação existente entre elementos tão importantes para o metabolismo humano – os halogênios e o enxofre – com uma classe de alimentos que faz parte da dieta de quase todas as culturas ao redor do mundo.

Palavras-chave: cromatografia de íons; espectrometria de massa; combustão iniciada por micro-ondas; biodisponibilidade de halogênios; especiação de PFAS.

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ABBREVIATIONS

ACN	Acetonitrile
AOAC	Association of Official Analytical Chemists
CGB	Graphitized carbon black
DHA	Docosahexaenoic acid
DOC	Dissolved Organic Carbon
DPA	Docosapentaenoic acid
EDXRF	Energy Dispersive X-ray Fluorescence
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
ESI	Electrospray Ionization
FAO/UN	Food and Agriculture Organization of the United Nations
FS	Full scan
HPLC-ESI-MS/MS	High Performance Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry
IC	Ion Chromatography
IC-MS	Ion Chromatography Mass Spectrometry
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
INAA	Instrumental Neutron Activation Analysis
IS	Internal standard
ISE	Ion-Selective Electrode
IUPAC	International Union of Pure and Applied Chemistry
LLE	Liquid-liquid extraction
LOD	Limit of Detection

LOQ	Limit of Quantification
m/z	Mass/charge ratio
MeOH	Methanol
m_f	Product ion
MIC	Microwave-Induced Combustion
m_p	Precursor ion
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
MTBE	Methyl t-butyl ether
n-3 LCPUFA	n-3 Long Chain Polyunsatured Fatty Acids
NIST	National Institute of Standards and Technology
PE	Polyethylene
PFAS	Per- and polyfluoroalkyl substances
PP	Polypropylene
PSA	Primary secondary amine
PTFE	Polytetrafluoroethylene
Q1, Q2, Q3	Quadrupole mass analyzers 1, 2, and 3
RCC	Residual Carbon Content
RSD	Relative Standard Deviation
SD	Standard Deviation
SLE	Solid-liquid extraction
SPE	Solid-Phase Extraction
TBAHS	Tetrabutyl ammonium hydrogen sulphate
TMAH	Tetramethylammonium hydroxide
UAE	Ultrasound-assisted extraction

UHPLC	Ultra high-performance liquid chromatography
w.b.	Wet basis

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1. INTRODUCTION

The consumption of aquatic animals by humans began before they could even write, since these animals were abundant and easily captured without offering risks for the hunters at the same time that their composition were a high-value nutritional source.[1] However, civilizations arose and later the world became highly industrialized. As all natural resources, aquatic organisms, now known as “seafood”, have been suffering with predatory fishing practices aiming maximum profit, which leads to species extinction and habitat destruction.[2]

Then, arises the need for good and sustainable fishing practices, which take other things than profit into account, in order to minimize these issues. However, beneficial and tracked fishing are good not only for the environment, but also for human health, since the chance of food contamination is also reduced. The illegal, unsustainable, and predatory fishing do not have a quality control, been performed in polluted water bodies. The pollutants incorporated into seafood can be several chemical compounds and elements.

Among these chemicals, the investigation on the presence of halogen species and other non-metals such as sulfur in foodstuff, both from natural or anthropogenic sources, is increasing exponentially. Both in inorganic or organic forms, and depending on their concentration, these elements can impact the human metabolism on very different ways.[3, 4] In this sense, determining total halogen and sulfur and its species on highly-consumed food, such as seafood, is essential to quality control and also understand the metabolic role of these elements/compounds.

Despite been essential, this kind of analysis is not enough. It is important that multi-areas approaches take place. In this sense, combined with total determination and speciation, it is recommended assess also the bioaccessible/bioavailable fractions of the analytes, to understand how each element will be released from the food matrix, as well as absorbed and metabolized by human organism. So, the use of proper and reliable analytical tools for each aim is imperative, since there is no universal analysis method.

There are several analytical challenges regarding the determination and speciation of elements such as the halogens and sulfur, and the choice of a suitable determination technique is the first. Plasma-based techniques, such as inductively coupled plasma mass spectrometry (ICP-MS) and optical emission spectrometry

(ICP-OES, respectively), and potentiometry with ion-selective electrode (ISE) are the most common techniques for Br, Cl, F, I, and S determination.[5-13] However, plasma-based techniques which use argon plasma have important limitations regarding F determination, and determination capability issues for the determination of Br, Cl, I, and S together. On the other hand, the ISE technique is more robust, but is a monoelemental technique, impairing the determination of all analytes in routine analysis. Besides, when considering speciation analysis, the technique choice is even more critical and it is often needed the coupling of two or more techniques – the first for separating and the second for quantifying the analytes.[14, 15]

The other analytical challenge regards the sample preparation step. Aiming to further total determination, the sample preparation must provide an efficient matrix decomposition (generally by oxidizing organic matter) and at the same time stabilize the analytes in a suitable solution.[16] On the other hand, aiming to further speciation analysis, the sample preparation must avoid reagents and other conditions that may lead to species interconversion, including the organic compounds (then, oxidize the organic matter is no longer an option). In this sense, several types of extraction can be employed, with slight modifications which provide better selectivity for the group of analytes and compatibility with the determination technique.[14]

Considering all these factors, there is a need for an extensive approach on analytical tools which enable the evaluation of: i) the halogen and sulfur content, ii) what species of these analytes are present, and iii) how much of them are effectively release from the food matrix and absorbed by the human organism. So, in the present thesis, analytical strategies were explored for accomplish these aims. Among these strategies, it is possible to highlight the combination of the microwave-induced combustion (MIC) with the ion chromatography coupled to mass spectrometry (IC-MS) for total halogen and sulfur determination in seafood; *in vitro* trials for assessing halogen (Br, I, and F) bioavailability, and an ultrasound-assisted extraction method combined with high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-ESI-MS/MS) for fluorine speciation both in seafood samples and bioavailable fraction.

2. LITERATURE REVIEW

2.1. General, nutritional, and composition aspects of seafood

Officially, the “seafood” term is defined as aquatic organisms, both vertebrate or invertebrate, from sea or freshwater, both cultivated or captured – with exception to mammals, reptilians, echinoderms, jellyfishes, and seaweeds.[17] The vertebrate (with spinal cord and brain) seafood are the fishes, while the most known invertebrate seafood are the crustaceans (e.g., shrimps, lobsters, prawns, crabs) and the mollusks (e.g., oysters, mussels, scallops, squids, octopus).

The Food and Agriculture Organization of the United Nations (FAO/UN) publishes every two years a report on “The State of World Fisheries and Aquaculture” to track the main aspects of this industry. In the last report, published in 2022, there are a few key points highlighted, such as: the new production record on fisheries and aquaculture, confirming the increasing tendency pointed in the last reports; the projection of a 14% increase on edible aquatic animal production up to 2030; the continuous growth of the consumption of aquatic food in the whole world; and the negative impact of the COVID-19 crisis on the sector.[18]

According to the FAO report, it is projected that up to 2030 the worldwide trade price of edible aquatic animals will increase about 33%, however the consumption will also increase from 20 to 21.5 kg per capita per year up to 2030.[18] At the same time, overfishing and pollution are increasing, which impacts the food quality. Fortunately, the cultivated (or “farmed”) aquatic food production is taking place as the main source of seafood, which allows better quality control and more sustainable practices.[18] In this scenario, food safety and composition evaluations of this products became even more relevant nowadays.

Assessments on seafood proximal composition demonstrates that, in average, 98% of the flesh total mass is composed by lipids and crude proteins. In lower contents seafood have about 1% of carbohydrates, and < 1% of vitamins and minerals. However, the exact content of these nutrients depends on several factors, such as the species, maturity, and nutritional conditions of the animals, as well as the quality of their habitat.[19]

In fishes (vertebrates), the total content of the high nutritional value lipids, the omega-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA), which can be the eicosapentaenoic acid (EPA), the docosahexaenoic acid (DHA) and the

docosapentaenoic acid (DPA), for example, can reach up to 2.5 g/100 g. These animals also contain in their composition significant contents of vitamins A and D, minerals and trace elements (mainly Ca, Se, Zn, P, Fe, Cu, and I). On the other hand, crustaceans and mollusks can present lower n-3 LCPUFA content than fishes (up to about 0.5 g/100 g), but higher concentrations of essential minerals, such as Ca, Se, Zn, and mainly I.[17]

However, the poor quality of waterbodies, with increasing pollution, is impacting the quality of different kinds of seafood, decreasing the nutritional and health benefits in consuming them. It is possible to find reports on the changing of proximal composition of seafood (decreasing proteins, lipids, and carbohydrates contents, as well metal accumulation) in function of acidification and increasing temperatures of the ocean.[20]

2.2. Halogens and sulfur and their relation with seafood and aquatic environments

Halogens (Br, Cl, F, and I) and sulfur are present in seafood composition due to its participation in several biogeochemical cycles, which allow lifeforms on different environments. Then, they are abundant in nature, mainly in waterbodies (with exception for the sulfur, which is present in higher amounts on dry lands).[3, 21] Since the seafood is obtained from waterbodies, they are susceptible to the chemicals present on the environment, which can be incorporated to their tissues and inserted on the food chain. It is well known that seafood are considered as natural sources of inorganic forms of halogens and sulfur for human diet.[3]

However, the use of several halogenated compounds in industry is also leading to an accumulation of halogenated substances in the environment. Persistent organic forms of halogens, mainly F and Br, can be mentioned, such as high-resistance polymers, flame retardants, pharmaceuticals and cosmetics, as well as pesticides and fertilizers.[22, 23] Similar to the halogen inorganic forms, the accumulation and intake of other halogenated species is still an open field to be explored.

It is worth mentioning that each one of this elements (Br, Cl, F, I, and S) have different roles and participates by different mechanisms of the human metabolism, and its deficiency or excess may lead to health disorders.[4] Briefly, the Br can modulate the transportation, activation or inhibition of some enzymes, compete for Cl^- and I^- receptors[24, 25], as well as some brominated species has a strong sedative and

antiepileptic action.[4] The Cl have physiological functions well established on the literature, participating of the control of osmotic pressure, acid/base equilibrium and blood pH.[26]

Other elements with some known functions are I and S. The I is an essential element, which participates of the thyroid hormones synthesis and, then, its excess or deficiency are directly correlated to hypo- and hyperthyroidism.[27] The S, on its turn, is a crucial element on the synthesis of several intermediate metabolites (e.g., glutathione), indicating the role of this non-metal in the biomolecules productions and even in antineoplastics defense mechanisms.[28, 29]

Other important halogen in the biologic perspective is the F. However, compared to the others halogens, the F behavior is very different since this element is capable of incorporate to mineralized tissues into their crystalline structure.[4] So, this micronutrient in its inorganic form is directly related to human development, participating on the bones and teeth growing. However, it excess (> 6 mg per kg of body mass per day) can also promote health issues, e.g., osteoporosis, or even be lethal [30, 31], as well as its deficiency may lead to teeth demineralization or other dental problems.[32] Nevertheless, there is a class of organofluorine compounds that have received the growing interest of the scientific community: the per- and polyfluoroalkyl substances (PFAS). In this sense, in the next topic in this literature review, some aspects about PFAS and its presence in seafood will be discussed.

2.2.1. Per- and polyfluoroalkyl substances (PFAS)

By definition, the PFAS are fluorinated non-aromatic organic compounds, in which the F atoms replace the H atoms completely (per-) or partially (polyfluorinated) in the carbon chain. The most common classes of these substances are the carboxylic and sulphonic acids, existing more than 12 thousand compounds divided into 15 groups classified as PFAS.[33]

Nowadays, the PFAS are popularly known as “forever chemicals”, and they are considered global emergent pollutants of higher relevance, of environmental and health concerns. They are nearly indestructible and they are basically everywhere, contributing significantly to fluorine concentrations in soil, water bodies and atmospheric air.[34] Besides, the exposure to PFAS is toxic in the long term, since these compounds easily bound to proteins and are capable of bioaccumulate in the organisms.[34, 35]

The C-F bonds present in such compounds promotes stability, chemical and thermal, besides the impermeability and both hydrophilicity and hydrophobicity. Due to these properties, the PFAS are used in several industrial applications, from waterproofing materials to pesticides.[36] As the use of PFAS has become very common, this has led to a worrying accumulation of these compounds in the environment, especially in seafood. Various reports in the literature have shown that PFAS are present in several species of fish, crustaceans, mollusks, and other organisms that are consumed by humans worldwide.[37-42]

With this in view, health authorities have started regulating or even restricting the use of some PFAS in different industries after the detection of these compounds in environmental and food samples. Regulations are mainly found in legal texts from the United States and European Union, and usually deal with the concentration limits of PFAS in water or food intended for human consumption.[43, 44] The perfluorooctanoic acid (PFOA) and the perfluorooctanesulfonic acid (PFOS) are the most commonly used PFAS and are therefore most cited in legislation, although thousands of PFAS have been cataloged.

To comply with these regulations, short- and ultra-short-chain PFAS (C1 to C4) are used as alternatives to restricted-use compounds. These compounds possess desirable characteristics for industries such as high chemical and thermal stability, as well as impermeability. However, from an environmental and health perspective, their presence can be even more harmful because they are highly mobile and pose an even more pronounced analytical challenge than medium- and long-chain PFAS.[45]

2.3. Bioaccessibility and bioavailability of halogens (Br, I, and F)

The bioaccessibility and bioavailability of a substance or chemical element refer to the portion that is released from the food matrix into the gastrointestinal environment, and the quantity of this fraction that will be absorbed, transported through the bloodstream, and metabolized, respectively. [46, 47]. These processes are influenced by various factors. When evaluating the bioavailability of macromolecules, for example, specific properties of the compounds can become determining factors, such as molecular mass, lipophilicity, reactivity with enzymes, among others.[48] On the other hand, when evaluating elemental bioavailability, the food matrix becomes a more significant influencing factor, also including the culinary treatment variables that can be employed to prepare each food.[49, 50]

This is because when it comes to food, generally, these are materials with high levels of complexity in terms of chemical composition and which, depending on the type of food, may contain fibers, proteins, carbohydrates (among which sugars), lipids, organic acids, minerals, incorporated water, among others – constituents that can affect the release of the elements of interest during digestion.[50] Regarding seafood specifically, it is worth mentioning that this influence of the food matrix and culinary treatments on elemental bioavailability has recently been evaluated in different studies. In three of these studies, the analytes investigated were potentially toxic elements: Hg and Se,[51] Cd, Cr, Cu, and Pb,[52] and As and its species.[53] In two other works, the authors carried out this evaluation for an element of the halogen family, I.[54, 55]

The two main assays for evaluating the bioaccessibility/bioavailability of a nutrient and/or contaminant are carried out based on: *i*) *in vitro* models and *ii*) *in vivo* models.[56] Considering the scope of this thesis, where experimental animal models will not be used, this review will focus on *in vitro* simulation models of the human digestive process. It is worth mentioning that *in vitro* models present advantages over in vivo methods, such as being faster, cheaper, less complex, and having fewer ethical restrictions, besides enabling better control in the experimental conditions - resulting in better reproducibility.[47, 56-58]

In general, *in vitro* methods for assessing bioaccessibility comprise the combination of a series of inorganic and organic reagents to mimic the physiological conditions of the human organism during digestion in three main phases: oral, gastric, and intestinal.[47] When the aim is to assess bioavailability, the model must include the use of a dialysis membrane to estimate the amount that will be able to cross the barrier of the intestinal epithelium to reach the blood circulation.[59]

Among the inorganic reagents used in these models, several of them have the elements Cl and S in their composition, impairing the evaluation of their bioaccessibility/bioavailability. In this sense, considering the analytes evaluated in this thesis (halogens and sulfur), it is only possible to make these assessments for Br, F, and I.

The release of elements with the characteristics of Br, F, and I is influenced, not only by the characteristics of the food matrix but also by the pH of the digests. For example, the gastrointestinal tract normally has pH ranges of 1.5 to 2.0 (fasting) and 3.0 to 7.0 (non-fasting),[60] which directly affects the absorption of F. Studies have

shown that the solubility of this micronutrient is more efficient in an acidic environment, i.e., during fasting state.[61] Also, when the F reaches the small intestine, which has a more alkaline pH due to the release of HCO_3^-, it is mainly in its dissociated ionic form (F^-). Due to the high electronegativity of this ion, it tends to form complexes of low solubility at this stage of the digestive process, making it difficult for the body to absorb.[61, 62]

Similarly, a study found that the absorption of I in the gastrointestinal phase reached the highest value (74%) at an acidic pH of around 2.0. However, the bioaccessibility rate of I decreased drastically at pH values above 2.54, which the authors attributed to the decrease in the catalytic activity of the pepsin enzyme.[63] Another factor that affects the bioaccessibility of I is the duration of the simulation phase of both the gastric phase and the small intestine phase in the *in vitro* model. The longer the digestion time, the higher the bioaccessible fraction of I, which increases from approximately 50% to approximately 70% when the digestion time is increased from 30 to 480 minutes.[63]

It is important to note that there is limited research on the bioaccessibility of Br, F, and I in seafood, and most studies have been conducted on edible vegetables and algae. The total concentration of Br, F, and I in food does not necessarily correlate with the proportion that will be bioaccessible. The release of these elements is more associated with the characteristics of the food matrix and the optimized parameters of the digestion model used. [57, 58, 64-66]

Therefore, it is crucial to develop studies specifically evaluating the bioaccessibility/availability of these elements in seafood. Additionally, it is essential to develop reliable analytical methods that consider the characteristics of the matrix of the bioaccessible/available and residual fractions resulting from these assays for the correct quantification of the analytes.

2.4. Analytical strategies for total halogen and sulfur determination

In view of the mentioned aspects regarding seafood, the need for developing better quality control tools for monitor halogen and sulfur, as well as bioaccessible/bioavailable halogens in food is highlighted in this work. However, this is only possible if these analyses are capable of producing reliable results, which is only possible to achieve with the development of suitable analytical methods (considering all analytical sequence) for each purpose. With this in view, in this topic

will be briefly presented and discussed sample preparation methods and determination techniques reported for total halogens and sulfur determination.

2.4.1. Sample preparation

As will be discussed in the next topic, most determination techniques require that sample is in the form of a compatible solution, since direct analysis techniques have limitations with sample homogeneity, matrix effects, and calibration, making them unsuitable for complex matrices analysis. So, before the determination, the sample preparation step is often necessary. In this step, solid or semisolid samples are converted into a suitable solution, containing all the analytes to be determined and ideally without the presence of any component that could generate interferences. There is no universal method for sample preparation, and the parameters that will be suitable depend on the characteristics of the sample and the analytes.[16]

Sample preparation for total elemental determination, particularly of non-metals, is not an easy task. For samples with mostly organic composition (as seafood), the commonly used strategies for enabling the determination of metals, for example, consists in destroy the organic matrix with procedures such as acid digestion. However, this strategy is often unsuitable for determining some halogens. These elements can form volatile compounds in an acidic medium and be lost during sample preparation.[67, 68]

As alternative to the mineral acids, it is possible to use alkaline solutions, such as tetramethylammonium hydroxide (TMAH), water-soluble tertiary amines, or other strong bases for sample preparation by extraction. However, this can lead to high residual carbon content (RCC) or high concentrations of dissolved carbon (DOC),[69] which can harm the achievement of adequate detection limits or cause interference and damage to the determination equipment. As an alternative, sample preparation based on combustion reactions, along with the use of diluted alkaline solutions for non-metals better stabilization, should be highlighted. This approach allows the use of reagents compatible with the characteristics of the analytes, without loss of sample decomposition efficiency.[70, 71]

There are well-stablished combustion methods, such as the combustion bomb or the Schöniger flask. However, since its first proposition in 2004,[72] the microwave-induced combustion (MIC) has been upstanding how the state-of-art for combustion methods. The MIC system details are shown in Figure 1.

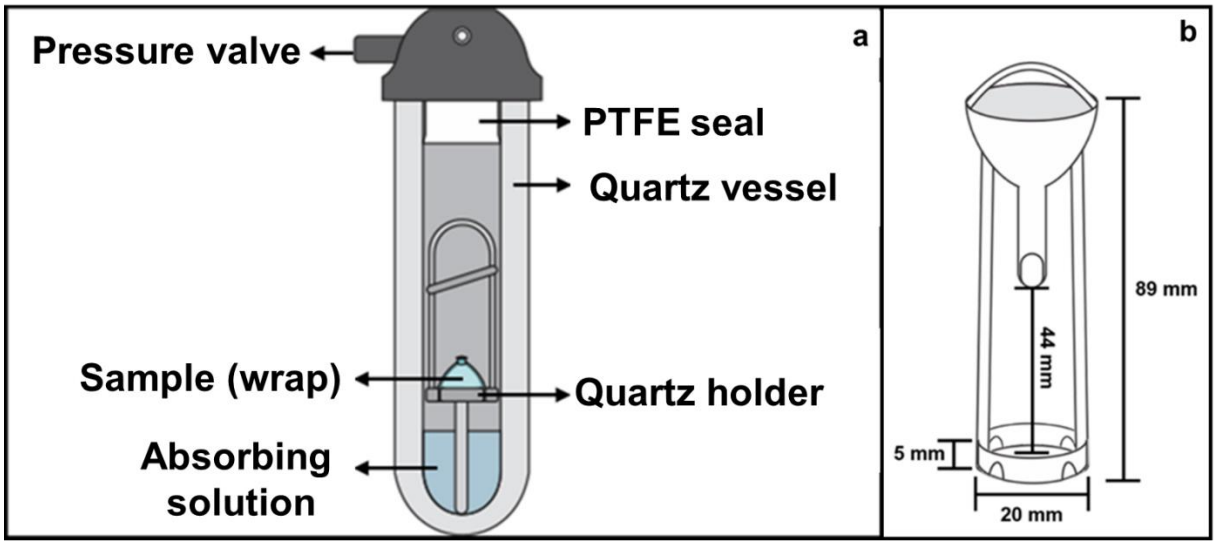

Figure 1. System used to perform the MIC method (a) and quartz holder dimensions (b). Adapted from Mesko, 2008.[73]

The combustion is a chemical reaction that involves a fuel (organic matter, as from the samples), an oxidizer (oxygen gas), and an ignition source (solution containing the NO_3^- ion interacting with microwave radiation, in the case of MIC). The general equation of combustion reactions is shown below[70]:

$$C_xH_{2y} + [O_2] \rightarrow xCO_{2(g)} + yH_2O_{(l)} + \text{Energy}$$

Complete consumption of organic matter in the reaction requires excess O_2, and the decomposition efficiency depends on the temperature reached. The closed and pressurized system used for MIC enable higher temperatures to be obtained, optimizing decomposition efficiency, even for high sample masses compared to the other combustion methods. Also, MIC contributes to decreasing the limits of detection (LOD) and quantification (LOQ) of the method by decomposing these higher sample masses and using less volume and less concentrated reagents.[72]

Another advantage of MIC is that when compared to other combustion methods, one of the main differences of this system concerns the possibility of carrying out a reflux step. This process represents a gain in the recovery efficiency of analytes in the absorbing solution. It is worth to mention that MIC is widely suitable for preparing samples for the subsequent determination of both metals and non-metals, and its digests are compatible with multiple determination techniques.[71] In face of all this features, MIC has been employed in the sample preparation of several

kind of samples, including foodstuff, for further halogens and S determination – always representing gains in terms of analytical merit figures.[10, 71, 74-81]

Other feature of MIC is its versatility, enabling the preparation of both solid or liquid samples. In this sense, this analytical tool can overcome some issues verified in the halogen bioaccessibility/bioavailability assessments. There is a lack when it comes to feasible analytical parameters in such evaluations. A few works evaluating halogen bioaccessibility/bioavailability in seaweeds or seafood used alkaline extraction with TMAH before analysis.[55, 66, 82-85] But more recently, a study evaluated the use of MIC for sample preparation after *in vitro* digestion procedure, broadly discussing it advantages when compared to the other work.[86]

2.4.2. Determination techniques

Several instrumental techniques can be used to determine the total concentration of non-metals, particularly halogens such as Br, Cl, F, and I, as well as S. These techniques are based on different chemical principles and include the spectrometric, such as X-ray dispersion fluorescence spectrometry (EDXRF), ICP-OES, and ICP-MS; the electroanalytic such as ISE, and radiochemistry-based such as instrumental neutron activation analysis (INAA). Furthermore, ion chromatography (IC) can be used for the total determination of these analytes through conductimetric detection.[67] Some of these techniques can also be used for speciation analyses, but only when combined with other determination techniques and after using a sample preparation method compatible with this type of determination.

The use of INAA technique is very restricted due to the need for a nuclear reactor and radioactive materials. It also takes a long time to provide results, and is a direct solids analysis technique – which can be challenging to ensure representativeness of the results.[87] On the other hand, ISE is a simple and low-cost electrochemical technique that can detect Br, Cl, F, and I ions, but it is monoelementary, time-consuming and requires a large sample volume – which increase the dilution factor require and, therefore, increase the LODs/LOQs.[67]

Nowadays, two spectrometric plasma-based techniques are very well-stablished for routine analysis: the ICP-OES and the ICP-MS. These techniques are multielementary, presenting high sensitivity for most elements, including halogens and non-metals like S. Their atomic/ionic, respectively, source is the same, i.e., the inductively coupled plasma, but ICP-MS separates and detects ions by their *m/z*

ratio, while ICP-OES measures the wavelengths (λ) emission from the analytes.[88] Using a plasma to promote the excitation/ionization of analytes, a high amount of energy is supplied over the sample, providing high efficiency in the number of atoms/ions generated and that arrive to the spectrometric system, contributing to the high sensitivity of these techniques.[88]

Despite these techniques present several challenges related to their use, such as the occurrence of various interferences,[89] strategies have been developed to overcome these inconveniences. Among these, it could be mentioned instrumental modifications and the use of sequence mass analyzers in ICP-MS, as well as vacuum optics purged with inert gas in ICP-OES.[89, 90] Also as strategy to overcome interferences, it also can be mentioned the correct development of a suitable sample preparation method, specific for the sample and analytes characteristics.[90]

Another limitation of ICP-based techniques is the low efficiency on excite/ionize F due to the ionization potential of this element being higher than the ionization potential of Ar. Besides, the F present a low *m/z* ratio and low emission lines (UV region), which practically impairs its determination using these techniques.[89] Few alternatives has been developed, such as the polyatomic BaF^+ ion formation before ICP-MS/MS sample introduction.[91]

When the use of ICP-based techniques is not possible, there is an alternative that has becoming more popular recently, i.e., the IC or the IC coupled to mass spectrometry (IC-MS).[78-80, 92] A brief review about IC and IC-MS will be presented in the next topic, since one of the studies developed in this thesis is specifically about a method development using this technique for sequential halogens and S determination.

2.4.2.1. Ion chromatography and ion chromatography coupled to mass spectrometry.

The IC is a liquid chromatography technique, and its mobile phase is an ionic solution. The ion-exchange IC is the most commonly employed IC, and on this technique the stationary phase is a resin activated with ionic groups.[93, 94] This enables the analyte separation with basis on the ionic exchange equilibrium. After separation, these ions are carried to the detector in different retention times.

There are various types of detectors that can be used in IC, which are based on different ionic properties. Common examples of detectors used in IC include

electrochemical (conductometric, amperometric, and potentiometric) and optical (spectrophotometric in the UV-Vis or fluorescence region). Among these, the conductimetric detector is the most commonly used for anions and cations quantification. In this detection mode, any charged species will generate a signal in the detector due to conductivity changes compared to eluent baseline conductivity.

These species are differentiated by their retention times, since the conductivity measure is not capable of differing one ion from the other.[88, 95] Also, as was mentioned, the detector has to be capable of differentiate changes in the conductivity generated by the analyte and the baseline. So, it is common the use of eluent conductivity suppressors in IC – which can be both chemical or electrolytic.[94] In Figure 2 is possible to observe the general system of an ion chromatograph.

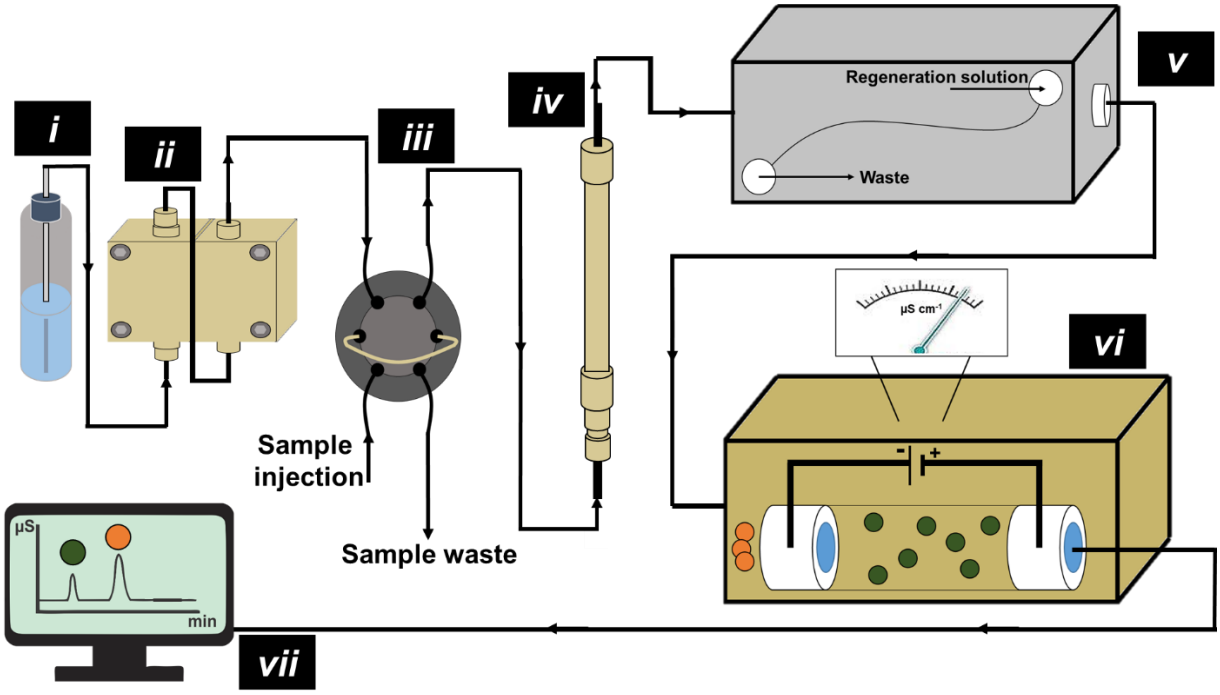

Figure 2. Instrumentation for IC with suppression and conductimetric detection. The basic components are: (i) reservoirs containing the eluent; (ii) pumping system (generally high pressure); (iii) sample injection loop and valve; (iv) column, containing the stationary phase; (v) suppressor; (vi) detector; and (vii) signal and data processing system. Adapted from Ion Chromatography, Metrohm, http://www.metrohm.com.br.

Despite the eluent suppression, the IC sensitivity for some analytes is still an issue. In some cases, this issue was overcome by more feasible sample preparation methods. However, there are some cases that better IC instrumentation is required. With this in view, a new coupling was developed, between the IC and the detection

by mass spectrometry (IC-MS). One factor that was impairing this coupling was the incompatibility between the constant liquid flow from the chromatograph and the injection into the mass analyzer. The strategy to enable this connection was the development of two ionization systems: atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI).[96] Given the technique used in this work, more details on ESI will be provided. A general representation of ESI mechanism is given on Figure 3.

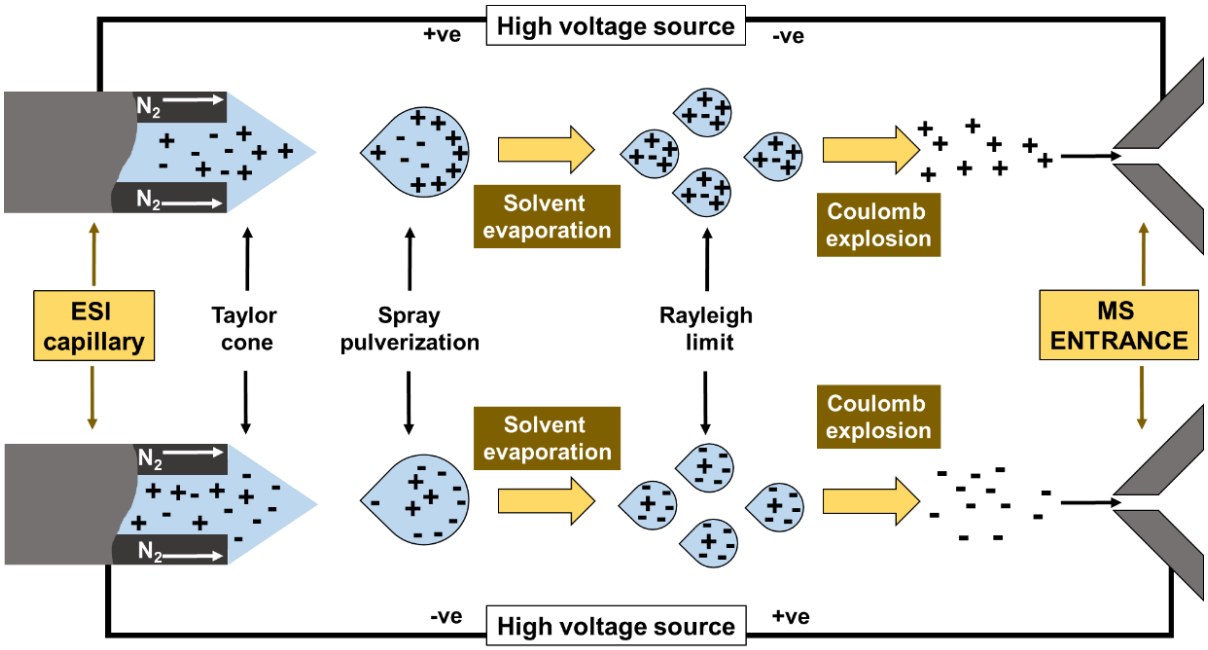

Figure 3. Schematic representation of ESI operation in positive (top) and negative (bottom) modes. Adapted from Ghosh, 2012.[97]

The ESI used electrical energy to *i*) convert the analytes into their ionic form (if they aren't already in the ionic form – which is the case in IC); and *ii*) transfer the ions in solution to the gas phase so that they can be easily injected into the mass spectrometer. There are certain factors that can impact the ESI process, such as the composition of the mobile phase and some physicochemical characteristics of the analytes, like their particle size.[98] One of the main advantages of this ionization method is that it can form both cations (positive ions) and anions (negative ions), making it highly versatile. Additionally, ESI can ionize and vaporize analytes with multiple charges. This means a greater range of analytes can be determined.[96, 99]

2.5. Analytical strategies for speciation in food

According to the International Union of Pure and Applied Chemistry (IUPAC), chemical species are defined as a specific form of an element, determined by its isotopic composition, electronic or oxidation state, and/or complex or molecular structure. Speciation analysis, on its turn, is the process that identifies and determines the different chemical and physical forms of an element existing in a sample.[100] The classification of speciation is mainly based on the type of differentiation made between the analytes. This includes isotopic speciation, redox speciation, and structure speciation, which exploit different physical and chemical properties of the analytes. These properties are responsible for the different characteristics observed in different species, such as solubility, mobility, permeability, and toxicity, among others.[15, 101]

However, carrying out speciation analyses can be challenging, especially considering the factors that influence the presence, release, and conversion of species in the environment. Determining the total concentration of non-metals, such as halogens and sulfur, is also difficult. Therefore, developing analytical tools and strategies is critical. To identify and quantify analytes, it is necessary to use different detection strategies and means of separating the analytes. The most commonly used separation techniques are chromatographic and electrophoretic. These techniques can be used with their detectors or coupled with other techniques.[14, 15]

The main factor in choosing the technique is to obtain adequate selectivity for different species, without compromising sensitivity that allows quantification at low concentrations. High selectivity is essential in speciation because analytes often differ little in their characteristics, increasing the probability of interference occurring. High sensitivity is also crucial, especially when dealing with the speciation of halogens, which are naturally present in low total concentrations in foods. The hyphenation between separation techniques, such as chromatography and electrophoresis, with a technique with better detection capacity, such as spectrometric techniques, is a suitable strategy.[14, 15]

In general, chromatography, whether liquid or gaseous, allows a level of modification of experimental parameters that makes it possible to separate an infinite number of species. However, one must pay attention to these parameters so that the conditions used do not cause or favor the interconversion of species. If the separation is adequate, the different species reach the detector separately depending on the retention time. Thus, the chromatographic technique must be

highly selective in regards to the analytes, while the detector must be highly sensitive to these species.

It is important to use different strategies for speciating analytes in the sample preparation stage than those used for determining their total content. This is because the decomposition methods used to determine total content are quite drastic and aim to completely eliminate the sample matrix.[16] However, these methods cannot be used for preparing samples for subsequent speciation. Analytes need to maintain their composition, distribution, shape, and state during their separation from the sample matrix to ensure correct evaluation of the results.[102]

For elemental speciation in solid samples, sample preparation processes such as extraction or dissolution are required. These methods are milder, which enable to avoid the interconversion of analyte species due to the action of reagents and temperature. [15] Optimization studies of analytical conditions, development of appropriate stabilization strategies, and presentation of various validation parameters are required. Strategies such as the addition of antioxidant or complexing substances and organic solvents can be used, depending on the analyte to be speciated and the sample matrix.

It is impossible to establish a universal sample preparation method suitable for the determination of all types of analytes, as is for determining their species. Therefore, several variations and adaptations can be made within the extraction and dissolution methods to make them suitable for their purpose. For example, solid-phase extraction (SPE) can use new porous materials such as nanometer-scale, ion-imprinted polymeric, magnetic, monolithic, and even biological materials as a solid phase to retain analytes and their species.[14]

This makes it possible to “customize” the method concerning properties such as adsorption capacity, selectivity, and adsorption/desorption kinetics. On the other hand, with regard to extraction using liquid solvents, it is possible to evaluate different solvents and different parameters such as process volume, time, and temperature, as well as evaluate the use of alternative forms of energy, such as ultrasound and microwave radiation.

There is a lack of reports in the literature of methods for the speciation of non-metals in foods, especially their inorganic forms. A single report was found in the literature on the speciation of inorganic forms of a halogen, iodine, in a species of seafood. In that study, the sample preparation method used for subsequent

speciation was not reported, and high-performance liquid chromatography (HPLC) coupled to ICP-MS was used as the determination technique.[54]

The vast majority of works found in the literature that differentiate halogenated species evaluate organic forms that contain at least one atom of elements from this family. For this purpose, the most used strategy is liquid extraction using solvents such as hexane, acetone, methanol, acetonitrile, among others; a clean-up stage with SPE, if necessary; and the determination of analytes using mass spectrometry after separation by liquid or gas chromatography.[103-110] The mentioned studies cover the determination of, mainly, pesticides based in brominated and chlorinated compounds.

Nevertheless, an emerging global pollutant has been increasingly determined in several samples, such as seafood – the PFAS. In Table 1 there are described the mainly methods used for these analyses.

Table 1. Analytical methods for PFAS determination in seafood.

Samples (origin location)	PFAS	Sample preparation	Determination technique	Ref.
Marine and farmed fish, crustaceans, bivalves and European eel (Netherlands)	PFBA; PFPeA; PFHxA; PFHpA; PFOA; PFNA; PFDA; PFUnDA; PFDoDA; PFTrDA; PFTeDA; PFBS; PFHxS; PFHpS; PFOS; PFDS	Fishes, crustaceans, and bivalves: sample + mass-labelled IS spiking + ACN (shaken and centrifuged). The supernatant was dried over Na_2SO_4, washed with hexane and cleaned up with activated carbon (centrifuged). The supernatant was collected and diluted with demineralized water. Eels: 1 g of sample + mass-labelled IS spiking + MeOH (pressurized liquid extraction). The cleaning up step was performed in a glass column filled with magnesia-silica gel + basic aluminum + Na_2SO_4. The PFAS were eluted with MeOH, and concentrated before analysis.	LC-MS/MS	[111]
Fish, crab, and Prawn (New South Wales – Australia)	PFBA; PFPeA; PFHxA; PFHpA; PFOA; PFNA; PFDA; PFUnDA; PFDoDA; PFTriDA; PFTreDA; PFBS; PFHxS; PFOS; PFDS; 8:2, 6:2, and 4:2 FTS; FOSA; N-MeFOSAA; N-EtFOSAA; N-MeFOSE; N-EtFOSE	1 g of samples were pre-digested with 200 mmol L^{-1} NaOH in MeOH. The extraction was performed with the digest + mass-labelled IS spiking + ACN. The clean-up step was performed using LLE with n-hexane, and subsequently the extract was submitted to clean up with activated carbon.	HPLC-ESI-MS/MS	[112]
Prawn (New South Wales – Australia)	PFBA; PFPeA; PFHxA; PFHpA; PFOA; PFNA; PFDA; PFUnDA; PFDoDA; PFTriDA; PFTreDA; PFBS; PFHxS; PFOS; PFDS; 8:2, 6:2, and 4:2 FTS		HPLC-ESI-MS/MS	[113]

Table 1 (continuation). Analytical methods for PFAS determination in seafood.

Shellfishes (France)	PFBS; PFHxS; PFHpS; PFOS; PFDS; PFHxA; PFHpA; PFOA; PFNA; PFDA; PFUnDA; PFDoDA; PFTrDA; PFTeDA	1 g of sample + mass-labelled IS spiking + 15 mL of 0.01 mol L^{-1} KOH in MeOH (shaken and left overnight). The supernatant was cleaned up using two SPE cartridges consecutively: the first with a stationary phase for weak anions exchange (elution using MeOH:NH_4OH 99.5:0.5 v/v) and the second with a stationary phase of activated carbon (elution with MeOH:acetic acid 80:1 v/v). The extract was dried under $N_{2(g)}$ and reconstituted in 50% MeOH, to which the injection IS was added previously the analysis.	UHPLC-MS/MS	[114]
Oyster, mussel, clam and other mollusks, crab, shrimp, and fishes (Bahia, Brazil)	PFDA; PFNA; PFOA; PFHpA; PFPeA; PFUnDA; PFDoDA; PFTrDA; PFTeDA; PFBS; PFDS; PFHxS; PFOS; FOSA; N-MeFOSA; N-EtFOSA; FOSAA; N-MeFOSAA; N-EtFOSAA	0.5 g of sample + mass-labelled IS spiking + ACN (sonicated, shaken, and centrifuged). The supernatant was collected and the sample was submitted to SLE again, but using 25 mmol L^{-1} NaOH in ACN (sonicated, shaken, and centrifuged). The combined supernatant was dried to 200 µL under $N_{2(g)}$ and reconstituted in 200 µL of a solution containing 20 mmol L^{-1} formic acid and 20 mmol L^{-1} ammonium formate.	UHPLC-MS/MS	[115]
Oyster, mussel, cockle, spotted babylon, squid, cuttlefish, and Pacific white shrimp (Thailand)	PFOA and PFOS	0.5 g of sample + mass-labelled IS spiking + 10 mL of MeOH (shaken at 30 °C for 90 min, sonicated at 60 °C for 30 min, and centrifuged). The supernatant was diluted in ultrapure water and pre-concentrated/cleaned up using SPE cartridges. The PFAS were eluted with 4 mL of MeOH and then with 4 mL (2x) of 0.1% NH_4OH in MeOH. The eluate was dried under $N_{2(g)}$ at 40 °C and reconstituted in 1 mL of 0.1% NH_4OH in MeOH: MeOH (1:1 v/v).	UHPLC-MS/MS	[116]
Shellfishes and crustaceans (South China)	PFBA; PFPeA; PFHxA; PFHpA; PFOA; PFNA; PFDA; PFUdA; PFDoA; PFTrDA; PFTeDA; PFHxDA; PFODA; PFBS; PFHxS; PFHpS; PFOS; PFDS; PFOSA; N-MeFOSA; N-EtFOSA; N-MeFOSE; N-EtFOSE	5 g of samples + 10 mL ACN + 2% formic acid. The mixture was purified using SPE with a C_{18} sorbent, graphitized carbon black, and $MgSO_4$. The extracts were dried under $N_{2(g)}$ at 45 ºC and then reconstituted in 1 mL of ACN:5 mmol L^{-1} ammonium acetate (1:1, v/v), which was ultrasonicated centrifuged.	UHPLC-MS/MS	[117]

Table 1 (continuation). Analytical methods for PFAS determination in seafood.

Mussels, crustaceans, shrimp, and fishes (India; Indonesia; Ecuador; Chile; Canada; Norway; Thailand; American Samoa; China; Costa Rica; Colombia; United States; Iceland; Mexico)	PFBA; PFPeA; PFHxA; PFHpA; PFOA; PFNA; PFDA; PFUdA; PFDoA; PFTrDA; PFTeDA; L-PFBS; L-PFPeS; L-PFHxS; L-PFHpS; PFOS; HFPO-DA; NaDONA; 9Cl-PF3ONS; 11Cl-PF3OUdS	5 g of sample (w.b.) + mass-labelled IS spiking + 5 mL of water/10 mL of ACN/150 µL of formic acid (shaken and centrifuged). $MgSO_4$ (6 g) and NaCl (1.5 g) were added (shaken and centrifuged again). The supernatant was collected and added to a centrifuge tube containing 900 mg of $MgSO_4$, 300 mg of PSA, and 150 mg of CGB (shaken and centrifuged). An aliquot of 1 mL of the supernatant was collected and cleaned up using a SPE cartridge. The PFAS were eluted with 4 mL of 0.3% NH_4OH in MeOH, which was reduced to 1 mL under $N_{2(g)}$ and spiked with the injection IS.	HPLC-ESI-MS/MS	[39]
Fishes, crustaceans, and mollusks (Tunisia)	11 PFCAs (C4–C14 PFCAs), 5 PFSAs (C4, C6–C8 and C10 PFSAs), 2 precursors compounds (6:2 FTS and 6:2 diPAP), and 4 emerging PFAS (NaDONA, HFPO-DA, 6:2 Cl-PFAES and 8:2 Cl-PFAES)	0.5 – 1.0 g of sample + mass-labelled IS spiking + ACN (shaken, sonicated in ultrasound bath, and centrifuged). The supernatant was collected and the procedure was repeated 2 more times. The combined supernatant was reduced to 1 mL under $N_{2(g)}$ and diluted up to 15 mL with ultrapure water. SPE cartridges were used to clean up step. The PFAS were eluted with 4 mL of MeOH and then with 4 mL (2x) of 1% NH_4OH in MeOH. The eluate was concentrated to 1 mL under $N_{2(g)}$. An additional clean-up was performed with activated carbon and acetic acid (shaken and centrifuged). The supernatant was again reduced to 200 µL under $N_{2(g)}$. Before the analysis, the injection IS was added.	Not informed	[38]

Table 1 (continuation). Analytical methods for PFAS determination in seafood.

Shrimp, clam, blood clam, oyster, conch, and fishes (Xiaoqing River, China)	PFBA; PFPeA; PFHxA; PFHpA; PFOA; PFNA; PFDA; PFUnDA; PFDoA; PFTriDA; PFTeDA; PFBS; PFHxS; PFOS; and emergent PFAS (HFPO-DA; HFPO-TrA; HFPO-TeA; PFMOAA; PF4OPeA; PF5OHxA; PFO2HxA; PFO3OA; PFO4DA; PFO5DoDA; 6:2 H-PFESA; 6:2 and 8:2 Cl-PFESA; H-PFMO2OSA; PFMO2OESA)	Sample was submitted to SLE using 10 mmol L^{-1} KOH in MeOH and, subsequently, to SPE. Target PFAS were eluted using 4 mL of MeOH and 4 mL of 0.5% NH_4OH in MeOH. The eluate was dried under $N_{2(g)}$ at 40 °C and reconstituted in 200 µL of 50% MeOH.	UHPLC-MS/MS	[118]

ACN: acetonitrile; CGB: graphitized carbon black; ESI: electrospray ionization; HPLC: high-performance liquid chromatography; IS: internal standard; LLE: liquid-liquid extraction; MeOH: methanol; MS/MS: tandem mass spectrometry; MTBE: methyl t-butyl ether; PSA: primary secondary amine; SLE: solid-liquid extraction; TBAHS: tetrabutylammonium hydrogen sulfate; UHPLC: ultra high-performance liquid chromatography; w.b.: wet basis.

As is possible to observe, there is a countless number of PFAS that can be determined in samples like seafood. Despite the differences in the sample preparation methods used, some key features common among them can be highlighted, such as the use of methanol or acetonitrile, often combined to an alkaline solution, as well as the clean-up step with activated carbon or SPE cartridges. It is also possible to find reports on the use of an alternative energy source, the ultrasound, to enhance the extraction efficiency. Regarding the determination technique used for target PFAS determination, the HPLC or UHPLC coupled to the tandem mass spectrometry using ESI is well-established. With this in view, the next two topics will cover two of these tools used in one of the chapters of this thesis: aspects about the ultrasound-assisted extraction (UAE) and about the MS/MS technique.

2.5.1. Ultrasound-assisted extraction (UAE)

In the same way that microwaves, the ultrasound energy has a broad application field on sample preparation. However, this non-conventional energy source is built of, unlike the microwaves, mechanical waves – more specifically acoustic waves, with frequency above 20 kHz up to 2 MHz. Ultrasounds above the 2 MHz present low energy, not being suitable for the require chemical processes in sample preparation methods.[119]

The key mechanism for ultrasound enhances chemical processes, such as chemical extractions, is the cavitation phenomenon that this kind of wave promote in a liquid environment. As the wave propagates through the solution, the dissolved gas begins to nucleate in bubbles, which interact with the different pressure regions created by the waves itself. After a few rarefaction and compression cycles, these bubbles achieve a critical diameter and implode.[120] This process leads to the fast and punctual release of energy, leading to high temperature (> 5000 K) and pressure (> 1000 atm) micro-spots.[119, 121, 122]

Nevertheless, this is far from a simple mechanism. Several factors may affect how the cavitation will behave and which effects it will generate. Besides the ultrasound parameters (e.g., frequency, power, and wave amplitude), cavitation effects are different in the presence of solids, varying also in function of the particle size of this solid, in different temperatures, in different pressures, and also vary according to the type of the gas dissolved in the liquid.[120]

The most common ultrasound systems are the ultrasonic bath, the probe, and the cup-horns (a hybrid between baths and probes). Regardless the system used, the parameters optimization is essential to obtain reliable and reproductible results.[16, 119, 123] Despite of this, there is a growing number of applications on ultrasound-assisted extraction for further determination of both elements and organic compounds.[119, 124, 125]

The main advantages on using ultrasound-assisted extraction methods are the enhancement of extraction efficiency without necessarily causing chemical changes in the analytes – which is very helpful for organic compounds analyses and speciation in general. Besides, it can be mentioned the reduction on volume and concentration of extraction reagents and the greater suitability for thermolabile/sensitive analytes.[126]

2.5.2. Tandem mass spectrometry (MS/MS)

As mentioned before, the coupling between separation and detection techniques is one of the most effective ways to identify and determine different species, as is the case of the HPLC-ESI-MS/MS technique. However, more attention will be addressed to the tandem mass spectrometry (MS/MS) in this topic. The mass spectrometry consists in measuring charged molecules or atoms according to their *m/z* ratio and abundance. For this, a mass spectrometer consists basically of an ion source, a mass analyzer, and a detector.[127]

Nowadays, there are several types of mass analyzers, based on different principles and designed for different applications. The most common are quadrupoles, time-of-flight, magnetic sector, and orbitrap, among others.[127] When two stages of mass analysis take place, i.e., sequential mass analyzers are used, it can be named as tandem mass spectrometry. The MS/MS is often performed with a step to dissociate ions or promote chemical reactions before the second mass analyzer.[128] This assembly can be designed in two forms: in space, when two different instruments are coupled; or in time, when a sequence of events is set in one device.[127]

The most employed MS/MS use linear quadrupoles in sequence, also known as triple quadrupole, a type of tandem MS in space. It is important to mention that the following considerations regards the analyses of organic molecules. Using the triple quadrupole configuration, there is some different scans modes that can be used. In

general lines, the first analyzer (Q1) is responsible for isolate or monitor the precursor ion (m_p), the second (Q2) is the space where collisions happen, and the third (Q3) is responsible for monitoring the product ions (m_f).[127, 128]

Taking into consideration the tools used to develop part of this thesis, more attention will be addressed to two scan modes: full scan (FS) and multiple reaction monitoring (MRM). A general scheme of FS and MRM modes can be observed in Figure 4.

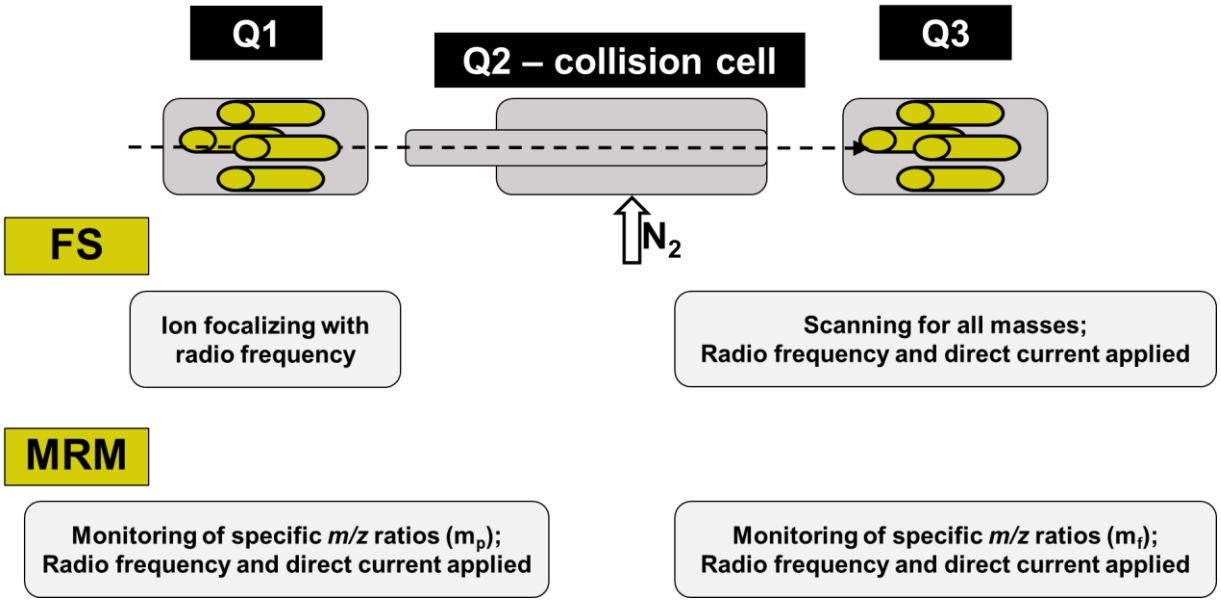

Figure 4. Schematic representation of Full Scan (FS) and multiple reaction monitoring (MRM) modes in triple quadrupole MS/MS configuration.

As is possible to observe, in the FS mode, the Q3 is used to scan and identify all masses present in the analyzer, which is useful to identify unknown compounds and components in a mixture. On the other hand, the MRM mode is more sensitive and more useful for target analysis, when the main transition reactions of the analyte are known. In the Q1, the specific *m/z* of the precursor ion is monitored, while the Q2 monitors the *m/z* of the main or the two main product ions from that molecule.[127, 128]

After covering the main theoretical aspects of analytical tools used in the development of the present work, the next chapters will be dedicated to present the main results obtained during the thesis project execution.

CHAPTERS

The results obtained in the development of this thesis are divided into three chapters and presented in the form of three papers. The sections: Introduction, Materials and Methods, Results and Discussion, Conclusions and References are found in each chapter, representing this study in its entirety. The studies are structured according to the rules of scientific journals.

3. CHAPTER 1

The paper "*Can minor aspects of sample preparation have major impacts on the reliability of analytical methods? A study for Br, Cl, F, I, and S in seafood*" show the results of a method development for total halogen and sulfur determination, which are organized in the following items: introduction, materials and methods, results and discussion, references, and supporting information. This article is been submitted to the *Talanta* journal.

Can minor aspects of sample preparation have major impacts on the reliability of analytical methods? A study for Br, Cl, F, I, and S in seafood

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Abstract

Background: The interest in assessing the presence of some chemical elements, such as halogens and sulfur, in seafood has been increasing. In this work, a novel analytical method was developed, enabling the determination of Br, Cl, F, I, and S in a single analysis in shellfish (brown-mussel, *Perna perna*), common octopus (*Octopus vulgaris*), and fish (argentine hake, *Merluccius hubbsi*), using ion chromatography coupled to mass spectrometry (IC-MS). **Results:** It was observed that sample pre-treatment by oven-drying at 100 °C can cause F losses by volatilization, compared to other drying methods, such as oven-drying at 60 °C and freeze-drying. Also, the pH of the digests demonstrated is not the main factor affecting halogen stabilization after MIC, and other factors must be considered, such as the concentration of the several analytes and the absorbing solution composition. Microwave-induced combustion (MIC) provided suitable recoveries (92% to 108%) for all analytes using 250 mmol L^{-1} NH_4OH as the absorbing solution when combined with IC-MS determination. The trueness of the proposed method was also evaluated by analyzing standard reference materials (SRMs NIST 1566a and 2976, oyster and mussel tissues). Results did not present statistical differences (Student's t-test, confidence level of 95%) compared to the certified/reference values (agreements varying from 95% to 109% for all analytes). Analyte concentrations varied in a large range: 7 to 181 mg kg^{-1} for Br; 1285 to 24,176 mg kg^{-1} for Cl; 5 to 34 mg kg^{-1} for F; < 3.3 to 7 mg kg^{-1} for I; and 7917 to 17,409 mg kg^{-1} for S. **Significance and Novelty:** The proposed method is a novel and reliable analytical tool and, at the same time, new insights on the influence of solution pH and drying method on the sample preparation step were provided.

Keywords: halogens; sulfur; ion chromatography; mass spectrometry; microwave-induced combustion; seafood.

1. Introduction

Seafood is a high-quality source of essential nutrients such as proteins, vitamins A and D, n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA), calcium, selenium, and iodine [1]. According to the last report published by the Food and Agriculture Organization of the United Nations (FAO/UN), seafood represents a large economic segment, since global fisheries production, both farmed and captured, reached almost 180 million tons in 2018. Out of this, 88% was destined for human consumption. This means an average consumption of 20 kg per capita per year, with an increasing rate of 3% per year worldwide, representing 17% of animal protein intake [2].

Given the relevance of this food matrix and the fact that it comes from natural ecosystems, the interest in better understanding how the presence of chemical elements and their species in the environment may affect the elemental composition of seafood is increasing constantly. Among these elements, the halogens (Br, Cl, F, and I) and S are of special concern. All of them play important metabolic roles in the human organism: Br and I are closely related to thyroid regulation, and the first also participates in the activation/inhibition of some enzymes [3-5]; Cl and S have well-established metabolic roles, the first in osmotic pressure and blood pH regulation, and the second in protein conformation as a component of several amino acids [6, 7]; F, in turn, has a unique biochemical behavior, being incorporated to mineralized tissues, such as bones and teeth [3].

Halogens and sulfur can be present in aquatic environments both naturally, participating in several biogeochemical cycles, as well as a result of anthropogenic activity, such as in the release of persistent pollutants [8]. Although studies already demonstrated the capability of aquatic organisms to bioaccumulate toxic and potentially toxic elements, such as As, Cd, Hg, Sn, and Pb [9, 10], no evidence was found in the literature on whether this is valid for halogens and S. However, depending on their concentration and chemical form in the aquatic

environment, it may be possible for these elements to be incorporated into seafood tissues and be ingested by humans – since aquatic organisms are considered a natural source for a few elements of this class in the human diet [8].

In this sense, the need for suitable analytical tools that enable the determination of the presence and concentration of elements such as halogens and sulfur in seafood is highlighted. In the past 10 years, the majority of studies regarding the presence of inorganic halogens and sulfur species in seafood were performed mainly for Br and I [11-20], while a few others reported Cl, F, and S determination [21-27]. None of the analytical tools employed in such cases enabled the determination of all these analytes in a single analysis, as is the case of the present study.

Most of those studies reported the determination of these analytes by spectrometric techniques, such as plasma-based mass spectrometry (ICP-MS) or optical emission spectrometry (ICP-OES), after sample preparation by extraction methods [13, 15, 16, 18-20]. The use of alkaline extraction procedures for further non-metals determination is understandable, since acid media in some cases may cause losses of a few halogens by volatilization [28]. However, the use of these non-destructible methods for sample preparation does not provide the elimination of the sample matrix. It is well-known that the presence of organic matter in the sample solution that will be analyzed may cause some inconveniences in the determination step and impair the accuracy of the results [29]. Among the published protocols for halogen determination in seafood, only a couple of them reported the use of a complete decomposition method, microwave-induced combustion (MIC) [11, 14], which may overcome the above-mentioned issues. However, the analytes in those cases were only Br and I, and one sample only was evaluated (shrimp).

In this sense, is necessary to evaluate the suitability of the MIC method for the determination of more analytes and be used for a wider scope of seafood samples. Recently,

the combination of the MIC with a chromatographic determination technique, the ion chromatography coupled with mass spectrometry (IC-MS), has been proposed to enable the determination of more analytes (halogens, such as Br, Cl, F, and I, as well as S) in a single analysis, presenting very suitable analytical figures of merit, in food matrices [30-32]. Despite that, it is worth mentioning that F determination is still a special challenge, due to the coelution of a few organic ions in close retention times. In this sense, in this work, a novel analytical method was developed and optimized, combining MIC and IC-MS (including an optimized elution gradient) for the determination of total halogens and sulfur in seafood. Besides, for the first time, the influence of the pH of the obtained solution after the sample preparation step, as well as the influence of the sample pre-treatment employed, were also evaluated.

2. Materials and Methods

2.1. Instrumentation

The separation of the ionic forms of Br, Cl, F, I, and S was performed through an analytical column (model IonPac^{TM} AS11-HC, 4 μm, 2 × 250 mm, Dionex/Thermo Fisher Scientific, USA) with an ion chromatograph (model ICS-5000+, Dionex/Thermo Fisher Scientific). A guard column (model IonPac^{TM} AG11-HC, 4 μm, 2 × 50 mm, Dionex/Thermo Fisher Scientific), a KOH eluent generator cartridge (EGC 500 KOH), and an electrolytic self-regenerating suppressor with a 2 mm membrane were also used in this system. A mass spectrometer (model MSQ Plus, Dionex/Thermo Fisher Scientific) equipped with a single quadrupole mass analyzer and electrospray ionization was used as the detector for Br and I. Operational conditions were further described in section 2.5.

The MIC procedures were performed on a microwave cavity oven (Multiwave 3000^{TM}, Anton Paar, Austria), using a setup of eight quartz vessels (80 mL internal volume, 80 bar

maximum pressure, 280 °C maximum temperature) and quartz holders to contain the samples. As a combustion aid for the MIC method, small discs of filter paper (15 mm diameter, 0.5% ash content, Prolab, Brazil) were used, as well as polyethylene (PE) films (8 x 8 cm) for wrapping the samples.

The sample pre-treatments (oven drying at 60 °C and 100 °C, and freeze-drying at -55 °C) were performed in a conventional oven (400/2ND, De Leo, Brazil) and a freeze-dryer (L101, Liobras, Brazil). The grinding of the samples was performed using a knife mill (226/2, Lucadema Científica, Brazil), and weighing was performed in an analytical balance (AY220, Shimadzu, Philippines).

2.2. Reagents and samples

The water used in all experiments and decontamination processes was the ultrapure type, ≥ 18.2 MΩ cm, obtained through a purification system (Simplicity UV®, Millipore, Merck KGaA, Germany). All concentrated reagents used were of analytical grade or higher. For the cleaning of sample preparation vessels and other materials, 14 mol L^{-1} HNO_3 (Synth, Brazil) was used. Absorbing solutions, i.e., ammonium hydroxide (NH_4OH) and ammonium carbonate ($(NH_4)_2CO_3$) in several concentrations, were prepared in ultrapure water by diluting 27% m v^{-1} NH_3 (Synth) and by dissolving the $(NH_4)_2CO_3$ (Synth) salt. The igniting solution, ammonium nitrate (6 mol L^{-1} NH_4NO_3, Merck), was prepared by the dissolution of the respective salt in water. For MIC procedures, oxygen gas (O_2, White Martins, Brazil) with 99.96% purity was used for vessel pressurization.

Standard stock solutions containing the analytes were obtained by dissolution of sodium chloride (NaCl, Dinâmica, Brazil), sodium sulfate (Na_2SO_4, Synth), potassium bromide (KBr, Synth), sodium fluoride (NaF, Merck), and potassium iodide (KI, Synth). Standard calibration solutions (1.0, 2.5, 5.0, 7.5, 10.0, and 12.5 mg L^{-1} of Cl^- and SO_4^{2-}; 10, 25, 50, 75, 100, and

140 150 µg L^{-1} of F^-; and 25, 50, 75, 100, 150, and 250 µg L^{-1} of Br^- and I^-) were obtained by
141 diluting the standard stock solutions in water. The salts were also used for obtaining the
142 solutions for standard addition and recovery tests.

143 The seafood samples were purchased in a fisheries market in Pelotas, RS, and
144 Florianópolis, SC, Brazil. The animal species used in this work are brown mussel (*Perna*
145 *perna*, sample M1, 1 kg unshelled and frozen), common octopus (*Octopus vulgaris*, sample
146 O1, eight tentacles, frozen), and argentine hake (*Merluccius hubbsi*, sample F1, eight fillets).
147 The moisture and ash contents were determined by the gravimetric method [33] in triplicate.
148 The standard reference materials (SRMs) NIST 1566a (Oyster tissue) and NIST 2976 (Mussel
149 tissue) were analyzed by the proposed method for accuracy evaluation.

150

151 2.3. Sample pre-treatments

152 Three sample pre-treatments for moisture removal were evaluated: *i*) oven drying at
153 60 °C (OD60); *ii*) oven drying at 100 °C (OD100); and *iii*) freeze-drying at -55 °C and -760
154 mmHg (FD). For OD60 and OD100 evaluations, samples were homogenized with a blender
155 and placed on Petri dishes, oven-dried for 48 h and 24 h, respectively, and then milled. For the
156 freeze-drying procedure, samples were frozen at -40 °C, fractioned in polypropylene (PP)
157 flasks, and then freeze-dried at -55 °C (for 32 h in total, 8 h day^{-1} for 4 days), and further
158 milling.

159 The analyte recovery tests were performed by adding 100 µL of a standard solution
160 containing F^- (48 mg L^{-1}, a final concentration of 8 mg kg^{-1}) to the octopus' sample
161 (homogenized with a blender, 20 g of the sample for each evaluated temperature) before
162 oven-drying at 60 °C (48 h) and 100 °C (24 h). After drying, each fraction of the sample was
163 then milled and analyzed by the proposed method (MIC, employing the optimized absorbing
164 solution, 250 mmol L^{-1} NH_4OH, followed by IC-MS).

165

166 2.4. Seafood sample preparation by MIC

167 The sample masses used in the sample preparation method were evaluated for each
168 sample matrix, from 100 to 700 mg for mussels and octopus, and from 100 to 900 mg for fish.
169 The samples were inserted into the MIC system after weighing on polyethylene (PE) films,
170 which were wrapped and sealed by heating. Then, the samples contained inside the PE wraps
171 were placed on quartz holders, above a filter paper disc previously moistened with igniting
172 solution (50 μL of 6 mol L^{-1} NH_4NO_3). These holders were then positioned in the quartz
173 vessels, which contained 6 mL of absorbing solution (NH_4OH or $(NH_4)_2CO_3$ in concentrations
174 of 100, 150, or 250 mmol L^{-1}). The vessels were pressurized with 20 bar of O_2. The irradiation
175 program used was the following: *i*) 1400 W/5 min (combustion and reflux); ii) 0 W/20 min
176 (cooling). The obtained digests were diluted up to 15 mL and conditioned in PP flasks for
177 further analysis.

178

179 2.5. Halogen and sulfur determination by IC-MS

180 The IC-MS technique provides the possibility of the separation and quantification of Br,
181 Cl, F, I, and S in a single analysis. However, in some cases, it is necessary to optimize the
182 elution conditions in order to accomplish suitable separation for all analytes. In this work, the
183 separation of the F^- required improvement, without impairing the separation of the other ions.

184 To achieve this condition, firstly the optimal temperature of the separation column was
185 evaluated, then the KOH concentration gradient was studied. Temperatures ranging from
186 26 °C to 40 °C (26, 28, 30, 32, 34, 36, and 40 °C) were applied in the column oven, and the
187 same KOH concentration gradient, previously defined, was used in all runs [34].

188 After choosing the temperature condition to be applied during the chromatographic run,
189 three gradients were tested, varying the KOH concentration until the achievement of suitable

separation of the analytes, in the shortest time possible. Fig. 1 displays the evaluated gradients.

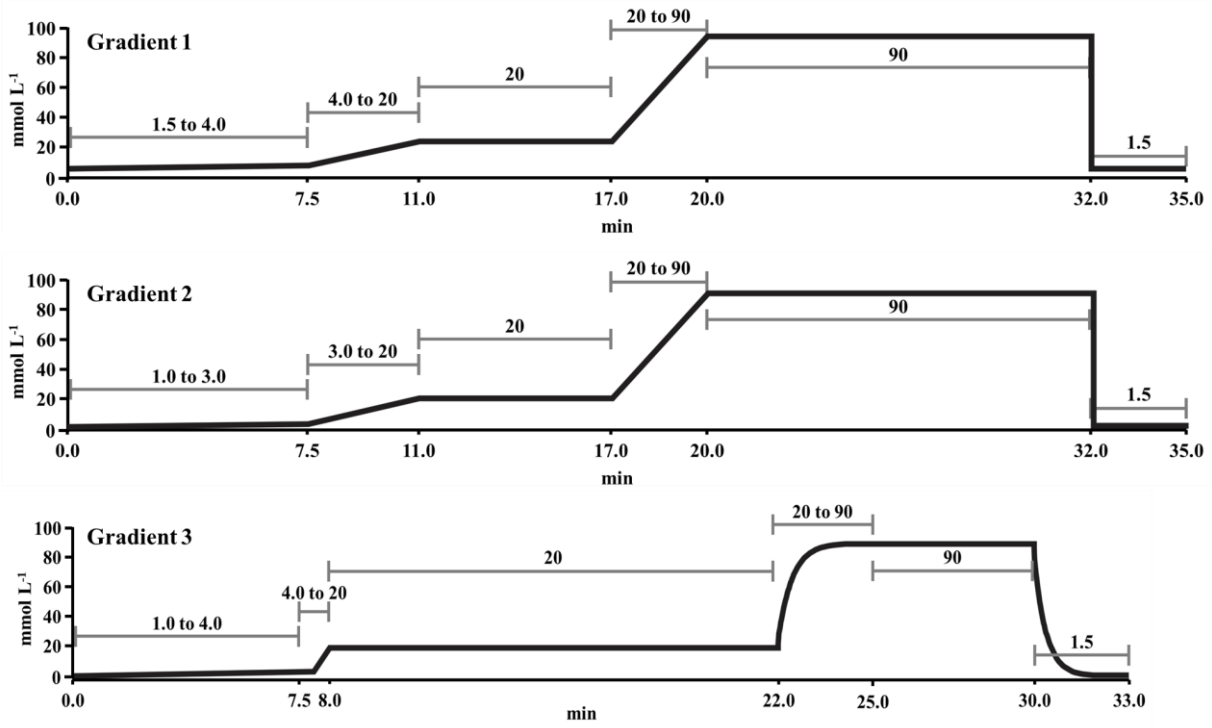

Fig. 1. Evaluated eluent gradients (KOH) for halogen and sulfur separation in MIC digests.

Table 1 describes other operational conditions used for halogen and sulfur determination by IC-MS. The mass spectrometer conditions were previously optimized [34]. Before sample injection into the system, polytetrafluorethylene (PTFE) microfilters (0.22 μm pore diameter, Unifil, Brazil) were used for filtering the solutions obtained after MIC.

Table 1. IC-MS operational settings for Br, Cl, F, I, and S determination in MIC digests.

Parameter	**Value**	
Eluent (mmol L^{-1})	KOH (1 to 90)	
Flow (mL min^{-1})	0.28	
Column temperature (°C)	36	
Injection volume (μL)	50	
Detection	*Conductivity detection*	*Mass detection*
	Cl^-, F^-, and SO_4^{2-}	^{81}Br and ^{127}I
	Suppression: electrochemical	Electrospray ionization (ESI)
		Probe temperature: 600 °C
		Needle voltage: 4.2 kV
		Cone voltage: 132 V

Limits of detection (LOD) and quantification (LOQ) of the method were obtained by calculating the average of the blank values plus three and ten times the value of the standard deviation for 10 blank replicates, respectively. The final solution volume and the dilution factor were also taken into account when necessary [35]. In the case of the analytes that were not detected in the blank solutions, the LOQs were calculated using the signal obtained for the first point in the calibration curve in ten chromatographic runs to obtain the average and standard deviation of the concentration value through the linear calibration equation. The final solution volume, dilution factor, and sample mass were also considered.

2.6. Trueness evaluation

In order to identify the most suitable absorbing solution, analyte recovery experiments were performed at low (S1, up to 25% of analyte concentration), intermediate (S2, up to 80% of analyte concentration), and high (S3, up to 200% analyte concentration) levels, using the M1 sample (oven-dried at 60 °C). These three addition levels were chosen to evaluate if the absorbing solutions could provide suitable recoveries in a wide range of analyte concentrations.

For S1, S2, and S3 recovery experiments, standard solutions containing Br^-, Cl^-, F^-, I^-, and SO_4^{2-} were added (100 µL) on the samples inside the PE wraps, before MIC. The final concentration (mg kg^{-1}) of the analytes added, concerning their concentration determined in the sample without standard addition, represented: *i*) in S1 level, 5% (Cl^-, I^-, and SO_4^{2-}), 10% (Br^-), and 25% (F^-); *ii*) in S2 level, 20% (Cl^- and SO_4^{2-}), 50% (Br^-), and 80% (F^- and I^-); and *iii*) in S3 level, 50% (SO_4^{2-}), 85% (Cl^-), 100% (Br^-), and 200% (F^- and I^-).

After choosing the absorbing solution that provided the most suitable recoveries, the method accuracy was evaluated by: *i*) performing the S1 and S3 spikes for a different sample

(F1, also submitted to OD60 pre-treatment); and *ii*) SRM analysis. The SRMs (tissues of oyster and mussel matrices) were submitted to the proposed method (MIC + IC-MS), using the optimized conditions. The obtained results were compared statistically with the certified/reference values for all analytes.

Statistical treatments were performed by using unpaired Student's t-test and Tukey one-way ANOVA test, both on a confidence level of 95% ($p > 0.05$). The software GraphPad Prism^{TM} (GraphPad Software Inc., Version 8.0.1, 2018) was used for statistical analyses.

3. Results and Discussion

3.1. Improvement of the separation and quantification of halogens and sulfur employing the IC-MS technique

In a previous study, in which Br, Cl, F, I, and S were determined in a human hair by IC-MS, a concentration gradient for the mobile phase was proposed [34]. However, matrix interferences caused coelution near the retention time of F, impairing its determination in several samples, as well as the achievement of lower LOQs [30-32, 34]. In that case [34], in order to obtain a satisfactory separation between the F and interferent peaks, high dilution factors had to be applied to the samples, which caused higher LOQs, with the interference independent of the sample preparation method.

In this sense, chromatographic conditions were evaluated in this study, aiming to improve F determination along with the other halogens, and sulfur. Firstly, different temperatures (26, 28, 30, 32, 34, 36, and 40 °C) were evaluated for the column oven, using the KOH eluent gradient optimized in the previous study: *i*) 5 mmol L^{-1} (0 to 4 min); *ii*) 5 to 30 mmol L^{-1} (4 to 8 min); *iii*) 30 to 10 mmol L^{-1} (8 to 13 min); *iv*) 10 mmol L^{-1} (13 to 19 min); *v*) 10 to 90 mmol L^{-1} (19 to 22 min); *vi*) 90 mmol L^{-1} (22 to 33 min); *vii*) 90 to 5 mmol L^{-1} (33 to 35 min) [34].

The chromatograms for a seafood digest (M1 sample) obtained after MIC using different column oven temperatures are demonstrated in Fig. 2. The red arrows indicate the F^- peak. As can be observed, the higher the temperature, the more apparent the tendency towards an improved separation between the F^- and the interferent. However, temperatures higher than 36 °C impaired the signal acquisition for this analyte.

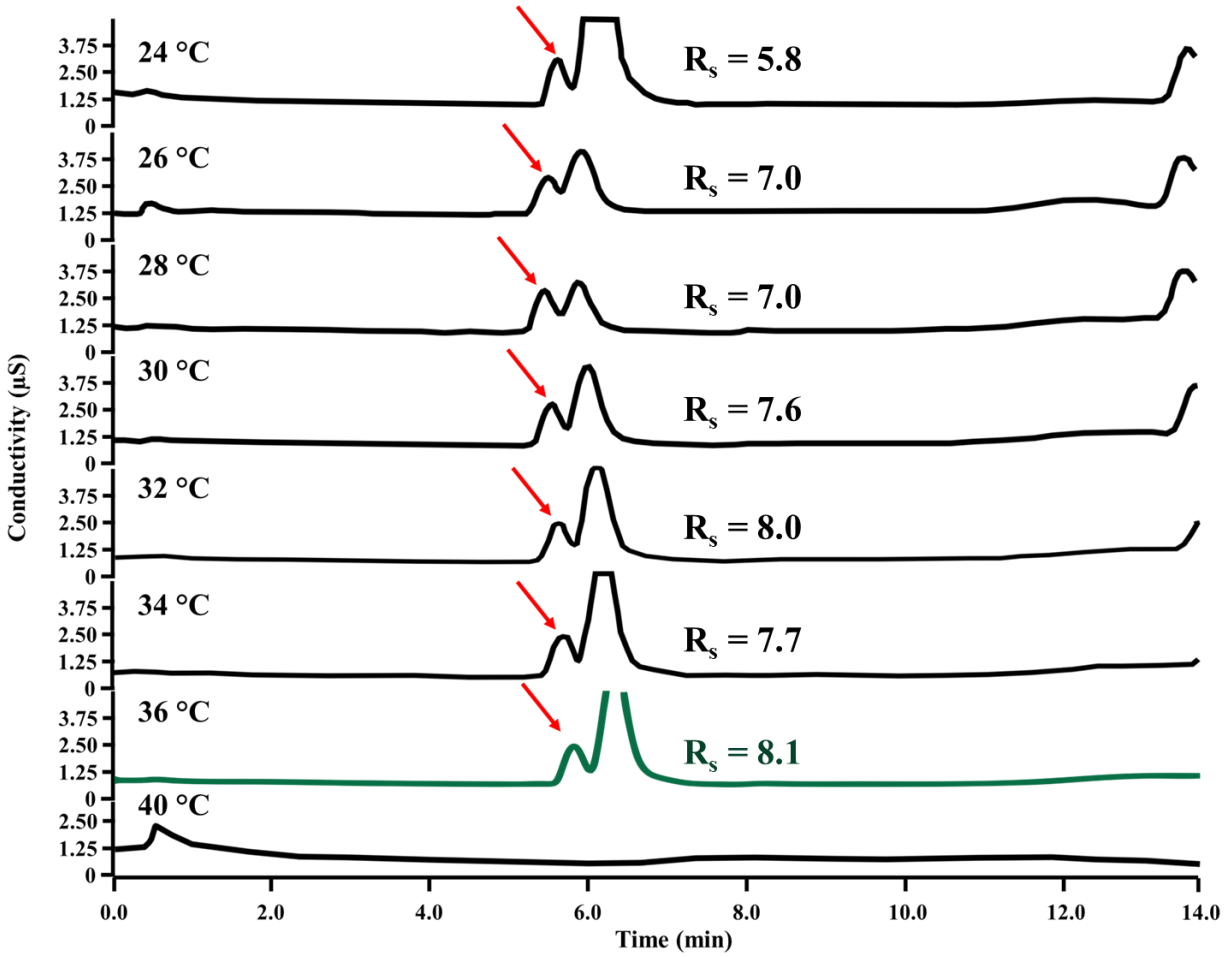

Fig. 2. Chromatograms obtained (0 to 14 min) for the seafood digest (150 mmol L^{-1} NH_4OH, three-fold dilution) by applying different temperatures in the column oven and using the previously optimized KOH concentration gradient. Red arrows indicate the F^- peak in each run. The separation resolution (R_s) is indicated in each run and the selected condition is highlighted (▬).

Based on the chromatograms, 36 °C was selected as the column oven temperature for F^- separation. Nevertheless, even after temperature optimization, peak resolution was not ideal, and higher temperatures in the column oven did not enable the separation of fluoride. Then,

other gradients of eluent concentration were evaluated as an alternative to overcome this issue. These gradients are described in section 2.5.

In Fig. 3, it is possible to observe the peak profiles in the chromatograms obtained after the analysis of the same sample digest obtained after MIC (absorbing solution composed of 150 mmol L^{-1} NH_4OH, ten-fold dilution factor), employing each of the described gradients, as well as the gradient used in previous work [34]. The best separation condition for F determination was achieved using Gradient 3. Fig. 4 shows the comparison between the chromatogram of the seafood sample, obtained by employing the optimized conditions (36 °C in the column oven, Gradient 3 of KOH concentration), and the chromatogram obtained with the analysis of a standard solution containing F^- (25 µg L^{-1}) in the same conditions.

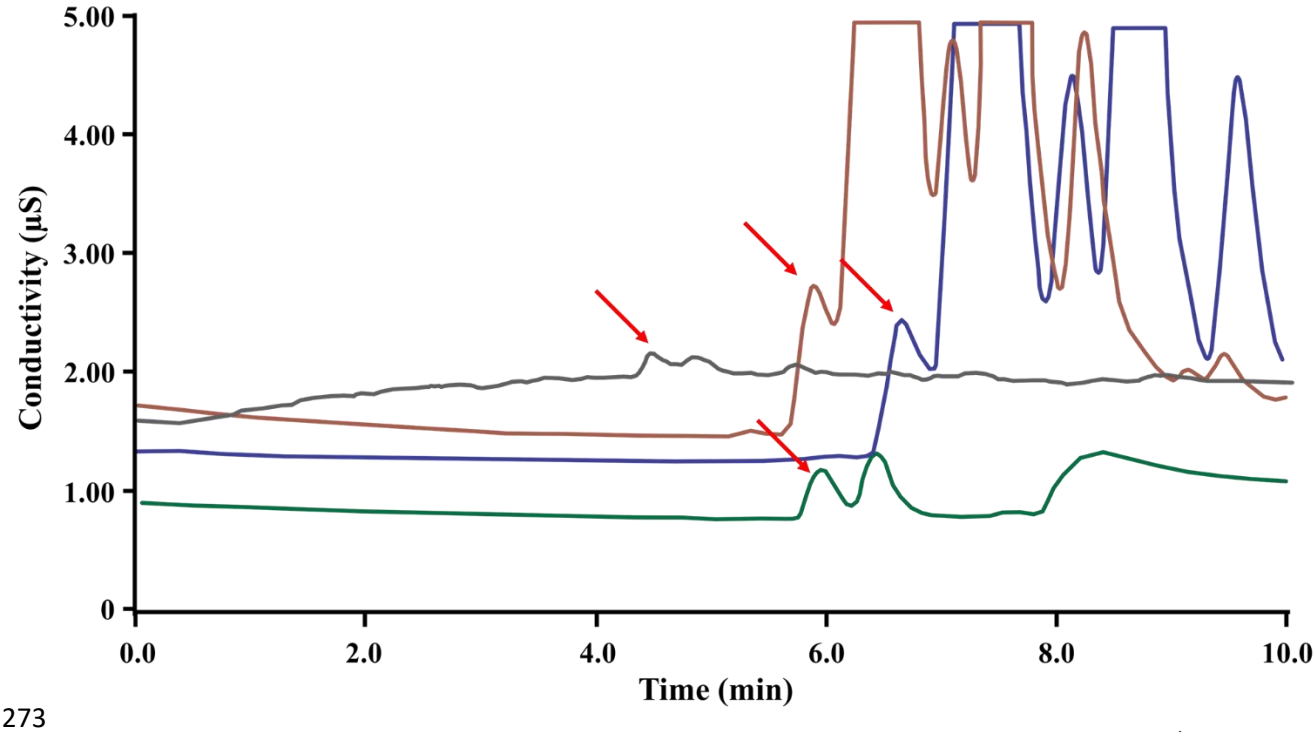

Fig. 3. Chromatograms obtained (0 to 10 min) for the seafood digest (150 mmol L^{-1} NH_4OH, ten-fold dilution) by applying the optimal temperature condition (36 °C) and using KOH concentration gradients 1 (▭), 2 (▭), and 3 (▭), as well by applying the KOH concentration gradient of previous work (▭) [34]. Red arrows indicate the F^- peak in each run.

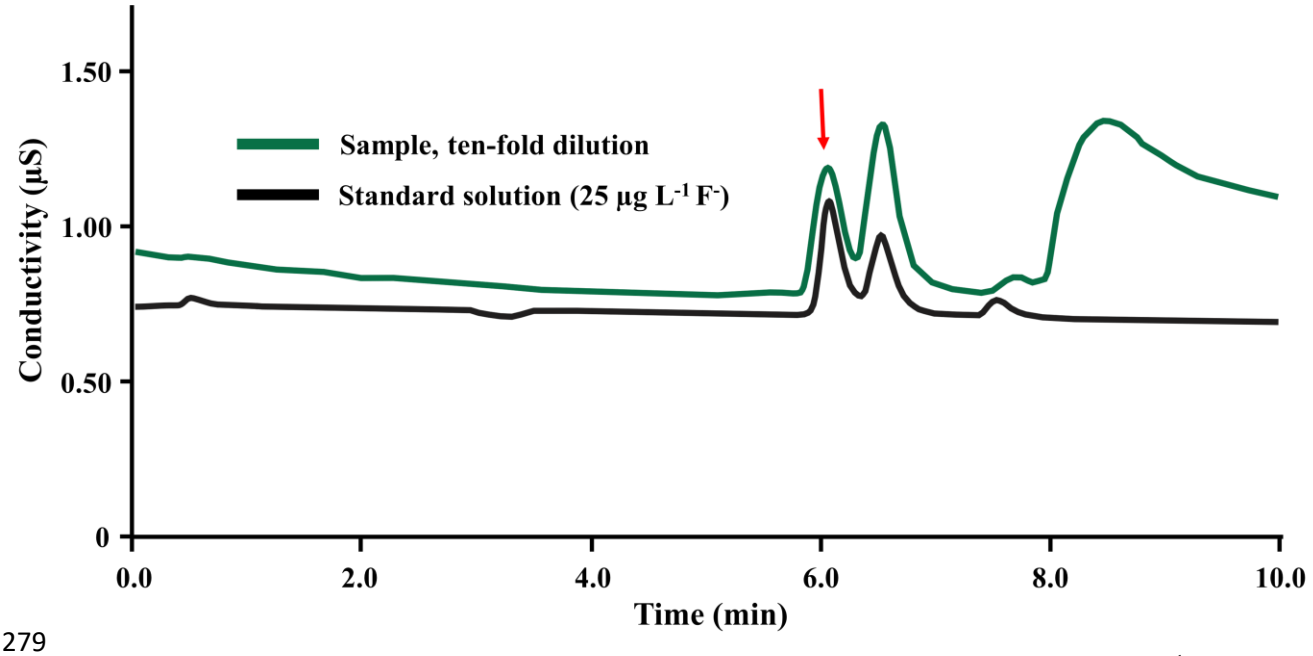

Fig. 4. Chromatograms obtained (0 to 10 min) for the seafood digest (150 mmol L^{-1} NH_4OH, ten-fold dilution, ▬) and for a standard solution containing 25 µg L^{-1} F^- (▬), by applying the optimized conditions (column oven temperature of 36 °C and Gradient 3 for KOH concentration). The red arrow indicates the F^- peak in both runs.

Comparatively, the main differences between the gradient optimized in previous work [34] and the new optimization were: a) in the first 7.5 min, initial KOH concentration was decreased (from constant 5 mmol L^{-1} to a ramp from 1 to 4 mmol L^{-1}); b) the KOH concentration from 8 to 20 min, which was kept constant; c) the last step, in which the final KOH concentration was decreased (from 5 mmol L^{-1} to 1.5 mmol L^{-1}); and d) the total time of the chromatographic run, which was reduced from 35 to 33 minutes. Moreover, the optimization of the initial eluent concentration was critical to enable the suitable determination of F^-.

In order to enhance the separation resolution for the F^- peak, the initial constant eluent concentration (5 mmol L^{-1} KOH) was replaced with a ramp gradually increasing from 1 to 4 mmol L^{-1} KOH. It is worth mentioning that this replacement did not compromise the total analysis time, once from 8 to 22 min, the KOH concentration was kept constant at 20 mmol L^{-1}, which is a higher eluent concentration than that used in the former gradient in the same

297 analysis period. This higher concentration in this point of the run promoted a faster elution of
298 some analytes, but without impairing their separation.

299 The main interference observed in this stage of the chromatographic run was the
300 overlapping between the peaks of SO_4^{2-} and NO_3^- ions. In the former method, this was the
301 reason for using lower eluent concentration from 8 to 22 min, as mentioned [34]. However, in
302 the present work, the peak generated for the NO_3^- (not an analyte in this case, but present in
303 MIC digests due to the use of NH_4NO_3 as igniter) did not interfere significantly in the
304 quantification of the SO_4^{2-}, as demonstrated in Fig. A.1, provided in Supplementary Material.
305 Probably, this interference did not occur in the analysis of seafoods once the SO_4^{2-}
306 concentration in those samples is significantly high (as will be seen in further sections) and
307 the signal generated for this ion was higher than the nitrate signal.

308

309 3.2. Optimization of the analytical method for Br, Cl, F, I, and S indirect determination in
310 seafood using IC-MS after MIC

311 Before to evaluate the conditions to be used in the sample preparation by MIC, the
312 proximal composition (moisture and ash) was determined. The knowledge about the moisture
313 and ash content in the samples is absolutely relevant in the optimization of the sample
314 preparation step using the MIC method. Based on this information, the analyst can establish
315 the best parameters for the sample pre-treatment and preparation steps, such as the drying
316 method and the need for using organic combustion aids, e.g., microcrystalline cellulose [32].

317 The moisture contents were about 77% (mussel), 84% (octopus), and 82% (fish). These
318 values can be considered high, but also expected, given the taxonomic characteristics and
319 visual aspects of these animals. On the other hand, the ash content was about 2.0% in mussels,
320 0.9% in octopus, and about 0.7% in fish. In this sense, considering that the samples were dried

before MIC and also presented a low ash content, there was no need for the use of any combustion aids.

Afterwards, the MIC method was optimized regarding the maximum mass that could be properly decomposed for each sample. This test was performed by increasing sample masses, in increments of 100 mg, starting at 100 mg. For samples M1 and O1, the maximum mass was kept at 600 mg, while for the F1 sample, the maximum mass was kept at 800 mg. This decision was based on the visual aspect of the obtained digests, the absence of soot in the vessels, as well as the flame pattern observed during the combustion step.

After selecting the sample masses, absorbing solution composition (NH_4OH or $(NH_4)_2CO_3$) and concentration (100 to 250 mmol L^{-1}) were evaluated by performing analyte recovery tests, using the M1 (OD60) sample. For both evaluated solutions, only the 250 mmol L^{-1} concentration provided suitable recoveries for the three standard addition levels for all analytes. The obtained recoveries, which varied from 93% to 107% (S1), 93% to 108% (S2), and 92% to 103% (S3), are described in Table 2. In this sense, the absorbing solution selected for further evaluation was 250 mmol L^{-1} NH_4OH.

336 **Table 2.** Recoveries (%) of Br, Cl, F, I, and S obtained in standard addition experiments at S1, S2, and S3 levels using the M1 (OD60) sample

337 and different absorbing solutions: 100, 150, and 250 mmol L^{-1} NH_4OH or $(NH_4)_2CO_3$ (average ± standard deviation, n=3).

Absorbing solution	Br	Cl	F	I	S
Standard addition level: S1					
100 mmol L^{-1}					
NH_4OH	107 ± 2	98 ± 13	106 ± 2	108 ± 5	50 ± 8
$(NH_4)_2CO_3$	60 ± 3	30 ± 2	46 ± 6	55 ± 2	60 ± 10
150 mmol L^{-1}					
NH_4OH	96 ± 4	93 ±7	99 ± 9	96 ± 6	96 ± 3
$(NH_4)_2CO_3$	99 ± 7	102 ± 7	47 ± 8	67 ± 4	105 ± 3
250 mmol L^{-1}					
NH_4OH	99 ± 4	107 ± 2	94 ± 3	94 ± 4	99 ± 4
$(NH_4)_2CO_3$	100 ± 5	106 ± 3	96 ± 1	92 ± 1	96 ± 7
Standard addition level: S2					
250 mmol L^{-1}					
NH_4OH	100 ± 2	105 ± 6	93 ± 7	99 ± 6	108 ± 3
$(NH_4)_2CO_3$	103 ± 6	101 ± 7	98 ± 5	93 ± 2	103 ± 6
Standard addition level: S3					
100 mmol L^{-1}					
NH_4OH	80 ± 5	33 ± 9	52 ± 1	29 ± 1	44 ± 12
$(NH_4)_2CO_3$	34 ± 1	87 ± 8	39 ± 1	66 ± 7	59 ± 3
150 mmol L^{-1}					
NH_4OH	62 ± 7	59 ± 22	60 ± 8	90 ± 10	93 ± 9
$(NH_4)_2CO_3$	50 ± 4	95 ± 5	42 ± 5	70 ± 8	105 ± 3
250 mmol L^{-1}					
NH_4OH	103 ± 4	93 ± 3	92 ± 1	93 ± 2	98 ± 9
$(NH_4)_2CO_3$	108 ± 2	102 ± 8	100 ± 6	94 ± 3	102 ± 3

338 S1: standard addition of 5% of Cl^-, I^-, and SO_4^{2-}, 10% of Br^-, and 25% of F^-.

339 S2: standard addition of 20% of Cl^- and SO_4^{2-}, 50% of Br^-, and 80% of F^- and I^-.

340 S3: standard addition of 50% of SO_4^{2-}, 85% (Cl^-), 100% of Br^-, and 200% of F^- and I^-.

3.3. Evaluation of the digest's pH on analyte stabilization after MIC

Although the difficulty of halogen and sulfur stabilization in solution is not often fully discussed in the literature, it is known that alkaline medium is often needed for adequate stabilization of these analytes, especially Br and I. Furthermore, it is well known that acidic solutions are generally unsuitable for halogen absorption, since they can be converted to their more volatile hydrogen halide forms in this medium, potentially causing analyte losses by volatilization [28]. Hence, one of the possible explanations for the low recoveries obtained for different absorbing solution concentrations (Table 2), could be the different pH values of the digests obtained after the MIC method.

With this in view, the relation between the digest pH and analyte recoveries was investigated. Fig. 5 shows the pH values of each digest obtained with the different absorbing solution composition (NH_4OH or $(NH_4)_2CO_3$) and concentration (from 100 to 250 mmol L^{-1}) after sample M1 decomposition without analyte addition and with analyte addition on the lowest (S1) and highest (S3) concentration levels.

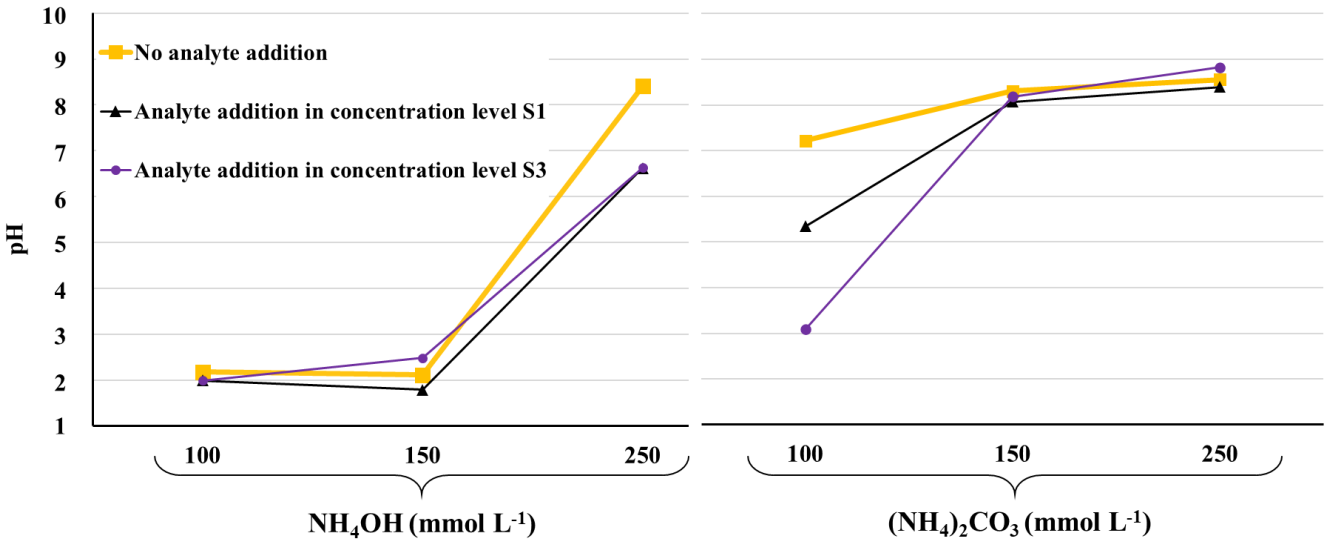

Fig. 5. Digests pH after decomposition of M1 sample using different absorbing solution composition (NH_4OH or $(NH_4)_2CO_3$) and concentration, without analyte addition and with analyte addition at low (S1) and high (S3) concentration levels.

These results show that the pH of the absorbing solutions varied greatly after performing the MIC method. Observing the pH of NH_4OH digests, the results could explain

why this solution only in the concentration of 250 mmol L^{-1} provided suitable recoveries for all analytes in all analyte addition levels, once this is the only case of digests with pH above 8. However, in the lowest level of analyte addition, the 150 mmol L^{-1} NH_4OH also provided suitable recoveries for all analytes, despite the pH value of approximately 2. It is important to notice that this solution was not chosen as the method condition, once in the highest level of analyte addition the recoveries were unsuitable, with an exception for S.

However, regarding the $(NH_4)_2CO_3$ solutions, even that the 150 mmol L^{-1} concentration maintained the pH at about 8, the obtained recoveries were considered unsuitable for some of the analytes (lower than 70% for Br, F, and I). Suitable recoveries for all analytes were only obtained when using 250 mmol L^{-1} $(NH_4)_2CO_3$ as absorbing solution, which also presented a pH value of approximately 8.

Based on these results, the pH values were considered not sufficient to explain the feasibility of a solution for stabilizing some elements after the MIC method, once the absorbing solutions with different compositions demonstrated uncorrelated behaviors between these two variables (pH vs recoveries). Thus, other factors such as ionic activity (increased with increased analyte concentration on the sample), sample matrix and absorbing solution composition (chemical characteristics of the counterion, for example) seems to have a significant influence on the stabilization of halogens (mainly Br, F, and I) after MIC.

Other interesting observations were made when comparing both solutions behaviors, regardless the recoveries provided by them. As observed in Fig. 5, the pH of the digests demonstrated that the NH_4OH solution seems to be more susceptible to acidification, once a concentration of 250 mmol L^{-1} - at least - was necessary to maintain the pH value above 7 after combustion without analyte addition. But when analyte addition was performed, the pH of the digests using 250 mmol L^{-1} NH_4OH was decreased again.

On the other hand, all evaluated concentrations of the $(NH_4)_2CO_3$ solution maintained a pH above 7 for M1 sample combustion without analyte addition, and had the pH decreased

after analyte addition only when the less concentrated solution (100 mmol L^{-1}) was employed – it is, when the concentration of the absorbing solution was not sufficient to maintain its pH. This is probably correlated with the buffer effect of the HCO_3^- ion from the $(NH_4)_2CO_3$ solution, and its dissociation equilibrium with the CO_2 (from the organic matter combustion) and H_2O present at the medium. So, it can be inferred that the pH behavior of $(NH_4)_2CO_3$ digests in particular is more affected by its concentration than by the concentration of analytes/ions in solution, unlike the NH_4OH solutions.

To verify this, another evaluation was carried out in order to test how the analyte/ion concentration in the samples (without considering the analyte addition) can influence the pH for the digests using each alkaline solution. For this, the pH of each solution and its different concentrations was measured after the combustion of the M1 and F1 samples, once it was observed that the mussel presents about 95% higher Cl^- and 55% higher SO_4^{2-} content on its composition than fish (as observed in Table 3, section 3.5). The results are demonstrated in Fig. 6.

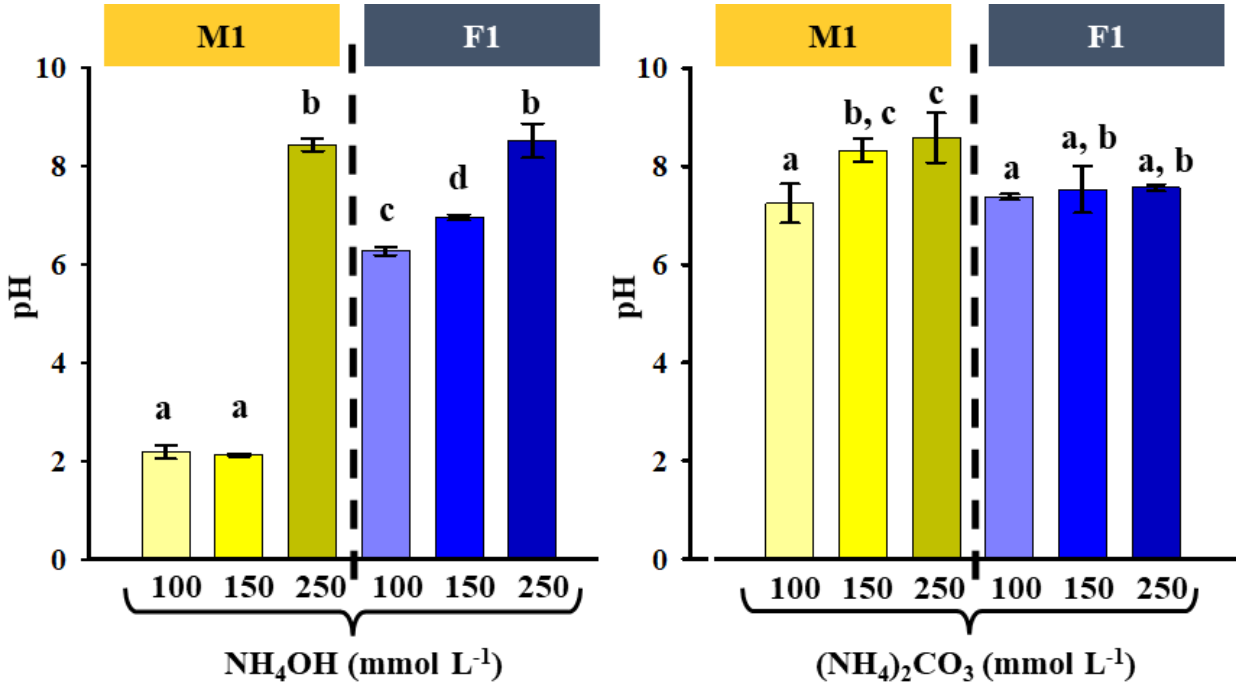

Fig. 6. pH of digests obtained after decomposition of M1 and F1 samples, using different absorbing solutions (NH_4OH and $(NH_4)_2CO_3$) and concentrations, without analyte addition. Indexes (a, b, c, or d) indicate statistical equality or difference (One-Way ANOVA Tukey's test, confidence level of 95%) for each alkaline solution.

These results shown that NH_4OH digests seems to be more susceptible to acidification (when the OH^- is decreased) in the sample with the highest analyte concentration (sample M1 in comparison to sample F1). These phenomena could potentially be associated with the relation between ionic strength and ionic activity (effective concentration) since the analytes are retained in the solution in their ionic forms. Briefly, in non-ideal solutions, the ionic activity is different from the ions' molar concentration, and the higher the ion's presence, the higher this difference. This can occur due to the shielding effect around each charge, modifying the electrostatic interaction between the ions in the solution. In this sense, one of the balances that could be affected by this phenomenon is alkaline or acid dissociation, modifying the pH of the digests. So, the seafood digest, which contained higher amount of Cl^- and, mainly, SO_4^{2-}, probably had the behavior of its pH influenced by ionic strength/activity.

On the other hand, using the $(NH_4)_2CO_3$ solutions, the differences on pH values are less significant, despite the sample digested and – then – the analyte concentration in the digests. This may corroborate the hypothesis of the buffer effect of the carbonate ion. With all this in view, for the first time, these relations between the stabilization of halogens (mainly Br, F, and I) after the MIC method with the characteristics of the absorbing solution (composition, concentration, and pH variation) as well as with the analyte concentration in the samples, were demonstrated

3.4. Accuracy evaluation for the proposed method

After choosing 250 mmol L^{-1} NH_4OH as the absorbing solution for the MIC method, the accuracy of the proposed method was evaluated by repeating S1 and S3 analyte recovery tests with a different sample used in this study, i.e., F1 (OD60). The analyte recovery test resulted in recoveries between 92% and 106% for all analytes in all levels, as described in Table A.1 in the Supplementary Material.

Then, the accuracy of the method was evaluated through SRM analysis. The matrices of analyzed SRMs were oyster and mussel tissues, with certified concentration values for all evaluated analytes (Br, Cl, F, I, and S). The agreement between the certified values and those determined using the proposed method ranged from 95% to 109% and no statistical differences (Student's t-test, confidence level of 95%) to the reference values were observed. Further details, such as the certified and determined concentration of each analyte in both SRMs, are demonstrated in Table A.2 in the Supplementary Material. These results make it possible to state that the proposed method presents suitable accuracy. Precision was considered suitable as relative standard deviations (RSDs) were lower than 10%. In this sense, the analytical method combining MIC and IC-MS provided suitable LOQs: 2.6 (Br), 18 (Cl), 3.3 (F), 2.1 (I), and 16 (S) mg kg^{-1} for mussel and octopus; and 2.0 (Br), 13 (Cl), 2.4 (F), 1.6 (I), and 12 (S) mg kg^{-1} for fish.

Briefly, the use of MIC for sample preparation enabled: *i*) an efficient decomposition of the samples, even using masses equal to or above 600 mg; *ii*) the use of an absorbing solution that provided suitable retention of the analytes and assuring low blank levels, minimization of interferences in IC analysis and suitable LOQs; *iii*) suitable sample throughput and low reagents consumption/waste generation were observed; *iv*) digests were fully compatible with injection into the IC-MS equipment for the determination of the analytes. On the other side, the use of the IC-MS technique enabled halogen and sulfur determination in a single analysis, in a wide range of concentrations, maintaining an excellent performance regarding analytical parameters. Thus, the proposed method was used to evaluate drying pre-treatments for the samples, focusing on possible influences on analyte concentration, e.g., volatilization, losses or contamination, prior to the sample preparation step.

3.5. Evaluation of drying pre-treatment and their influence on the concentration of Br, Cl, F, I, and S in seafood.

In the analytical sequence, before the sample preparation for converting a solid matrix into a suitable solution, the samples can be submitted to various pre-treatments for moisture removal and homogenization, such as drying and milling. However, these procedures can be performed in several forms, according to the characteristics of the sample matrices and/or analytes. Employing an unsuitable sample pre-treatment can affect directly the result reliability, possibly leading to volatilization and analyte losses, contamination by using inappropriate materials in the milling step, sample mass losses, inappropriate homogenization, etc.

So, the evaluation of the most suitable pre-treatment procedure for each case is needed. In this sense, three drying methods were evaluated using samples M1, F1, and O1 (see section 2.3). The samples M1 and F1 were dried using the three methods for comparison, while the O1 sample was dried using OD60 and OD100 methods, and its third fraction was used to evaluate the oven-drying temperatures with standard addition. In Table 3, the determined analyte concentrations in each sample after each drying method can be observed.

Table 3. Analytes concentration, determined by IC-MS after MIC (250 mmol L^{-1} NH_4OH) in mussel, fish, and octopus submitted to different drying methods (average ± standard deviation, n=3, mg kg^{-1}, (RSD)).

Sample	Drying method	Br	Cl	F	I	S
M1	OD60	181.2 ± 8.8 (5%) [a]	24176 ± 1630 (7%) [a]	7.68 ± 0.29 (4%) [a]	7.07 ± 0.06 (1%) [a]	14253 ± 911 (6%) [a]
	OD100	193.0 ± 5.5 (3%) [a]	24635 ± 830 (4%) [a]	4.80 ± 0.50 (11%) [b]	7.75 ± 0.15 (2%) [a]	13895 ± 695 (5%) [a]
	FD	183.8 ± 9.5 (5%) [a]	24683 ± 1471 (6%) [a]	7.35 ± 0.42 (6%) [a]	7.51 ± 0.66 (9%) [a]	13961 ± 824 (6%) [a]
F1	OD60	7.33 ± 0.28 (4%) [a]	1285 ± 74 (6%) [a]	34.39 ± 2.52 (7 %) [a]	< 1.6*	7917 ± 328 (4%) [a]
	OD100	7.01 ± 0.46 (7%) [a]	1290 ± 16 (1%) [a]	24.01 ± 1.89 (8%) [b]	< 1.6*	8493 ± 396 (5%) [a]
	FD	7.00 ± 0.19 (3%) [a]	1219 ± 33 (3%) [a]	34.12 ± 0.30 (1%) [a]	< 1.6*	7641 ± 403 (5%) [a]
O1	OD60	48.54 ± 2.33 (5%) [a]	19972 ± 517 (3%) [a]	5.52 ± 0.39 (7%) [a]	< 2.1*	17409 ± 346 (2%) [a]
	OD100	45.46 ± 2.40 (5%) [a]	20292 ± 444 (2%) [a]	3.99 ± 0.14 (3%) [b]	< 2.1*	17569 ± 1443 (8%) [a]

M1: Mussel; F1: Fish; O1: Octopus; OD60: Conventional oven drying at 60 ºC; OD100: Conventional oven drying at 100 ºC; FD: Freeze-drying at -55 °C.
[a, b]: Indexes indicating statistical equality or difference. Values in the same column regarding each sample that presents the same superscript letter as index do not have a statistical difference, while those presenting different superscript letters as index have statistical differences from each other.
* LOQ (mg kg^{-1}).

As can be seen, Br, Cl, I, and S concentrations have not been influenced by the different drying methods. On the other hand, F concentration was about 30% lower and statistically different (Student's t-test, confidence level of 95%) after the OD100 pre-treatment when compared to the other evaluated pre-treatments (OD60 and FD). In order to verify this phenomenon, analyte addition tests were carried out using the O1 sample. The addition of a standard solution containing about 1.4 mg kg^{-1} of fluorine (4.5 times the LOQ) in F^- form (from NaF salt diluted in ultrapure water) was performed before the OD60 and OD100 pre-treatments.

After F determination in spiked samples after the drying pre-treatment, a similar behavior was observed. A recovery of 92 ± 5% was observed for OD60, and only 40 ± 10% for OD100. As observed for the samples without spiking oven-dried at 100 °C, the F loss in the spiked samples was about 30% too. The determined F concentrations in the O1 spiked sample submitted to each drying method, 6.8 ± 0.3 (OD60) and 4.5 ± 1.3 (OD100) mg kg^{-1}, were statistically different (Student's t-test, confidence level of 95%).

It is worth to highlight that no liquid-solid separation differences were observed among the samples submitted to both drying methods during the sample manipulation for performing this recovery test, which could explain the differences on the results. Nevertheless, more careful evaluations must be carried out to verify if this phenomenon is related to F concentration for all kinds of matrices, or if sample composition also influences the loss of this analyte.

4. Conclusions

A suitable and reliable analytical tool was developed for halogen and sulfur determination in seafood. Microwave-induced combustion was proposed as the sample preparation method, and parameters such as sample mass (600 mg for mussels and octopus, and 800 mg for fish) and absorbing solution composition and concentration (250 mmol L^{-1}

503 NH_4OH) were optimized. These conditions provided suitable recoveries for all analytes in
504 three standard addition levels (92% to 108%), good agreement with certified reference values
505 (95% to 109%), and suitable LOQ values.

506 The IC-MS was proposed as the determination technique, enabling the determination of
507 five analytes: Br, Cl, F, I, and S, in a single analysis (reported for the first time in seafood),
508 even with widely varied concentration ranges, without impairing the achievement of suitable
509 analytical parameters, such as accuracy and precision. Given the results obtained in the
510 present work, it is possible to state that MIC, combined with IC-MS, can be a very reliable
511 analytical tool for halogen and sulfur determination in seafood.

512 In addition, the influence of digest pH on the stabilization and recovery of the analytes
513 was explored, as well as its relation with the concentration range of the ions in the solution.
514 Finally, the influence of the drying method on analyte recovery was also demonstrated, and it
515 was observed that, when seafood samples were oven-dried at 100 °C, F concentration
516 decreased significantly when compared to the other evaluated drying methods.

517

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521

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Supplementary Information

Can minor aspects of sample preparation have major impacts on the reliability of analytical methods? A study for Br, Cl, F, I, and S in seafood

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627 **Figure captions**

628 **Fig. A.1.** Chromatogram (14 to 21 min) obtained for the fish (250 mmol L^{-1} NH_4OH,
629 hundred-fold dilution, ▬) and mussel samples (250 mmol L^{-1} NH_4OH, three-hundred-fold
630 dilution, ▬), and a standard solution (7.5 µg L^{-1} SO_4^{2-}, ▬), by applying the optimized
631 conditions. The red arrow indicates the SO_4^{2-} peak in the three runs.

Tables

Table A.1. Bromine, Cl, F, I, and S recoveries (%) obtained in S1 and S3 standard addition experiments using 250 mmol L^{-1} NH_4OH solution in the MIC method, used for F1 (OD60) sample decomposition (average ± standard deviation, n=3).

Sample	Br	Cl	F	I	S
	Standard addition level: S1				
F1 (OD60)	94 ± 2	106 ± 1	98 ± 1	99 ± 3	94 ± 1
	Standard addition level: S3				
F1 (OD60)	95 ± 3	92 ± 1	98 ± 7	96 ± 5	97 ± 6

S1: standard addition of 5% of Cl^-, I^-, and SO_4^{2-}, 10% of Br^-, and 25% of F^-.

S3: standard addition of 50% of SO_4^{2-}, 85% (Cl^-), 100% of Br^-, and 200% of F^- and I^-.

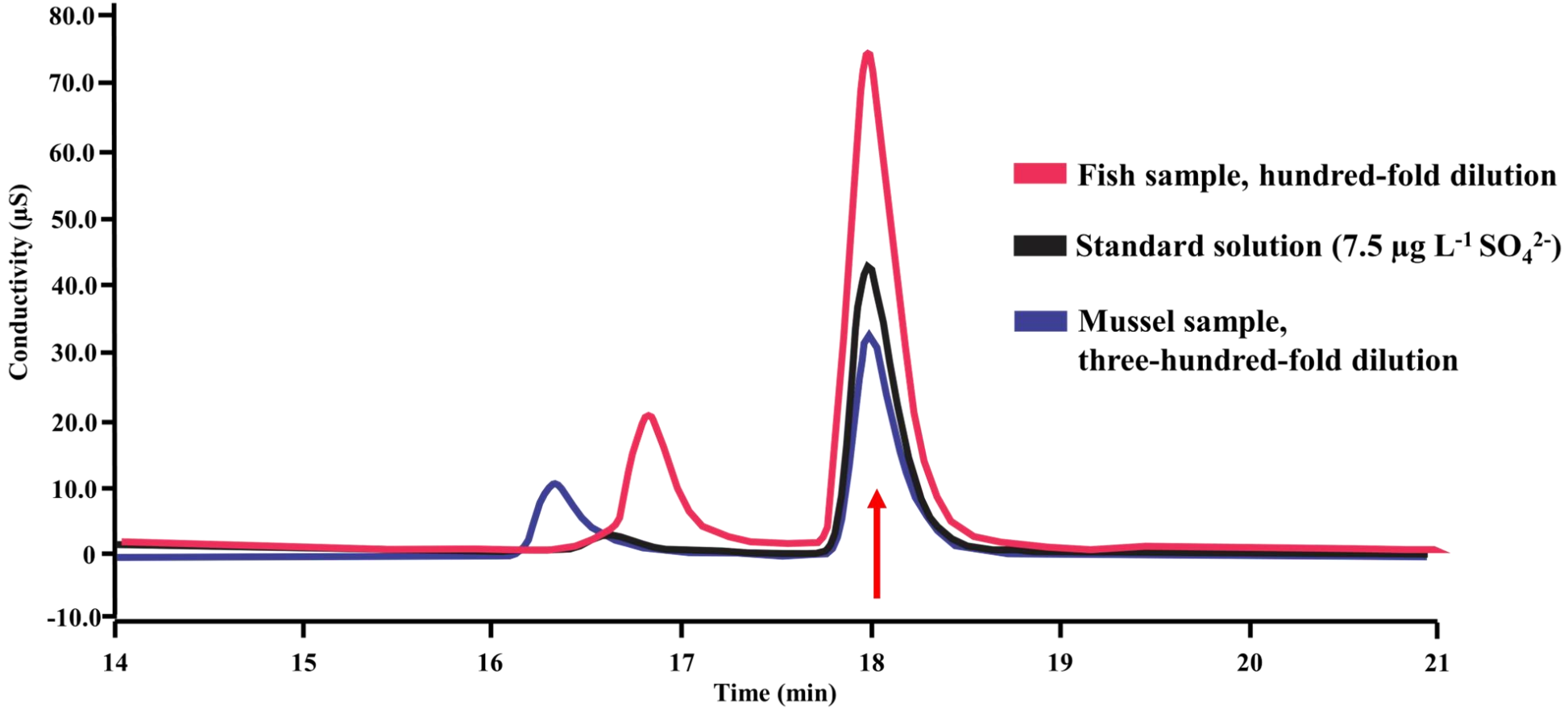
638 **Table A.2.** Comparison between the certified reference values for the analytes in the SRMs and
639 the values obtained using the proposed method (average ± standard deviation, n=3).

Analyte (concentration)	Certified / Reference values	Determined	Agreement
	SRM NIST 1566a Oyster Tissue		
F (μg g^{-1})	240*	255 ± 14	106%
Cl (% w/w)	0.829 ± 0.014	0.874 ± 0.037	105%
S (% w/w)	0.862 ± 0.019	0.882 ± 0.058	102%
I (μg g^{-1})	4.46 ± 0.42	4.27 ± 0.38	96%
	SRM NIST 2976 Mussel Tissue		
Cl (% w/w)	5.7 ± 0.5	6.2 ± 0.2	109%
S (% w/w)	1.9*	2.0 ± 0.1	106%
Br (mg kg^{-1})	329 ± 15	314 ± 11	95%

640 *Informed (noncertified) values.

641 **Figures**

642 **Fig. A.1**



643

4. CHAPTER 2

The paper “Halogens and sulfur in seafood: influence of culinary treatments and halogen bioavailability assessments” show the results of three assessments on seafood: fractionation, culinary treatments influence, and halogen bioavailability. The manuscript is divided into introduction, materials and methods, results and discussion, conclusion, and references. This manuscript is in process for submission in the *Food Chemistry* journal.

Halogens and sulfur in seafood: influence of culinary treatments and halogen bioavailability assessments

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Abstract

Nowadays, it is known that total amount of substances and chemical elements in food is far from enough to correctly interpret their nutritional and/or toxic potential. So, fractionation, culinary treatment, and bioavailability assessments are more helpful to obtain this information. In this work, the microwave-induced combustion (MIC) was used as strategy for sample preparation of different matrices (solid and liquid) to enable this kind of evaluations regarding halogens and sulfur in oyster, mussel, fishes, and shrimp (whole, muscle, and shell/head), as well as their bioavailable fraction. As the MIC provide digests compatible with multiple determination techniques, all analytes were determined after the same sample preparation procedure, by ion chromatography (IC) and inductively coupled plasma mass spectrometry (ICP-MS). Analytes were determined using MIC combined to these both determination techniques. Concentrations ranged from 6 to 187 mg kg^{-1} of Br; and 0.5 to 7.4 mg kg^{-1} of I, determined by ICP-MS; < 3.3 to 36 mg kg^{-1} of F; 1084 to 24176 mg kg^{-1} of Cl; and 1404 to 22824 mg kg^{-1} of S, determined by IC. It was observed, in the fractionation study, that Br, I, and F accumulate more in the shell of shrimps, while Cl and S accumulate in higher concentrations in the muscle tissue. Regarding the culinary treatments, the analyte loss pattern was different between matrices with different compositions. The MIC method evaluated for preparation of the bioavailable (liquid) fraction provided suitable recoveries (92% to 102%), ensuring the reliability of bioavailability results for F, Br, and I. The Br was the most bioavailable element from oyster, mussel, and shrimp; while I remained almost totally in the residual fraction in half of the evaluated samples.

Keywords: sample preparation; microwave-induced combustion; *in vitro* digestion; halogen bioavailability; nutritional assessments.

1. Introduction

According to the European Food Safety Authority (EFSA), seafood can be defined as aquatic organisms, both vertebrate or invertebrate, from salt and fresh water (with exception for mammals, reptilians, echinoderms, jellyfish and plants) [1]. About 90% of all seafood produced for commercialization in the world is destined to human consumption, which represents – according to the last data found – a consumption of approximately 20 kg *per capita* for year [2]. The high and increasing consumption of seafood in the human diet, as well as the high nutritional value of this foodstuff [1], highlight the need of knowledge about the presence of major and minor elements in their composition and their amount that the human organism is capable of absorb from each food matrix.

It is well stablished that halogens, such as F, Br, and I, are present in high amounts in both fresh and salt water, due their participation in several biogeochemical cycles. Besides of this natural occurrence, such elements can be present in water-bodies from anthropogenic activities and persistent pollutants use (mainly F)[3]. Hence, naturally, the organisms that inhabit these environments, present F, Br, and I in their composition [4-8].

However, the presence of any compound or element in the food composition do not necessarily mean that this compound or element will be effectively released from the food matrix to the human organism during the digestion (bioaccessibility). In the same way, not necessarily the total amount of the compound or element that is released for the gastrointestinal tract will be metabolized (bioavailability) [9].

Then, evaluate halogen bioaccessible/bioavailable fractions from seafood is extremely relevant to the correct evaluation of the nutritional role of this foodstuff in the human diet. It is possible mentioning a few studies that approached this topic,

performing fluorine [10] and iodine [11] bioaccessibility assessment from fishes, indicating different behaviors of these elements depending on several factors, such as pH and the presence of cations in the gastrointestinal medium, the bile salts concentration [10], as well as the culinary treatment [11]. Also, there are evidences that factors such as food matrix major composition, the chemical form (specie), the state of the metabolism (fasted or fed), among others, can influence in the bioaccessibility/availability of the analytes [12].

Regarding specifically the culinary treatments, the losses of Br and I after cooking fish and shrimp were demonstrated [4, 13]. However, no studies were found about the influence of these treatments over the bioaccessibility/availability of these elements. The lack of evaluations regarding halogen bioavailability, especially from seafood, and the influence of culinary treatments over it, highlights the need of developing studies in this field.

One of the alternatives to assess halogen bioavailability is performing *in vitro* digestion protocols, which simulate the processes of human digestion, mimetizing oral and gastrointestinal chemical characteristics [14]. Compared to *in vivo* methods, *in vitro* digestion protocols require less time, money and effort, and mainly do not present any ethical restriction. Even so, the possibilities of controlling the experiments conditions and processing several samples at the same time, guarantee a suitable reproducibility in most cases [9].

However, regarding elemental determination, the protocols used to assess bioavailability may impair the suitable analytical performance, since the most of the instrumental techniques require the sample to be in a suitable form (liquid, with analytes available and free of interferences) to be analyzed. After performing bioavailability *in vitro* assessment, it is necessary to analyze three fractions with different characteristics:

i) the whole sample, normally solid, as is the case of seafood; ii) the bioavailable fraction, a solution with a very complex matrix (composed for many organic and inorganic substances); and iii) the residual fraction, a solid residue with a matrix similar to the sample plus the substances present in the *in vitro* assay reagents. So, employing a suitable sample preparation method is crucial in order to obtain reliable results, especially regarding the further determination of elements such the halogens.

As a possible strategy, the microwave-induced combustion is an efficient decomposition method for several kinds of samples – including foodstuffs, both in solid or semi-solid forms. Then, the versatility of the MIC method is very advantageous for bioavailability evaluations. Besides, employing MIC allow the choice for the most suitable absorbing solution in function of the elements to be determined. Regarding the further halogen determination, this possibility is extremely important, since the use of acid solutions may lead to volatilization losses of these elements.

In the present work, a MIC method used for assessing halogens bioaccessibility from seaweeds [15] was adapted and evaluated for assessing the bioavailability (after dialysis) of these elements from seafood. After MIC, the halogen (Br, I, and F) determination was performed using inductively coupled plasma mass spectrometry (ICP-MS) and ion chromatography (IC). To ensure the method reliability for the samples evaluated in this work, analyte recovery experiments were performed, using two different levels of added concentration.

2. Materials and Methods

2.1. Instrumentation

The separation of F^-, Cl^-, and SO_4^{2-} was performed using an analytical column (model IonPac™ AS11-HC, 4 μm, 2 × 250 mm, Dionex/Thermo Fisher Scientific,

117 USA) along with an ion chromatograph (model ICS-5000+, Dionex/Thermo Fisher
118 Scientific) equipped with conductivity detector. For Br and I determination, an
119 inductively coupled plasma mass spectrometer (Elan DCR II, Perkin Elmer SCIEX,
120 Canada) was used. Samples were injected through a concentric nebulizer and a quartz
121 torch with a quartz injector tube. The instrumental parameters are described in Table 1.
122 **Table 1.** Operational parameters for F, Cl, and S determination by IC, and Br and I by
123 ICP-MS.

Parameter	**IC**
Eluent (mmol L^{-1})	KOH (1 to 90)
Flow (mL min^{-1})	0.28
Column temperature (°C)	36
Injection volume (µL)	50
Detection	*Conductivity detection:* F^-, Cl^-, and SO_4^{2-} Suppression: electrochemical
	ICP-MS
RF Power (W)	1300
Plasma gas flow rate (L min^{-1})	15.0
Auxiliary gas flow rate (L min^{-1})	1.2
Nebulizer gas flow rate (L min^{-1})	1.0
Spray chamber	Cyclonic
Nebulizer	Concentric
Sample and skimmer cones	Pt
Isotopes (*m/z*)	^{79}Br; ^{127}I

124

125 For sample preparation by MIC, a microwave oven was used (Multiwave 3000™,
126 Anton Paar, Austria). The samples were combusted inside quartz vessels (80 mL
127 internal volume, 80 bar maximum pressure, 280 °C maximum temperature) using quartz
128 holders to support the samples. For *in vitro* bioavailability experiments, an orbital
129 shaker (TE-4200; Tecnal, Piracicaba, Brazil) with temperature control, a centrifuge (80-
130 2b centrifuge, Daiki, South Korea) and a pH-meter (MPA-210, Tecnopon, Brazil)
131 equipped with a glass combined electrode were also used.

For culinary treatments experiments, hot plates (RH Basic 2, IKA®, Germany) were used. An analytical balance (Shimadzu, Philippines) with a resolution of 0.0001 g, was used to weigh both seafood samples and reagents. A ball mill (Pulverisette 0, Fritsch GmbH, Germany) was used for sample milling. The samples were then dried using a conventional oven (400/2ND, DeLeo, Brazil). To obtain ultrapure water with a resistance of 18.2 MΩ cm, a purification system (Simplicity UV, Merck Millipore, Germany) was used.

2.2. Reagents and samples

Ultrapure water (18.2 MΩ cm) was used in all experiments. Concentrated reagents were analytical grade or higher purity. Nitric acid (HNO_3, Synth, Brazil) was used for cleaning. As absorbing and ignition solutions in the MIC method, ammonium hydroxide (NH_4OH) was obtaining by diluting 27% m v^{-1} NH_3 (Synth), and 6 mol L^{-1} ammonium nitrate (NH_4NO_3) was obtained by dissolving the respective salt (Merck, Germany). The gases used were oxygen gas 99.96% (O_2, White Martins, Brazil) for vessels pressurization in the MIC, and argon 99.998% (Ar, White Martins, Brazil) as plasma and nebulizing gas.

Standard solutions used in spiking experiments and instrumental IC and ICP-MS calibration were obtaining dissolving salts of analytical purity (Synth) in ultrapure water. Pharmaceutical grade microcrystalline cellulose was used as a combustion aid in the MIC method, previously decontaminated by immersion with a 10% (v/v) HNO_3 solution.

The *in vitro* digestion protocol was performed using solutions mimetizing the saliva, gastric juice, and duodenal/biliary juice. These solutions were composed by inorganic, organic, and enzymatic fractions. In the preparation of these solutions, the

following reagents, all analytical purity (Sigma, Merck KgaA, Germany), were used. Inorganic fraction: NaCl, $NaHCO_3$, $CaCl_2.2H_2O$, KCl, KSCN, NaH_2PO_4, Na_2SO_4, NH_4Cl, KH_2PO_4, $MgCl_2$, and HCl. Organic and enzymatic fractions: urea, d-(+)-glucose, glucuronic acid, glucosamine hydrochloride, uric acid, albumin from bovine serum, α-amylase, mucin from porcine stomach type III, pepsin from porcine gastric mucosa, lipase, pancreatin from porcine pancreas, and bile bovine. For dialysis after the *in vitro* digestion, a TRIS-HCl buffer (pH 7.0, Sigma, Merck KgaA) and a dialysis membrane (SnakeSkin^{TM}, ThermoFisher Scientific) with a molecular weight cut-off of 10 kDa, 22 mm of internal diameter, and capacity of approximately 4 mL cm^{-1}.

Seafood samples were purchased in local fishery markets or supermarkets in Pelotas/RS, Brazil. The evaluated species were oyster (*Crassostrea gigas*, sample Oy1), brown mussel (*Perna perna*, sample M1), argentine hake (*Merluccius hubbsi*, samples F1 and F2), and pink shrimp (*Farfantepenaeus brasiliensis*, samples WS1 - whole shrimp, SS1 – shrimp shell/head, and SM1 – shrimp muscle).

2.3. Culinary treatments

Besides the evaluations of raw seafood, samples F2, WS1, and SM1 were submitted to different culinary treatments before analysis. The mass correction, i.e., the sample mass before and after the culinary procedure, was considered in the results calculations. The fish sample was roasted in an oven wrapped in an aluminum foil (150 ± 10 °C/15 min), and fried by submerging in soy oil (180 ± 10 °C/2 min). The shrimp (both whole and only muscle) was cooked in boiling ultrapure water (100 ± 10 °C/10 min) and fried performing the same procedure used for fish. After all culinary treatments processes, samples were oven-dried and milled before storage and analysis.

182 2.4. *In vitro* digestion protocol for F, Br, and I bioavailability assessment

183 The protocol used to assess halogen bioavailability from seafood is adapted from
184 previous work [15, 16]. Briefly, approximately 1 g of seafood (dry and milled) was
185 weighed and mixed with 1.5 mL of artificial saliva (pH of 6.8 ± 0.2). The mixture was
186 stirred for 5 minutes at 37 ± 2 °C. Next, 3 mL of artificial gastric juice (pH 1.3 ± 0.02 at
187 37 ± 2 °C) was added to the mixture and the pH was adjusted to 2.5 ± 0.5 using HCl.
188 The mixture was then stirred for 2 h in a head-over-heel motion (37 ± 2 °C). Afterward,
189 3 mL of artificial duodenal juice (pH 8.1 ± 0.2 at 37 ± 2 °C), 1.5 mL of bile (pH 8.2 ±
190 0.2 at 37 ± 2 °C), and 0.5 mL of 1 mol L^{-1} $NaHCO_3$ solution were added. The pH of the
191 mixture was then adjusted to 6.5 ± 0.5 and stirred for 2 h under the same conditions as
192 in the stomach phase. This mixture was then centrifuged at 2750 *g* for 5 min, resulting
193 in a residual (undigested) fraction and a bioaccessible fraction. For reaction suspension,
194 after the time of each step, the samples were cooled in an ice bath.

195 The bioaccessible fraction was loaded into a tubular membrane and dialyzed to a
196 buffer (0.1 mol L^{-1} TRIS-HCl, pH 7.0) at 37 °C and 150 rpm for 4 h. The solution
197 obtained after dialysis was stored until analysis. The procedure blanks were obtaining
198 mixing the saliva, gastric juice, and duodenal/biliary juice, and then submitting the
199 mixture to dialysis in same conditions as the samples.

200

201 2.5. Microwave-induced combustion sample preparation

202 The sample preparation method for total halogens and sulfur in seafood is
203 described elsewhere ("*Can minor aspects of sample preparation have major impacts on*
204 *the reliability of analytical methods? A study for Br, Cl, F, I, and S in seafood" –*
205 submission process to *Talanta* journal), as the method for decomposition for the
206 bioavailable fraction adapted to the present work [15]. For the analyte determination in

207 the total sample and residual fraction, the solid was weighed (0.6 and 0.3 g,
208 respectively) over polyethylene (PE) films, which were sealed by a heating source. For
209 the analyte determination in the bioavailable fraction, an 800 µL aliquot was taken and
210 dispensed over 0.3 g of microcrystalline cellulose on the PE films, also sealed by
211 heating.

212 The wraps containing the samples were placed in the quartz holders over a filter
213 paper disc moistened with the ignition solution and positioned inside the quartz vessels.
214 Also inside the quartz vessels, it was contained the absorbing solution, i.e., 250 mmol L^{-1}
215 NH_4OH (6 mL). After assembling this system, the vessels were pressurized at 20 bar
216 with O_2 and the samples were submitted to a microwave irradiation program (1400 W/5
217 min for combustion and reflux; and 0 W/20 min for cooling). The digests were filled up
218 with ultrapure water to 15 mL and conditioned in PP flasks before the analysis by IC.
219

220 2.6. Analytical parameters

221 Since the sample preparation parameters were adapted from another study, to
222 verify if it was suitable to the analyte determination in the bioavailable fraction from
223 seafood, spiking experiments were performed. Two concentration levels were used: S1,
224 containing the equivalent for a final concentration of 2.5 mg kg^{-1} of F, Br, and I; and S2
225 containing the equivalent for a final concentration of 8 mg kg^{-1} of F, Br, and I. The
226 standard solutions S1 and S2 were added (100 µL) together with the bioavailable
227 fraction on the cellulose before MIC. The sample used for this assessment was the
228 bioavailable fraction of the SM1 raw sample, randomly chosen.

229 The limits of detection (LOD) and quantification (LOQ) of the method was
230 calculated considering both sample preparation and determination techniques, and,
231 therefore, it was obtained values for each fraction: total sample, bioavailable and

residual fractions. For limits calculations, were used the average of the blank values plus three (for LOD) and ten (for LOQ) times the value of the standard deviation of 10 blank replicates. In the case analytes not being detected in the blanks, the LOQs were calculated using the average and standard deviation of the concentration value calculated from the signal obtained for the first calibration point in ten chromatographic runs. Then, factors such as dilution factors, final digest's volume, sample mass, and, for the bioavailable fraction, volume of the sample aliquot and volume of reagents used for the *in vitro* digestion and dialysis, were also considered. The LOQ obtained for each analyte and for each fraction is demonstrated in Table 2.

Table 2. Limits of quantification for the methods used in this work.

Analyte	Total (mg kg^{-1})	Bioavailable (mg of analyte)	Residual (mg kg^{-1})
Br*	0.2	0.03	0.8
I*	0.1	0.02	0.6
F**	3.3	1.4	3.0
Cl**	18	--	--
S**	16	--	--

* Determination by ICP-MS; ** Determination by IC.

For the result expression in the fractionation study with the shrimp sample, a ratio between the shell/head fraction and the muscle tissue fraction (45%:55% of dry weight) was considered for the analyte concentration calculation.[4,17] Also, the mass loss after the culinary treatments was considered for all respective calculations.

Statistical analyses were performed using the following tests: unpaired Student's t-test and One-Way ANOVA and post-hoc Tukey test, both on a confidence level of 95%. The software GraphPad Prism™ (GraphPad Software Inc., Version 8.0.1, 2018) was used.

3. Results and discussion

3.1.Total halogens in seafood and influence of culinary treatments

Oyster, mussel, fishes and shrimp samples were analyzed using a previously optimized method for halogens and sulfur determination. This method was also applied aiming to evaluate different fractions of shrimp (whole and divided into shell/head and muscle), as well as the influence of using culinary treatments on halogens and sulfur concentration from seafood. The obtained results are demonstrated in Table 3.

259 **Table 3.** Total concentrations (mg kg^{-1}) of Br and I (determined by ICP-MS), and F, Cl, and S (determined by IC) in seafood.

Sample	Culinary treatment	Bromine	Iodine	Fluorine	Chlorine	Sulfur
Oyster	Raw	98.9 ± 0.5	3.3 ± 0.3	< 3.3*	22289 ± 1320	18067 ± 1132
Mussel	Raw	187 ± 8	7.4 ± 0.4	7.7 ± 0.3	24176 ± 1630	14253 ± 1132
Fish 1	Raw	7.9 ± 0.5	0.51 ± 0.05	34.4 ± 2.5	1285 ± 74	7917 ± 328
Fish 2	Raw	7.0 ± 0.3[a]	0.73 ± 0.07[a]	< 3.3*	1334 ± 48[a]	9774 ± 433[a]
	Roasted	6.7 ± 0.4[a]	0.72 ± 0.01[a]	< 3.3*	1409 ± 80[a]	9578 ± 311[a]
	Fried	5.9 ± 0.2[b]	0.56 ± 0.02[b]	< 3.3*	1084 ± 61[b]	8659 ± 238[b]
Whole shrimp	Raw	182.9 ± 0.2[a]	5.77 ± 0.01[a]	35.7 ± 1.9[a]	4827 ± 269[a]	10918 ± 326[a]
	Cooked	157.9 ± 3.3[b]	3.65 ± 0.16[b]	27.6 ± 2.0[b]	1205 ± 54[a]	10849 ± 438[a]
	Fried	67.8 ± 1.1[c]	1.24 ± 0.08[c]	16.8 ± 1.2[c]	1150 ± 95[b]	2756 ± 127[b]
Shrimp shell/head	Raw	295.5 ± 8.4	8.90 ± 0.40	40.3 ± 3.2	1090 ± 38	1404 ± 75
Shrimp muscle	Raw	40.7 ± 2.1[a]	1.48 ± 0.03[a]	25.3 ± 0.9[a]	9130 ± 407[a]	22824 ± 1585[a]
	Cooked	11.6 ± 0.3[b]	0.99 ± 0.05[b]	17.9 ± 1.1[b]	1461 ± 122[c]	19452 ± 1036[b]
	Fried	7.1 ± 0.4[c]	0.54 ± 0.05[c]	12.0 ± 0.9[c]	4610 ± 182[b]	18191 ± 1217[b]
Average shrimp shell/head + muscle (raw)		168.1	5.19	32.8	5110	12114
Agreement with whole shrimp (raw)		92%	90%	92%	106%	111%

260 * LOQ (mg kg^{-1}).

261 [a,b,c] Different letters in the same column for each sample group indicate statistical difference (One-Way ANOVA and Tukey test, confidence level of 95%).

In general, the LOQs of the method were suitable, since it was possible to quantify all analytes in the evaluated samples, with exception for F in oyster and the fish 2. Also, the method used presented suitable precision, with all RSD's ≤ 9.4%. Regarding analyte concentrations, as expected, the most concentrated analytes in the evaluated samples are Cl and S, followed – generally – by Br, F, and I.

Regarding the fractionation of the shrimp sample, the knowledge about the analyte distribution is important since this food can be consumed both with or without shell. This type of chemical information is relevant by the nutritional aspect, as well as for food safety and quality control. As demonstrated by the results obtained, while the higher concentrations of Br, I, and F were observed in the shrimp's shell/head, the muscle presented the higher percentages of Cl and S. Similar concentrations of Br and I in shrimps and its fractions are reported in the literature [4, 17]. However, it was not found reports on fractionation evaluation of F, Cl, and S in these samples. It is worth mentioning that the concentration sums in shell/head and muscle agreed in 90% to 111% with the concentration determined in the whole shrimp for all analytes.

Another relevant information regarding foodstuff is the influence of culinary treatments over the concentration of chemicals present on its composition. With this in view, different culinary treatments were employed for fish, whole shrimp, and shrimp muscle samples. The fish was roasted and fried, while the whole shrimp and its muscle were cooked and fried. Regarding the fish sample, the concentration of all analytes did not differ statistically comparing the raw and roasted sample. On the other hand, by frying the fish, about 11% to 23% of the analytes was lost.

The behavior of analyte concentration in whole shrimp and its muscle tissue after the culinary treatments was different. The frying process promoted higher losses (from 53% to 79%) of all analytes compared to the cooking (14% to 37%) in the whole shrimp. In the case

of the tissue, frying promote more losses compared to cooking for Br, I and F (83% vs 72, 64% vs 33%, and 53% vs 29%, respectively). For S, similar amounts were lost with both culinary treatments (about 15% to 20%). Finally, for Cl, the higher loss was verified after cooking (84%) compared to the loss after frying (50%).

These differences are probably related to the presence of the shell, which may act like a protect cover, due to its major composition and higher inorganic content. This cover may impair the analytes diffusion to the media when cooking, only when more drastic conditions are employed, i.e., higher temperatures during the frying process. On the other hand, in the soft tissue, elements such as Cl and S can diffuse more easily to the water during the cooking process.

3.2.Bioavailability of F, Br, and I from seafood

After evaluating the presence of total halogens and sulfur content in seafood, as well as the influence of culinary treatments in the concentration of these analytes, bioavailability assessments were performed to better understand how the presence of such elements in food may impact the human metabolism. It is important to mention that only the bioavailability of F, Br and I was evaluated, since Cl and S are elements that are present in the gastrointestinal medium and, therefore, are used in the *in vitro* digestion protocol, which impair evaluating the bioavailability of these elements from any food.

Firstly, analyte recovery tests were performed in the sample preparation step for the bioavailable fraction. The analyte recovery tests enable to ensure if the sample preparation method used is suitable to eliminate these matrix interferences and totally release the analytes in the digests. The recovery results are shown in Table 4.

Table 4. Recoveries (%) obtained for Br, I, and F after using MIC as sample preparation for the bioavailable fraction in two levels of analyte addition (average standard deviation, n=3).

	Bromine	**Iodine**	**Fluorine**
Low level	92 ± 2	99 ± 11	99 ± 11
High level	99 ± 3	102 ± 5	98 ± 1

It is important to highlight that a sample preparation step is necessary despite the bioavailable fraction have a liquid matrix, since its direct injection can lead to interferences during the measure. Taking into account the recoveries obtained in both analyte addition levels for all analytes, the sample preparation method was considered suitable for the further assessments on halogen bioavailability from seafood.

Nevertheless, the existing *in vitro* protocols for bioavailability assessments were developed taking into account analytes that are present usually in higher concentrations in food than the halogens, e.g., metals and transition metals. With this in view, despite the sample preparation method being suitable to overcome matrix interferences and stabilize the analytes in solution, it was not possible to obtain any concentration value for F in both bioavailable or residual fractions. This is related to the inherent challenge on determining this analyte in very low concentrations, due to the limitations of the available techniques.

Combined to this, it must to be highlighted that the *in vitro* digestion and dialysis procedures requires large amounts of reagents, contributing significantly to increase the LOD/LOQ values, that must to be taken into consideration. Despite of this, it was possible to obtain results above the LOQ values for Br and I in the bioavailable (only for Br) and residual fractions. It is explained by the determination technique used to quantify Br and I, the ICP-MS, differently of F, which was quantified by IC. These results are demonstrated in Table 5.

In Table 5 it is also possible to verify the percentages of Br and I in each fraction (bioavailable and residual) compared to the total analyte concentration in seafood samples.

334 **Table 5.** Bioavailable amount and residual concentration of Br and I from seafood after *in vitro* bioavailability trials (average ± standard deviation, n=3).

Sample	Culinary Treatment	Bioavailable (mg of analyte)	Bioavailability (%)	Residue (mg kg^{-1})	Residue (%)	Sum Bioavailability + Residue (%)
Bromine						
Oy1		0.039 ± 0.001	39	61 ± 1	62	101
M1		0.118 ± 0.008	63	85 ± 1	45	108
F1		< 0.03*	--	3.2 ± 0.3	41	--
F2	Raw	< 0.03*	--	2.2 ± 0.1	31	--
	Roasted	< 0.03*	--	2.3 ± 0.2	34	--
	Fried	< 0.03*	--	1.4 ± 0.1	24	--
WS	Raw	0.062 ± 0.005	34	84 ± 10	46	80
	Cooked	0.051 ± 0.001	32	54 ± 9	34	66
	Fried	< 0.03*	--	14 ± 1	21	--
SM	Raw	< 0.03*	--	3.5 ± 0.3	09	--
	Cooked	< 0.03*	--	2.7 ± 0.3	23	--
	Fried	< 0.03*	--	3.2 ± 0.4	45	--
Iodine						
Oy1		< 0.02*	--	3.0 ± 0.2	91	--
M1		< 0.02*	--	7.4 ± 0.3	100	--
F1		< 0.02*	--	< 0.6**	--	--
F2	Raw	< 0.02*	--	0.61 ± 0.02	84	--
	Roasted	< 0.02*	--	< 0.6**	--	--
	Fried	< 0.02*	--	< 0.6**	--	--
WS	Raw	< 0.02*	--	2.6 ± 0.1	45	--
	Cooked	< 0.02*	--	3.3 ± 0.2	89	--
	Fried	< 0.02*	--	< 0.6**	--	--
SM	Raw	< 0.02*	--	0.63 ± 0.03	43	--
	Cooked	< 0.02*	--	< 0.6**	--	--
	Fried	< 0.02*	--	< 0.6**	--	--

335 * LOQ in mass (mg) of analyte; ** LOQ in concentration (mg kg^{-1}) of analyte.

As is possible to observe, it was possible to quantify iodine only in the residual fraction after the bioavailability experiments. The LOQ for Br and I were significantly lower than for F, as discussed before. Even so, it is also possible to observe that the percentage of I in the sample's residual fraction correspond to 43% to 100% of the total I concentration in the seafood evaluated. This may suggest that, even if a lower LOQ was obtained for this analyte, the I percentage in the bioavailable is considerably low.

On the other hand, Br was quantified both in bioavailable (from oyster, mussel and whole shrimp samples) and residual fractions (from all samples). In the cases where the Br concentration was above the LOQ only in residue, the percentage in this fraction ranged from 9% to 45%. However, if the remaining Br is in the bioavailable fraction, the theoretical concentration is < 0.03 mg, in most cases. Nevertheless, it must to be highlighted that part of the analyte can be only bioaccessible (before dialysis), and not bioavailable (after dialysis).

In the cases that was possible to quantify Br in both fractions the sum of percentages of this analyte bioavailable and residual corresponded to 66% to 108% of total Br in samples. The lower percentages of the sum bioavailable and residual Br in such cases correspond to the whole shrimp samples, i.e., 80% for the raw sample and 66% after cooking. This corroborates the previous hypothesis that, for this sample specifically, a fraction of Br is retained in the bioaccessible fraction, being not available after dialysis.

4. Conclusions

In this work, three different assessments were performed aiming to provide new information about halogens in seafood: the fractionation of shrimp samples; the influence of culinary treatments in analyte concentration; and the halogen bioavailability. These assessments were possible using MIC for the sample preparation, a strategy that enabled the decomposition of the several types of matrices used in this work. Feasible method parameters

were achieved, such as suitable recoveries after analyte addition and precision, besides suitable LOQs for total halogens and sulfur in seafood, and for Br and I in the bioavailable and residual fractions.

The shrimp fraction with higher concentrations of Br, I, and F is the shell/head, while the higher concentrations of Cl and S are present in the muscle tissue. Seafood with different major composition, presented different behavior when submitted to culinary treatments. Finally, the most bioavailable halogen from seafood is Br, while almost all I present in samples remains in the residual fraction.

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5. CHAPTER 3

The paper “Target analysis of per- and polyfluoroalkylated substances (PFAS) in seafood: what about the sample preparation?” regards fluorine speciation, shown the development of a target method for the most common PFAS compounds, with a very suitable analytical performance. Besides, the unprecedent results regarding the influence of culinary treatments on PFAS concentration in seafood, as well as PFAS fractionation in shrimps are demonstrated. This article is in process for submission in the *Analytical Chemistry* journal.

Target analysis of per- and polyfluoroalkylated substances (PFAS) in seafood: what about the sample preparation?

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Abstract

The Per- and Polyfluorinated Substances (PFAS) are compounds of growing interest in the most diverse knowledge areas, due to its unique characteristics and harmful potential to the environment and human kind. This work shows the presence of, at least, 10 PFAS in several seafood. Total PFAS concentration in the evaluated samples ranged from 3 to 1086 μg kg^{-1}. For this, a reliable sample preparation method was specifically developed for PFAS extraction from these samples, considering the variations on the characteristics of sample matrices and molecular characteristics of the analytes. A Design of Experiments protocol was used for mixture planning to optimize the extraction solution composition, enabling the prediction of the best extraction solution mixture to this aim, i.e., the mixture of acetonitrile with an ammonium hydroxide solution (250 mmol L^{-1}) in a proportion of 5:1 v v^{-1}. This extraction solution enabled obtain suitable analyte recoveries (83% to 115%) of 11 PFAS, also showing a good extraction efficiency providing recoveries of 77% to 120% of the mass-labelled PFAS added to the sample before extraction procedures. Besides, a method previously optimized was used to quantify total fluorine in the samples, using the microwave-induced combustion for sample preparation and ion chromatography as determination technique. Then, the PFAS concentrations were compared to the obtained results for total F. The rate of PFAS ranged from 0.02% to 6.67% of fluorine present in the samples.

Keywords: Forever chemicals; Design of Experiments; seafood; speciation; sample preparation.

The concern about the presence of Per- and Polyfluorinated Substances (PFAS) in the environment and food is constantly growing, as the number of uses of these compounds.[1] Nowadays, the United States Environmental Protection Agency (US-EPA) list over 12,000 PFAS[2], which are used in the most diversified industrial applications (more than 200 categories).[1] The PFAS are anthropogenic-source compounds, synthetized with properties such as very high thermal and chemical stability, and also both hydro- and lipophilicity. These characteristics are obtained replacing all hydrogen atoms of a carbon chain with fluorine atoms (general molecular formula -C_nF_{2n+1}).[3] Unfortunately, the high stability of these compounds also means that they are highly persistent in the environment, besides being easily bioaccumulated.[4-6]

In view of this, feasible analytical strategies are necessary to evaluate the presence of PFAS in several types of samples, enabling the assessment about the presence, mobility or bioaccumulation of these species. In one hand, PFAS are frequently determined by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS)[7], or by this technique with electrospray ionization (HPLC-ESI-MS/MS).[8-12] However, the sample preparation step is frequently neglected in this kind of analysis.

Several factors and phenomena occurring during the sample preparation may influence the reliability of the final result in speciation analysis. Besides the traditional challenges faced in the sample preparation of solid samples for total elemental determination, this step before speciation analyses requires even more caution and optimizations.[13] At the same time that the procedure adopted have to present certain selectivity to the analytes, be capable of separate them of the sample matrix and provide

a solution compatible with the determination technique, it can't cause changes on analyte's distribution or specie.

One of the most critical parameters that have to be evaluated in solid-liquid extractions, is the extraction solution. This is even more challenging for PFAS-matrix separation since these compounds have chemical affinity with both polar or non-polar solvents, and this affinity also changes as the carbon number in the molecule vary. Due to the high relevance of this theme and aiming to overcome this challenge, this work shows the relevance on develop and evaluate a sample preparation method, which have to be specific and suitable for the aim of the analysis. For this, a Design of Experiments (DoE) protocol for mixture planning [14] was used to fully understand the influence of the most common extraction solution on obtaining reliable results for several PFAS in seafood.

Materials and Methods

Instrumentation

The PFAS separation, detection and quantification was performed using the HPLC-ESI-MS/MS technique (Agilent 1200 infinity HPLC, Agilent Technologies, Germany), through an analytical column (BrownLee SPP C18 column, 2.7 μm, 3 × 100 mm, PerkinElmer, UK) coupled to a mass spectrometer equipped with a triple quadrupole mass analyzer and electrospray ionization (Agilent 6465 Triple Quadrupole MS/MS, Agilent Technologies, Germany). Operational conditions, as well as analyte names and acronyms, are described in Table S1 in the Supporting Information.

For the sample preparation method, a solid-liquid extraction, an ultrasound bath (model Transsonic T 700/H, Elma Schmidbauer GmbH, Germany), two centrifuges (model Rotina 420 R, Andreas Hettich GmbH & Co. KG, Germany and model

MiniSpin, Eppendorf SE, Germany), and a conventional oven (model UF30, Memmert GmbH, Germany) were used.

The total fluorine determination was performed using an ion chromatograph (model ICS-5000+, Dionex/Thermo Fisher Scientific), equipped with an analytical column (model IonPac^{TM} AS11-HC, 4 μm, 2 × 250 mm, Dionex/Thermo Fisher Scientific), and a KOH eluent generator cartridge (EGC 500 KOH). For sample preparation, a microwave cavity oven (Multiwave 3000^{TM}, Anton Paar, Austria), equipped with a setup of eight quartz vessels (80 mL internal volume, 80 bar max. pressure and 280 °C max. temperature) was used.

Reagents and standards

The water used in all experiments and decontamination processes was the ultrapure type, ≥ 18.2 MΩ cm. The methanol (MeOH) and acetonitrile (ACN) used in the extraction procedures and chromatographic analysis were HPLC-grade. All other reagents used were of analytical grade or higher. For the cleaning of sample preparation tubes and other materials, 1 mol L^{-1} KOH (Merck KGaA, Germany) was used.

The chromatographic mobile phase solutions used were 100% ACN and 5 mmol L^{-1} ammonium acetate (CH_3COONH_4) in ultrapure water. Extraction solutions were 250 mmol L^{-1} ammonium hydroxide (NH_4OH), 100% ACN, and 100% MeOH, or their mixtures. The NH_4OH solution was prepared by diluting 27% m v^{-1} NH_3 (Merck KGaA) in ultrapure water. The PFAS standards were obtained by diluting in MeOH the PFAC-MXC mix (Wellington Laboratories, Canada), which contained perfluoroalkyl carboxylic acids (PFCAs) and perfluorosulfonic acids (PFSAs), both ranging from C_4 to C_{18}. Mass-labelled PFAS extraction standard and internal standards were also used (MPFAC-C-ES and MPFAC-C-IS mixes, Wellington Laboratories), containing ^{13}C

112 labelled C_4–C_{12}, C_{14} PFCAs and C_4, C_6 and C_8 PFSAs; and ^{13}C labelled C_4, C_8, C_{10}
113 PFCAs and C_8 PFSA, respectively.
114
115 *Samples, pre-treatments and culinary procedures*
116 The seafood sample used for method optimization was the Giant River-prawn
117 (also known as Malaysian prawn) from an aquaculture farm in Bangladesh, purchased
118 still fresh in Graz, Austria. Other seafood samples evaluated in this work (fish, mussel,
119 and shrimp) were purchased still fresh in fisheries markets, with exception of one of the
120 fishes – obtained frozen in a supermarket, in Pelotas, Brazil. In Table 1 is possible to
121 observe more details about these samples.
122

123 **Table 1.** Details and acronyms of seafood samples evaluated in this work.

Species of seafood	Commercial name	Collection date and processing	Culinary treatment	Fraction	Acronyms
Macrobrachium rosenbergii	Giant River-prawn	June 2023	Raw	Muscle	PM1
Merluccius hubbsi	Argentine hake	October 2021	Raw	Fillet	F1
Merluccius hubbsi	Argentine hake	June 2022	Raw	Fillet	F2R
			Oven-cooked	Fillet	F2C
			Fried	Fillet	F2F
Perna perna	Brown-mussel	October 2021	Raw	Muscle	M1
Farfantepenaeus brasiliensis	Pink shrimp	February 2023	Raw	Shell + head	SS1
				Whole	SW1R
				Muscle	SM1R
			Cooked	Whole	SW1C
				Muscle	SM1C
			Fried	Whole	SW1F
				Muscle	SM1F

The samples F2, SW1, and SM1 were submitted to different culinary treatments. Besides the raw samples analysis, the fish sample was also oven-cooked and fried and the shrimp sample (both whole and only the muscle) was also cooked in boiling water and fried. All samples were rinsed with ultrapure water before culinary procedures. All samples were weighed before and after culinary treatments for mass correction in the results calculations.

Regarding the fish sample, each fillet (eight) was sectioned in three parts (one for each treatment). For oven-cooking, the fillet fractions were wrapped with an aluminum foil and placed inside a conventional oven at 150 ± 10 °C for 15 min. For frying, the fillet fractions were submersed in 100 g of soy oil at 180 ± 10 °C for 2 min. The shrimp sample was subdivided in three fractions each (whole and muscle) for culinary treatments. The cooking was performed by submerging the sample in 200 mL of ultrapure water at 100 ± 10 °C for 10 min. The frying procedure was performed by submerging the shrimps in 100 g of soy oil at 180 ± 10 °C for 2 min. After the described procedures, all samples were oven-dried and milled in a ball mill.

PFAS extraction from seafood

The general sample preparation procedure consisted in an ultrasound-assisted extraction (UAE), using 300 mg of each sample in triplicates. The MPFAC-C-ES mix was added (20 µL) over the solid samples, followed by the addition of 10 mL of the extraction solution. This mixture was mechanically homogenized, submitted to ultrasound energy for 15 min, centrifuged (4500 RPM for 5 min), and the supernatant was collected. These steps were repeated other two times, resulting in a total volume of 30 mL of extracts for each replicate of all samples.

The extracts were then heated (60 °C) in a conventional oven until completely dry. For extracts reconstitution, 1 mL of MeOH was used. The clean-up step was performed adding the extracts in 2 mL tubes containing activated carbon. This mixture was mechanically

homogenized, centrifuged (13400 RPM for 5 min). The supernatant was collected in HPLC glass vials, and then analyzed by HPLC-ESI-MS/MS.

The extraction solution was optimized using a Design of Experiments (DoE) protocol for mixtures planning in a ternary diagram (three components). The components evaluated were MeOH (component A), ACN (component B), 250 mmol L^{-1} NH_4OH (component C), and their mixtures. The diagram with the experimental regions tested is demonstrated in Fig. 1.

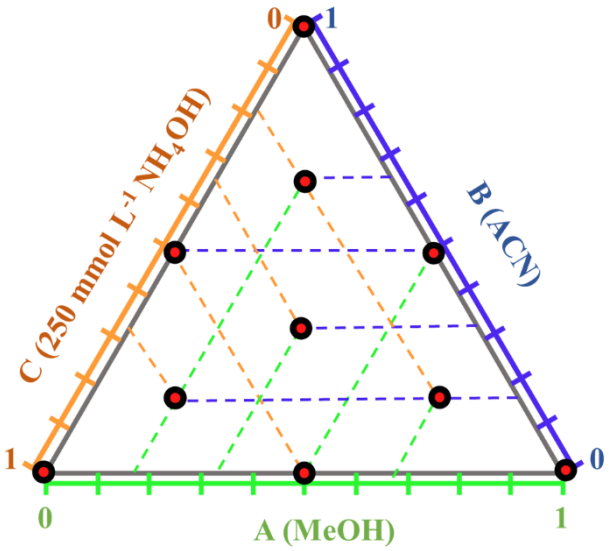

Fig.1 Ternary diagrams used for mixture planning on extraction method development.

After modelling refinement and excluding the analytes that did not fit in the model, new experimental regions were tested aiming to better evaluate the interaction between the organic extraction solvents and the alkaline solution. The new extraction solutions evaluated (in duplicates) are shown in Table 2.

Table 2. Mixture planning used for extraction method optimization.

Exp	Component A (MeOH)		Component B (ACN)		Component C (250 mmol L^{-1} NH_4OH)	
	V (mL)	Encoded	V (mL)	Encoded	V (mL)	Encoded
1	60	1.0	0	0.0	0	0.0
2	0	0.0	60	1.0	0	0.0
3	54	0.9	0	0.0	6	0.1
4	42	0.7	0	0.0	18	0.3
5	0	0.0	54	0.9	6	0.1
6	0	0.0	42	0.7	18	0.3

The monitored response for the experiments was the extraction recovery, and the target response was 100% of analyte recoveries. For this, all experiments were performed without and with analyte spiking before extraction procedures. The model was used to assess more than one analyte recovery (multiple responses) using the desirability function.

Total fluorine determination in seafood

The total fluorine (F) in seafood samples was determined using a method proposed elsewhere ("*Can minor aspects of sample preparation have major impacts on the reliability of analytical methods? A study for Br, Cl, F, I, and S in seafood"* – under review at *Talanta*), using microwave-induced combustion (MIC) for sample preparation and ion chromatography (IC) in the determination step. Briefly, 600 mg of sample was weighed over polyethylene (PE) films, wrapped and sealed by heating. Then, the wraps were positioned in quartz holders over a filter paper disc moistened with 50 μL of 6 mol L^{-1} NH_4NO_3 (for ignition). The holders were placed inside quartz vessels containing the 6 mL of absorbing solution (250 mmol L^{-1} NH_4OH), which were closed and pressurized (O_2, 20 bar), and the samples submitted to a microwave irradiation program (1400 W/5 min for combustion and reflux; and 0 W/20 min

for cooling). The digests were filled up with ultrapure water to 15 mL and conditioned in PP flasks before the analysis by IC.

Analytical parameters and data processing

The limits of detection (LOD, μg kg^{-1}) and quantification (LOQ, μg kg^{-1}) were obtained using the regression equation (y = ax + b) of the calibration curve, by the reason of the standard error of the predicted peak area (y) to each calibration concentration (x) and the curve slope (a), multiplied by 3 (for LOD) or 10 (for LOQ). Then, the obtained values were divided by the sample mass. The LOD and LOQ obtained for all analytes are described in the Table S2 (Supporting Information).

The trueness of the proposed method and extraction efficiency was assessed by the recoveries of mass-labelled PFAS, added before sample preparation procedures and compared to a reference solution containing these compounds. Instrumental quality control was also performed in every 20 analyses, using standard solutions containing 0.5 and 5.0 μg L^{-1} of the analytes.

Data obtained from HPLC-ESI-MS/MS technique was processed using MassHunter Qualitative Analysis (Agilent Technologies Inc., Version 10.0, 2019) and MassHunter Quantitative Analysis for QQQ (Agilent Technologies Inc., Version 10.2, 2019) software. The treatment of DoE results and mathematic modelling was performed using Octave GNU (Version 6.1.0, 2020) software. The statistical analyses (Student's t-test, One-Way ANOVA, and Tukey's test, all on a confidence level of 95%, $p > 0.05$) were performed using the software GraphPad Prism™ (GraphPad Software Inc., Version 8.0.1, 2018).

Results and discussion

Sample preparation method development for PFAS extraction from seafood

Initially, ten solutions, combining the components A, B, and C from mixture planning, were evaluated as extraction solutions (Fig. 1). The calculated coefficients for the mathematical model, before and after data refinement, as well as their confidence interval, were demonstrated in the Table S3 in the Supporting Information. Also in the Supporting Information, is possible to find the calculated effects and their percentages for each component and their interactions in Fig. S1.

The valid coefficients, calculated based in the desirability values, were only those corresponding to the component B (ACN) and its interaction with component C (NH_4OH solution). At the same time, the effect percentage for the component C alone is virtually null. Then, the valid coefficients were used to obtain the optimal condition for PFAS extraction from seafood. The model indicated the area corresponding to using only the component B, i.e., 100% ACN as extraction solution, as the best condition. However, the recoveries for all analytes using this extraction condition ranged from 50% to 300%, which is considered unsuitable.

With basis on that, aiming to enhance the recovery range for the analytes, as well as investigate the real influence of using or not the NH_4OH solution with the extraction solvent, the experimental region was redefined and new experiments were performed (Table 2). The coefficients for the new mathematical model were calculated and refined, as demonstrated in Table S4 in the Supporting Information, together with Table S5 containing the ANOVA table with regression parameters, as well as Fig. S2 showing the effect values and percentages for each component and their interactions. In Fig. 2 is demonstrated the surface obtained with the valid coefficients for the model.

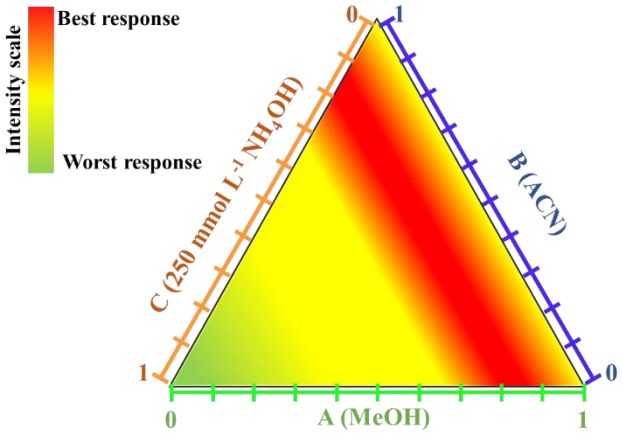

Fig. 2. Response surface obtained with the data from the second mixture planning.

Firstly, in both models, using only NH_4OH as extraction solution is the less suitable condition for this method. Also, is possible to observe with these results is that both MeOH and ACN combined with the 250 mmol L^{-1} NH_4OH solution have similar effect on PFAS extraction from seafood. With basis on the global model, the most suitable mixture, i.e., the optimal condition for the extraction method is the combination of ACN with the alkaline solution in a proportion of 5:1 v v^{-1}.

It is important to point it out that this exact condition was not tested during the design of experiments, but is an experimental region in the between of the evaluated conditions (9:1 and 7:3 v v^{-1} of organic solvent:alkaline solution). Then, new spike experiments using the optimized condition for the method were performed for modelling validation. The recoveries obtained for each analyte in these three conditions are demonstrated in Table 3. Also, in Table 4 are demonstrated the parameters used to assess the trueness of the proposed method.

244 **Table 3.** Analyte recoveries obtained in the best extraction solution conditions tested according to the mixture planning.

Extraction solution mixture proportion (v v^{-1})*	Recoveries (%)										
	PFHxA	**PFHpA**	**PFOA**	**PFNA**	**PFDA**	**PFuDA**	**PFBS**	**PFPS**	**PFHxS**	**PFHpS**	**PFOS**
(9:1)	90 ± 4.6	93 ± 0.1	93 ± 1.2	93 ± 2.8	108 ± 4.8	71 ± 1.5	108 ± 3.5	66 ± 5.1	81 ± 4.3	96 ± 3.3	95 ± 5.2
(5:1)**	115 ± 4.7	113 ± 4.9	100 ± 0.1	91 ± 0.1	106 ± 0.1	101 ± 1.0	109 ± 0.1	83 ± 5.0	97 ± 0.1	103 ± 0.1	108 ± 1.0
(7:3)	87 ± 2.1	91 ± 0.4	94 ± 1.8	94 ± 0.9	95 ± 3.5	123 ± 2.6	65 ± 1.4	57 ± 4.6	80 ± 6.4	88 ± 7.4	91 ± 0.1

245 * Proportion between ACN and 250 mmol L^{-1} NH_4OH solution.
246 ** Optimal method condition according to the mathematical model.
247

248 **Table 4.** Analyte recoveries obtained in trueness evaluation using the optimized condition for PFAS extraction from seafood.

Trueness parameter	Recoveries (%)										
	PFHxA	**PFHpA**	**PFOA**	**PFNA**	**PFDA**	**PFuDA**	**PFBS**	**PFPS**	**PFHxS**	**PFHpS**	**PFOS**
Mass-labelled PFAS 50 µg L^{-1}	105	120	101	99	106	81	92	77	77	109	109
Quality control 0.5 µg L^{-1}	100	99	108	121	93	98	117	78	115	93	107
Quality control 5.0 µg L^{-1}	121	118	99	103	91	94	101	79	102	97	100

249

As is possible to observe, suitable recoveries values were obtained (83% to 115%) for all 11 analytes included on the model, using the optimal condition obtained mathematically. Also using this method condition, i.e., ACN:250 mmol L^{-1} NH_4OH (5:1 v v^{-1}), the trueness parameters obtained were considered suitable. The extraction efficiency, measured by mass-labelled PFAS recovery, ranged from 77% to 120%. The instrumental quality control was also suitable, with recoveries from 78% to 121% considering the two checked points. To show the relevance on obtaining this method condition, in Table S6 in Supporting Information is possible to observe the unsuitable analyte recoveries obtained using the other conditions.

So, the calculated model can be considered validated and the developed extraction method suitable for further PFAS (C_4 to C_{11}) quantification in seafood. Then, the proposed method was used for sample preparation of several seafood and subsequent PFAS determination. The PFAS content was also compared to the total fluorine concentration in each sample.

Concentration of PFAS in seafood

The first evaluation performed in this study was regarding the individual concentration of each analyte. Among all samples, ten PFAS were detected and/or quantified in seafood samples. The results are demonstrated in Table 5.

268 **Table 5.** Individual PFAS concentration (µg kg^{-1}, average ± standard deviation, n = 3) in seafood.

Sample	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFuDA	PFBS	PFPS	PFHxS	PFHpS	PFOS
PM1	35.9 ± 2.0	11.2 ± 0.7	< 0.5*	11.1 ± 0.5	22.7 ± 2.3	857.2 ± 67.2	< 0.4*	27.6 ± 1.6	31.1 ± 2.7	34.9 ± 4.1	54.3 ± 1.5
F1	Detected	14.2 ± 1.9	< 0.5*	< 0.7*	< 0.7*	< 0.5*	< 0.4*	< 0.3*	< 0.7*	< 0.4*	2.6 ± 0.3
F2R	Detected	< 1.8*	< 0.5*	< 0.7*	< 0.7*	< 0.5*	< 0.4*	< 0.3*	< 0.7*	< 0.4*	Detected
F2C	Detected	< 1.8*	< 0.5*	< 0.7*	< 0.7*	< 0.5*	< 0.4*	< 0.3*	< 0.7*	< 0.4*	Detected
F2F	Detected	< 1.8*	< 0.5*	< 0.7*	< 0.7*	< 0.5*	< 0.4*	< 0.3*	< 0.7*	< 0.4*	Detected
M1	< 0.4*	< 1.8*	< 0.5*	< 0.7*	< 0.7*	< 0.5*	< 0.4*	< 0.3*	< 0.7*	Detected	Detected
SS1	< 0.4*	Detected	Detected	Detected	5.6 ± 0.6	< 0.5*	< 0.4*	< 0.3*	< 0.7*	Detected	37.2 ± 0.8
SW1R	< 0.4*	10.4 ± 1.5	2.0 ± 0.4	Detected	2.5 ± 0.03	< 0.5*	< 0.4*	Detected	< 0.7*	Detected	23.6 ± 0.6^{Aa}
SW1C	< 0.4*	Detected	Detected	< 0.7*	Detected	< 0.5*	< 0.4*	Detected	< 0.7*	Detected	13.9 ± 0.9^{b}
SW1F	< 0.4*	Detected	< 0.5*	< 0.7*	< 0.7*	< 0.5*	< 0.4*	< 0.3*	< 0.7*	< 0.4*	3.1 ± 0.2^{c}
SM1R	< 0.4*	25.6 ± 1.5	Detected	Detected	Detected	< 0.5*	< 0.4*	< 0.3*	< 0.7*	< 0.4*	10.5 ± 0.1^{Ba}
SM1C	< 0.4*	13.9 ± 3.9	< 0.5*	< 0.7*	Detected	< 0.5*	< 0.4*	< 0.3*	< 0.7*	< 0.4*	8.9 ± 0.3^{b}
SM1F	< 0.4*	Detected	< 0.5*	< 0.7*	Detected	< 0.5*	< 0.4*	< 0.3*	< 0.7*	< 0.4*	9.6 ± 0.5^{b}

269 * LOD (µg kg^{-1})
270 A, B: different capital letters indicate statistical difference (Student's t-test, confidence level of 95%).
271 a, b: different lowercase letters indicate statistical difference for culinary treatments comparison (One-Way ANOVA, Tukey test, confidence level of 95%).

The sample used for the method optimization, i.e., prawn muscle, presented the highest concentration of practically all analytes, but especially PFuDA. The PFAS content on other seafood is significantly lower. This is probably related to the geographical origin of this sample, which is different from the other evaluated samples (Bangladesh and Brazil, respectively). Also, with exception for prawn sample, the higher PFAS concentrations correspond to the PFOA and, especially, PFOS compounds in seafood. Given the obtained results, the PFOS was the only analyte for which a statistical comparison was possible, and only for the shrimp sample.

The results obtained regarding this analyte for the whole animal are statistically higher than those obtained for the shrimp muscle. At the same time, the mass balance between PFOS concentration in the whole shrimp (23.6 ± 0.6 μg kg^{-1}) versus its average in muscle and shell/head (23.9 μg kg^{-1}) presents an agreement of 101% between these two values. The whole animal also presented the highest concentration of PFOA and PFDA compared to only the muscle fraction. On the other hand, only the PFHpA compound was present in a higher concentration in the muscle fraction.

Regarding the influence of culinary treatments in the PFOS concentration, both cooking and frying promoted analyte loss for the whole shrimp; however, the frying treatment decreased significantly more the content of this compound. This behavior is not observed for the shrimp muscle, which lost the same amount of analyte after cooking or frying. A possible explanation for this behavior is that the shell provides a protection to the animal, accumulating most of the PFAS from the environment, at the same time that its structure facilitates the compounds extraction in the oil used for the frying. Besides PFOS, the tendency of PFAS loss after culinary treatments was also observed for the PFHpA, PFOA, and PFDA compounds.

296 After this, the sum of total PFAS (∑PFAS) in each sample was compared to the total
297 fluorine concentration. The ∑PFAS was obtained by the sum of averages of each quantified
298 compound. The results are shown in Table 6.

299 **Table 6.** Comparison of total fluorine (average ± standard deviation, n = 3) and ∑PFAS
300 concentrations (μg kg^{-1}) in seafood.

Sample	Total F	∑PFAS	∑PFAS (%)
PM1	16275 ± 849	1086	6.67
F1	34390 ± 2520	16.7	0.05
F2R	< 3300*	< 6*	--
F2C	< 3300*	< 6*	--
F2F	< 3300*	< 6*	--
M1	7680 ± 290	< 6*	0.00
SS1	40300 ± 3200	42.7	0.11
SW1R	35700 ± 1900^{Aa}	38.5	0.12
SW1C	27600 ± 2000^{b}	13.9	0.05
SW1F	16800 ± 1200^{c}	3.1	0.02
SM1R	25300 ± 900^{Ba}	36.0	0.14
SM1C	17900 ± 1100^{b}	22.7	0.13
SM1F	12000 ± 900^{c}	16.0	0.13
Average shrimp shell/head + muscle (raw)	32800	39.4	--
Agreement with whole shrimp (raw)	92%	102%	--

301 * LOQ (μg kg^{-1})
302 A, B: different capital letters indicate statistical difference (Student's t-test, confidence level of 95%).
303 a, b, c: different lowercase letters indicate statistical difference for culinary treatments comparison (One-Way
304 ANOVA, Tukey test, confidence level of 95%).

305 Firstly, is interesting to notice that one of the fish samples, which not presented PFAS
306 concentration above the LOQ, also did not present enough total fluorine concentration for
307 quantification. At the same time, the other evaluated fish presented both PFAS and total
308 fluorine above the methods LOQs. Despite being the same species, these two samples were

obtained from different sources. The F1 sample was obtained fresh, from a local producer in a fisheries market; the F2 sample was obtained frozen in a supermarket, already industrialized. Also, the two samples were collected in different times, with a 6-months interval. All these factors can explain the analyte concentration variation between F1 and F2 samples.

Another observation is that no PFAS were quantified in the mussel sample, but the total fluorine was quantified in ppm levels. This may suggest that almost the total fluorine content in this sample is in the inorganic form, possibly fluoride (F^-). The F^- presence in food obtained from salt water is expected. The absence of PFAS in this sample can be a result of a protection provided by the mussel shell.

As the prawn sample presented the highest concentration of all PFAS evaluated, the ∑PFAS was also the highest among the evaluated samples. However, the total fluorine content was not the highest compared to the other seafood in this work. This means that the ∑PFAS represents a significatively higher relative (percentage) content of the fluorine present in this sample (almost 7%) compared to the others (0% to 0.14%).

In the same way that for individual compounds, the whole shrimp presented more total fluorine and ∑PFAS than only the muscle of the animal. The mass balance shows suitable agreements between the whole shrimp versus the sum of muscle and shell/head content of total fluorine (92%) and ∑PFAS (102%).

Finally, regarding the influence of the culinary treatment, despite both cooking and frying decrease the concentration values, this is more significant after the second treatment. However, a different behavior was observed between total fluorine and PFAS. Both whole and muscle shrimp lost approximately the same amount of fluorine after cooking (23% to 29%) and frying (53%). In the other hand, as observed for PFOS individually, the PFAS content lost after cooking and frying was way higher for the whole shrimp (64% and 92%, respectively) compared to only the muscle (15% and 09%, respectively).

Conclusion

A sample preparation method was studied, specifically optimized for PFAS extraction from seafood. The studies were carried out using a mixture planning performed through a DoE protocol. The development of the sample preparation method was essential to achieve suitable analytical parameters. The parameters assessed demonstrated the reliability of the method despite the lack of certified reference material suitable for this analysis. Using the calculated mathematical model, an optimal condition for the extraction solution composition was obtained. This condition provided very suitable recoveries (83% to 115%) for all analytes fitted in the model, as well as a good extraction efficiency, demonstrated by the mass-labelled PFAS recoveries after sample preparation (77% to 120%).

All analytes, with exception of PFBS, were detected or quantified in some seafood samples. The higher concentrations of total PFAS were observed in prawns, followed by shrimps (especially in the shell/head). In these samples, the PFAS content was about 6.67% and 0.12% of total fluorine, respectively. Finally, it was observed that culinary treatments, e.g., cooking and frying, may decrease both total fluorine and PFAS concentration in the evaluated seafood. At the best of the author's knowledge, this is one of the first times that is reported the presence of PFAS in Brazilian seafood. Also, no other reports on the influence of culinary treatments in the concentration of these compounds on seafood were found.

Supporting Information

In the Supporting Information file there is tables and figures containing relevant data about the values used for the mathematical model calculation, the observed effects for the components evaluated as extraction solution, and some extra data besides the optimized method condition. Besides, some details of the instrumentation, reagents and standards used are presented (DOC).

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Supporting Information

Target analysis of per- and polyfluoroalkylated substances (PFAS) in seafood: what about the sample preparation?

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Figure captions

Fig. S1. Mixture components effects (values and percentage) from the first mathematical model of PFAS extraction from seafood.

Fig. S2. Mixture components effects (values and percentage) from the second mathematical model of PFAS extraction from seafood.

436 **Tables**

437 **Table S1.** Operational conditions for PFAS analysis by HPLC-ESI-MS/MS.

Parameter	**Value**							
Eluent (%)	(5 mmol L^{-1} CH_3COONH_4 / ACN): 75/25; 40/60; 0/100; 75/25							
Flow (mL min^{-1})	0.3							
Detection with ESI in negative polarity mode	**Compound**	**Acronyms**	**Precursor ion (m/z)**	**Product ion (m/z)**	**Ret. Time (min)**	**Fragment (m/z)**	**Collision Energy**	**Cell accelerator voltage**
	Perfluorohexanoic acid (C_6)	PFHxA	313	269	5.3	72	12	4
		PFHxA	313	119	5.3	72	88	4
		M5PFHxA	318	273	5.3	68	28	4
	Perfluoroheptanoic acid (C_7)	PFHpA	363	319	7.5	74	16	4
		PFHpA	363	169	7.5	74	28	4
		M4PFHpA	367	322	7.5	74	20	4
	Perfluorooctanoic acid (C_8)	PFOA	413	369	9.1	76	16	4
		PFOA	413	169	9.1	76	28	4
		M8PFOA	421	376	9.2	68	8	4
	Perfluorononanoic acid (C_9)	PFNA	463	419	10.7	84	16	6
		PFNA	463	219	10.7	84	28	6
		M9PFNA	472	427	10.7	84	12	6
	Perfluorodecanoic acid (C_{10})	PFDA	513	469	12.1	92	20	6
		PFDA	513	219	12.1	92	24	6
		M6PFDA	519	474	12.1	106	16	6
	Perfluoroundecanoic acid (C_{11})	PFuDA	563	519	13.4	52	16	5
		PFuDA	563	169	13.4	52	92	5
		M7PFuDA	570	525	13.4	82	24	5

438

439 **Table S1(continuation).** Operational conditions for PFAS analysis by HPLC-ESI-MS/MS.

Detection with ESI in negative polarity mode	Compound	Acronyms	Precursor ion (m/z)	Product ion (m/z)	Ret. Time (min)	Fragment (m/z)	Collision Energy	Cell accelerator voltage
	Perfluoro-1- butanesulfonic acid (C_4)	PFBS	299	99	5.6	158	88	4
		PFBS	299	80	5.6	158	112	4
		M3PFBS	302	80	5.6	162	88	4
	Perfluoro-1-pentanesulfonic acid (C_5)	PFPS	349	99	7.9	164	84	4
		PFPS	349	80	7.9	164	108	4
	Perfluoro-1-hexanesulfonic acid (C_6)	PFHxS	399	99	9.8	186	76	4
		PFHxS	399	80	9.8	186	108	4
		M3PFHxS	402	80	9.8	176	108	4
	Perfluoro-1-heptanesulfonic acid (C_7)	PFHpS	449	99	11.4	200	120	4
		PFHpS	449	80	11.4	200	92	4
	Perfluoro-1-octanesulfonic acid (C_8)	PFOS	499	99	12.9	208	104	6
		PFOS	499	80	12.9	208	108	6
		M8PFOS	507	80	12.9	196	100	6

440

441 **Table S2.** Limits of detection and quantification of PFAS using the proposed method.

	PFHxA	**PFHpA**	**PFOA**	**PFNA**	**PFDA**	**PFuDA**	**PFBS**	**PFPS**	**PFHxS**	**PFHpS**	**PFOS**
LOD (μg kg^{-1})	0.4	1.8	0.5	0.7	0.7	0.5	0.4	0.3	0.7	0.4	0.4
LOQ (μg kg^{-1})	1.2	6.0	1.8	2.2	2.5	1.6	1.2	0.9	2.2	1.3	1.3

Table S3. Coefficients obtained provided by the first mathematical model calculated for PFAS extraction solution optimization.

Coefficient number	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Components and interactions	**A**	**B**	**C**	**A,B**	**A,C**	**B,C**	**A,B,C**
Before refinement							
Coefficient value (b)	0.080903	0.72013	-0.05998	-0.57276	0.17355	-1.4128	3.4028
Confidence interval (Ci)	0.21388	0.21351	0.20236	1.0779	1.053	1.0171	6.547
b - Ci	-0.13298	0.50662	-0.26234	-1.6506	-0.87942	-2.4299	-3.1442
b + Ci	0.29479	0.93364	0.14238	0.50511	1.2265	-0.39573	9.9499
After refinement (valid coefficients)							
Coefficient value (b)		0.69217				-1.2052	
Confidence interval (Ci)		0.2496				1.1498	
b - Ci		0.44256				-2.355	
b + Ci		0.94177				-0.0554	

445 **Table S4.** Coefficients obtained provided by the second mathematical model calculated for PFAS
446 extraction solution optimization.

Coefficient number	**1**	**2**	**3**	**4**	**5**
Components and interactions	**A**	**B**	**C**	**A,C**	**B,C**
Before refinement					
Coefficient value (b)	-0.0020059	0.0020059	-12.746	18.208	18.208
Confidence interval (Ci)	0.011042	0.011042	0.36125	0.5203	0.5203
b - Ci	-0.013048	-0.0090364	-13.107	17.688	17.688
b + Ci	0.0090364	0.013048	-12.385	18.728	18.729
After refinement (valid coefficients)					
Coefficient value (b)			-12.746	18.199	18.217
Confidence interval (Ci)			0.44871	0.62319	0.62319
b - Ci			-13.195	17.576	17.594
b + Ci			-12.297	18.823	18.84

447

448 **Table S5.** ANOVA table of mathematical model employed for PFAS extraction solution mixture optimization.

	Squares sum	Degrees of freedom	Mean squares (MS)	F (MSr/MSR)	$F_{tab(0.05;\ 2,9)}$	F/Ftab	R^2	R^2 max
Regression (R)	0.15456	2	0.077279	19.239	4.26	4.5	0.81044	0.88464
Residual (r)	0.036151	9	0.0040168					
Total (T)	0.19071	11	0.017337	**F (MSlof/MSe)**	**$F_{tab(0.05;\ 2,7)}$**	**F/Ftab**		
Error (e)	0.022	7	0.0031429	2.2514	4.74	0.5		
Lack of adjustment (lof)	0.014151	2	0.0070757					

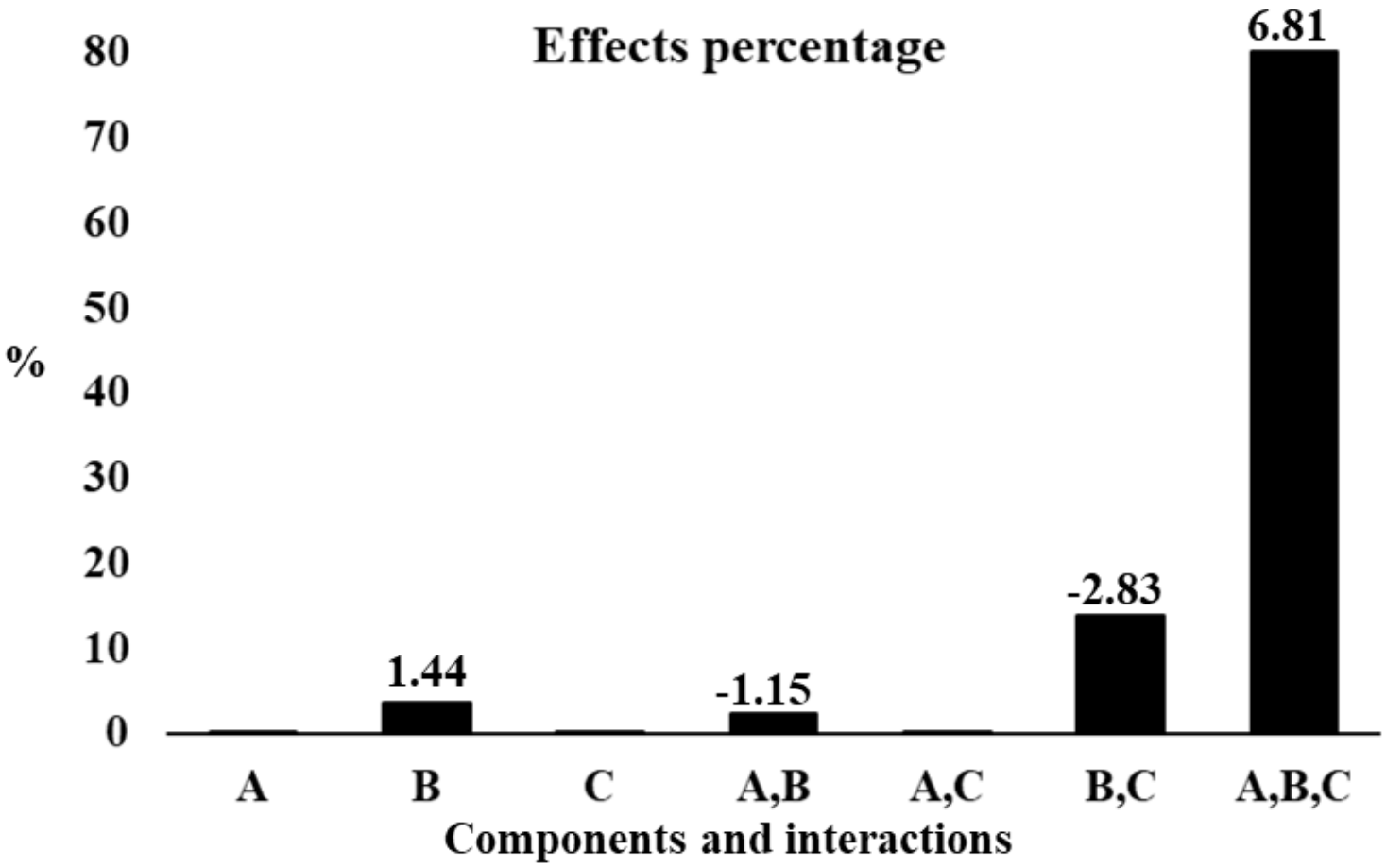
449

Table S6. Analyte recoveries obtained in different conditions tested according to the mixture planning.

Extraction solution condition	Recoveries (%)										
	PFHxA	**PFHpA**	**PFOA**	**PFNA**	**PFDA**	**PFuDA**	**PFBS**	**PFPS**	**PFHxS**	**PFHpS**	**PFOS**
100% MeOH	86 ± 4.7	91 ± 0.7	92 ± 1.1	93 ± 1.2	98 ± 6.7	0	91 ± 35.2	63 ± 4.4	83 ± 6.1	81 ± 5.2	86 ± 7.3
100% ACN	201 ± 7.8	181 ± 7.4	166 ± 3.7	148 ± 2.3	151 ± 11.0	207 ± 4.3	164 ± 12.8	112 ± 1.6	141 ± 4.7	127 ± 3.5	139 ± 4.5
MeOH:250 mmol L^{-1} NH_4OH (9:1 v v^{-1})	94 ± 6.1	98 ± 2.7	99 ± 1.6	98 ± 1.2	104 ± 0.6	58 ± 1.2	61 ± 11.4	59 ± 7.8	81 ± 11.4	90 ± 8.0	97 ± 11.3
MeOH:250 mmol L^{-1} NH_4OH (7:3 v v^{-1})	89 ± 3.5	89 ± 4.3	93 ± 1.1	93 ± 3.6	109 ± 1.3	27 ± 0.6	0	50 ± 4.8	73 ± 6.6	88 ± 1.6	94 ± 7.7

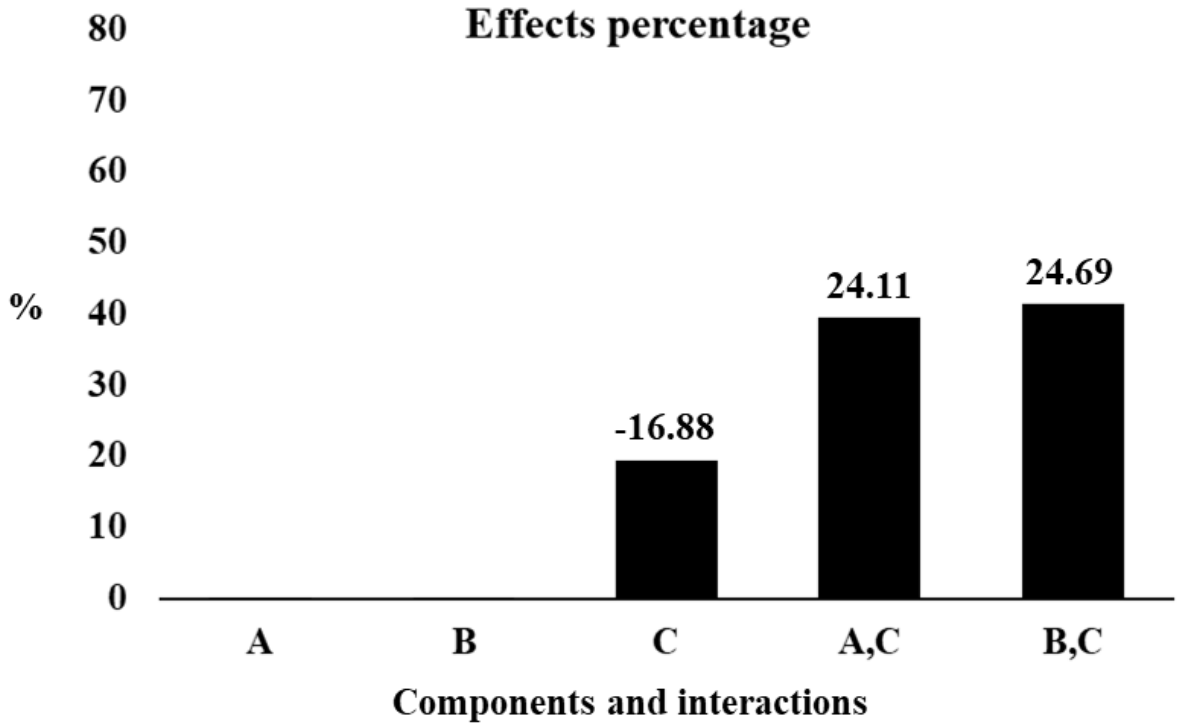
452 **Figures**

453 **Fig. S1.**



454
455

Fig. S2.


Effects percentage
80
70
60
50
%
40
30
20
10
0
-16.88
24.11
24.69
A
B
C
A,C
B,C
Components and interactions

6. FINAL REMARKS

The results obtained with the development of this thesis demonstrate, firstly, that even the minor details in sample preparation can affect the reliability of the results produced, especially concerning the determination of halogens and sulfur. It was observed that not only the composition and concentration of the absorbing solution was a key factor for analyte retainment, but also was the analyte concentration in the sample, since it affects the ionic activity in the final digests, altering the solution pH. Despite this, with the optimized parameters, the MIC was a very useful and versatile tool for decomposing several kinds of seafood without losing the efficiency of eliminating the organic matter and stabilizing the analytes into a solution.

At the same time, the combination of this sample preparation method with the IC-MS technique made it possible to obtain suitable LOQs for the analytes and, therefore, quantify all of them (Br, Cl, F, I, and S) in a single chromatographic run. So, this analytical method presents itself as an alternative to routine analysis when it comes to a less expensive technique (compared to the ICP-based ones, for example) without losing the sample throughput.

After obtaining the total halogen and sulfur concentration in seafood, further evaluations were carried out, such as shrimp fractionation studies, assessments about the influence of culinary treatments on the analyte concentration, as well as bioavailability assays. There are a few remarks that worth highlighting after these analyses. It was again demonstrated that Br and I are mostly present in the shrimp shell, but also was demonstrated for the first time that F is also more concentrated in the shell while Cl and S are more present in the muscle tissue. Also, it was demonstrated how roasting, cooking, and frying seafood may or may not affect the content of each one of the analytes. Both culinary treatment and its interaction with the matrix characteristics influence the decreasing concentration of some analytes.

Regarding the bioavailability assays, firstly it must be pointed out that this is the first time that MIC is proposed as a sample preparation method to enhance the reliability of the results obtained from the bioavailable (liquid fraction). The proposed method was based on a previous work that used MIC for decomposing the bioaccessible fraction (liquid fraction, but before dialysis) from seaweeds and further F, Br, and I determination. Nevertheless, analyte recovery tests were performed to ensure the trueness of the expressed results. Despite F concentrations being always

below the LOQ for this fraction – suggesting further method development is still necessary – the combination of MIC with ICP-MS enabled the evaluation of Br and I bioavailability. Bromine was the analyte with higher ratios of bioavailability, while iodine was present mainly on the residual fraction in the evaluated seafood.

Finally, concerning the F speciation study – i.e., the PFAS determination –, the development and evaluation of a specific sample preparation method was again demonstrated to be imperative. After optimizing the better extraction solution using a design of experiments for planning mixtures, very suitable analytical parameters were achieved for PFAS determination in seafood. Up to 11 PFAS were detected and accurately quantified in several samples, and the sum of PFAS concentration ranged from < 6 to 1086 µg kg-1.

Studies of shrimp fractionation and culinary treatment evaluation were also performed regarding PFAS. It was observed that PFAS accumulated more in the shell, probably due to its affinity to bond to proteins. The culinary treatment assessment was possible only regarding the PFOS compound, which was the only analyte quantified in whole shrimp and its muscle, before and after cooking and frying. It was observed that frying decreased the PFOS concentration more than cooking the whole shrimp, while both cooking and frying had a similar influence on the concentration of this analyte in the muscle tissue.

Given these results, this thesis presents a comprehensive overview of the presence of halogens and sulfur in seafood, factors that may influence these analytes content, how much of some of them the human organism can absorb, and different species that F can be present in these samples. This was possible using methods developed specifically for each purpose, using alternative energy sources such as microwave and ultrasound, and both new and well-established determination techniques. In summary, the analytical tools and the studies presented in this thesis provided unprecedented results and insights, revealing new fields to explore – as science always should do – in the interface of analytical chemistry and food sciences.

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